Bangladesh J. Plant Taxon. **21**(1): 1-12, 2014 (June)

RELATIONSHIPS OF *ASTRAGALUS* L. IN SECTION SESAMEI BASED ON MORPHOLOGICAL CRITERIA AND MOLECULAR MARKERS

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Keywords: *Astragalus*; Fabaceae; ISSR; RAPD; Section Sesamei.

Abstract

The relationships among five species and two varieties of *Astragalus* L. in the section Sesamei (Fabaceae) from Egypt and Saudi Arabia have been reassessed based on morphological variation and molecular polymorphism as revealed by RAPD and ISSR fingerprinting. The analysis of morphological variation delimited the examined taxa into two groups; one comprising samples representing *A. sinaicus*, *A. asterias* and *A. schimperi*, and the other is comprised of two samples of *A. stella* and six samples representing *A. tribuloides*. The grouping of *A. asterias* and *A. schimperi* based on morphological criteria indicates affinities between them that were not reflected in their previous treatments. Both morphological criteria and molecular markers indicated considerable distance between the samples of *A. stella* and *A. tribuloides*. The multiform nature of *A. tribuloides* is confirmed as *A. tribuloides* var. *mareoticus* is clearly differentiated from the type *A. tribuloides* and *A. tribuloides* var. *minutus*.

Introduction

Astragalus L. is the largest and most diverse genus of all angiosperms with more than 2,500 species distributed in arid and temperate regions of the Northern Hemisphere and South America (Podlech, 2008). It is particularly abundant in south western (SW) and south central (SC) Asia, western North America and South America (Maassoumi, 1998). The centre of origin and diversity of the genus is the drier mountainous parts of SW and SC Asia and the Himalaya (Maassoumi, 1998; Wojciechowski, 2005). In Egypt, *Astragalus* is represented by 32 species (Boulos, 1999) and in Saudi Arabia by 25 species (Migahid, 1996). The species in both countries are distributed in different phytogeographical regions and are delimited in several sections.

In the first comprehensive classification of the genus *Astragalus* presented by Bunge (1868), the annual species were assigned to two subgenera, *Trimeniaeus* Bunge and *Pogonophace* Bunge based on glabrous and barbellate stigma, respectively. In that classification subgenus *Trimeniaeus* included most of the species while subgenus *Pogonophace* contained only seven species. In recent taxonomic treatments of the genus, all annual species of *Astragalus* in the Old World were classified under subgenus *Trimeniaeus*, which has been considered to be monophyletic (Taeb *et al.*, 2007). Podlech (2008) classified the annual species of *Astragalus* into 14 sections including the section Sesamei DC. The section Sesamei is represented by five species in Egypt and five species in Saudi Arabia (Migahid, 1996; Boulos, 1999).

The molecular approaches to the taxonomy of *Astragalus* have been useful in constructing phylogenetic clades that help understand the evolutionary relationships and diversification in the genus (Wojciechowski, 2005; Kazempour Osaloo, *et al.*, 2005). Wojciechowski *et al*. (1999) have shown that some of the species-rich sections are monophyletic but other works indicated that none of the subgenera and large sections of the genus are monophyletic (Kazempour Osaloo, *et al*., 2005).

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Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) markers are used for detecting genetic variation and species relationships (Williams *et al.*, 1990; Zietkiewicz *et al.,* 1994). In the genus *Astragalus* L., RAPD and ISSR markers have been applied in recent studies at the intra- and inter-specific relationship. ISSRs were chosen to assess genetic differentiation among population of the endemic species *Astragalus oniciformis* Barneby in the upper Snake River Plain of central Idaho in the USA (Alexander *et al*., 2004). Intra- and inter-specific relationships within the *Astragalus microcephalu*s complex were studied using RAPD (Mehrina *et al.*, 2005). High levels of genetic diversity were observed in three morphological types of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao as revealed by ISSR (Xie *et al*., 2009). Comparative analysis of molecular diversity of *Astragalus adsurgens* germplasm from north China was made using RAPD and ISSR Markers (Huang *et al*., 2009). Anand *et al*. (2010) used ISSR, RAPD and DAMD (Directed amplification of mini-satellite DNA) to address the relationships among four closely related species of the *Astragalus rhizanthus* complex (i.e. *A. rhizanthus, A. candolleanus, A. malacophyllus* and *A. pindreensis*) from different parts of the Indian Himalaya and proved that these markers are potential to distinguish the closely allied species and to analyze the genetic diversity within and between the species of *Astragalus*. The objective of the present study is to clarify the systematic status of some taxa of *Astragalus* section Sesamei growing in Egypt and Saudi Arabia based on RAPD and ISSR polymorphism in addition to morphological variations.

Materials and Methods

Plant materials and scoring of morphological traits

The materials used in this study include 14 samples representing seven taxa of *Astragalus* section Sesamei collected from different localities in Egypt and Saudi Arabia (Table 1). The plant specimens have been identified following Boulos (1999) and Migahid (1996). The specimens of the examined taxa are deposited at the Herbarium of Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt and at the Museum of Biology Department, Faculty of Science, Hail University, Hail, Saudi Arabia.

A total of 45 morphological characters were considered, which include 32 two-state characters and 13 multi-state characters. The measurements and description of these characters were scored from at least five plants of each taxon. The characters and their states for morphological analysis are appended in Table 2.

DNA extraction

For DNA extraction, seeds of bulked samples of each of the studied taxa were germinated at 20ºC for 15 days. Young seedlings were collected on ice and DNA was extracted from fresh young leaves using the CTAB method following the protocol of Saghai-Maroof *et al*. (1984).

RAPD fingerprinting

RAPD fingerprinting was performed using 20 arbitrary 10-mer random primers (Operon Technologies, Inc., USA). However, only ten primers gave clearly defined fingerprinting which are shown in Table 3. PCR was carried out using a Biocycler TC-S thermal cycler from HVD, Austria. The PCR reactions were developed in a total volume of 50 µl with the following components: 5 µl of 10X reaction buffer (75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 nM $(NH_4)_2SO_4$ and 0.001% bovine serum albumin), 2 µl of 25 mM of each primer, 1 µl of Taq DNA polymerase (1U/µl), and 2 µl template DNA. The volume was completed to 50 µl with deionizied diethylpyrocarbonate (DEPC) water. The following PCR program was used: an initial denaturation of DNA was carried out at 94ºC for 1 min, followed by 40 cycles of annealing at

37ºC for 1 min, extension at 72ºC for 2 min and a final extension at 72ºC for 7 min. The RAPD products were resolved in 1.4% agarose gel in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA; pH=8) at 100 volt for 60 min. A molecular size marker ranging from 530 to 1950 bp was used to estimate the size of resolved RAPD products. The gels were stained in 0.2 µg/ml ethidium bromide and photographed using a gel documentation system (Gel Doc BioRad 2000). Each experiment was repeated twice and only stable bands were scored.

Table 1. List of *Astragalus* L. taxa of the section Sesamei examined along with their locality.

Sl. No.	Taxon	Locality
1.	*Astragalus asterias* Stev. *ex* Ledeb. 1	Burg El-Arab, Egypt
2.	*A. asterias* Stev. *ex* Ledeb. 2	Hail-Al Jouf road, Saudi Arabia
3.	*A. schimperi* Boiss. 1	Saint Catherine, South Sinai, Egypt
4.	*A. schimperi* Boiss. 2	Aja Mountain, Hail, Saudi Arabia
5.	*A. sinaicus* Boiss. 1	Wadi El Arish, North Sinai, Egypt
6.	*A. sinaicus* Boiss. 2	Aja Mountain, Hail, Saudi Arabia
7.	*A. stella* L. 1	Wadi El Arish, Sinai, Egypt
8.	*A. stella* L. 2	Al Madinah-Makkah road, Saudi Arabia
9.	*A. tribuloides* Del. 1	Alexandria-Matruh Road, Egypt
10.	*A. tribuloides* Del. 2	Hail- Al Madinah road, Saudi Arabia
11.	*A. tribuloides* var. *mareoticus* Sirj. 1	Alexandria-Matruh Road, Egypt
12.	*A. tribuloides* var. *mareoticus* Sirj. 2	Hema Faid region, Hail, Saudi Arabia
13.	*A. tribuloides* var. *minutus* Boiss. 1	Saint Catherine, South Sinai, Egypt
14.	*A. tribuloides* var. *minutus* Boiss. 2	Al Madinah-Makkah road, Saudi Arabia

ISSR fingerprinting

Eight ISSR primers manufactured by the UBC (University of British Columbia, Canada) were used in the present study; the sequences of these primers are listed in Table 3. The amplification of ISSR markers was performed according to Nagoka and Ogihara (1997). The reaction mixture consisted of 12.5 µl Hot Start Master Mixture, 2.0 µl of primer (10 mM), 1.0 µl of template DNA (50 mg/µl), and filled up to 25 µl by ddH_2O. Amplification was carried out in a HVD thermocycler programmed as follows: 40 cycles after an initial cycle for 5 min at 94°C and each cycle consisted of a denaturation at 94°C for 2 min, annealing at 36°C for 1 min, extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. The ISSR products were resolved in 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate buffer, pH=8) at 100 volt for 60 min. A 1 kb ladder was used as DNA molecular size standard. ISSR bands were visualized on UV-trans-illuminator and photographed using gel documentation system (Gel Doc-BioRad 2000). Each experiment was repeated twice and only stable bands were scored.

Data analyses

The relationship among the examined taxa was estimated based on differences among them in morphological traits as well as ISSR and RAPD fingerprinting separately and in combination. The morphological traits were given codes ranging between 0 and 3 depending on the variation in the average value for the measured traits (Table 2). The RAPD and ISSR bands were scored as '1' and '0' for presence or absence, respectively. In order to construct trees elucidating the relationships among the examined taxa, the coded data were analyzed using UPGMA (Sokal and Michener, 1958) and the Neighbor-joining (Saitou and Nei, 1987) methods based on a distance matrix. All analyses were performed with NTSYS-pc (Rohlf, 2000).

Table 2. Morphological characters and their state used in the numerical analysis.

No.	Characters	Characters states
1.	Habit	Erect herb (0), prostrate herb (1)
2.	Length (cm)	0 – 10 (0), 10.1 – 20 (1), > 20 (2)
3.	Stem hairness	Tomentose (0), canescent (1), appressed (2), villous (3)
4.	Colour of stem hairs	White (0), white and black (1)
5.	Stipule length (cm)	0.5 (0), 0.51 – 1 (1), > 1 (2)
6.	Stipule width (cm)	0.1 – 0.5 (0), > 0.5 (1)
7.	Adnation of stipules	Free (0), adnate (1)
8.	Shape of stipules	Ovate (0), lanceolate (1), triangle (2)
9.	Stipule apex	Acute (0), acuminate (1)
10.	Stipule hairs	White (0), white and black (1)
11.	Leaf length (cm)	1 – 10 (0), > 10 (1)
12.	Leaf width (cm)	0.1 – 1 (0), > 1 (1)
13.	Leaf rachis	Imparipinnate (0), paripinnate (1)
14.	Colour of leaf hairs	White (0), white and black (1)
15.	Leaflet length (cm)	< 0.5 (0), 0.51 – 1 (1)
16.	Leaflet width (cm)	0.1 – 0.5 (0), > 0.5 (1)
17.	Leaflet upper surface	Glabrous (0), hairy (1)
18.	Leaflet arrangement	Opposite (0), alternate (1)
19.	Leaflet shape	Ovate (0), elliptic (1), lanceolate (2)
20.	Leaflet apex	Obtuse (0), acute (1), notched (2)
21.	Number of leaflets	1 – 10 (0), 11 – 20 (1), > 20 (2)
22.	Inflorescence type	Raceme (0), capitate (1)
23.	Peduncle length (cm)	0.1 – 5.0 (0), > 5 (1)
24.	Inflorescence hairs	White (0), black and white (1)
25.	Flower colour	White (0), purple (1), violet (2)
26.	Flower length (cm)	0.1 – 1 (0), 1.1 – 1.5 (1), > 1.5 (2)
27.	Calyx length (cm)	< 0.5 (0), 0.51 – 1.0 (1)
28.	Colour of calyx hairs	White (0), white and black (1),
29.	Stamen length (cm)	0.1 – 0.5 (0), 0.5 – 1 (1), > 1 (2)
30.	Ovary length (cm)	0.1 – 0.5 (0), 0.5 – 1 (1), > 1 (2)
31.	Ovary width (cm)	0.1 (0), 0.2 (1)
32.	Pod length (cm)	0.1 – 2 (0), > 2 (1)
33.	Pod width (cm)	0.1 – 0.5 (0), > 0.5 (1)
34.	Pod pedicel	Absent (0), shorter than pod (1), longer than pod (2)
35.	Pod texture	Glabrous (0), hairy (1)
36.	Pod surface	Membranous (0), wrinkled (1)
37.	Pod dorsal suture	Obtuse (0), grooved (1), furrowed (2)
38.	Pod ventral suture	Obtuse (0), furrowed (1)
39.	Pod apex	Acute (0), beaked (1)
40.	Number of seeds	1–10 (0), > 10 (1)
41.	Seed length (cm)	0.1 – 0.2 (0), > 0.2 (1)
42.	Seed width (cm)	0.1 – 0.2 (0), > 0.2 (1)
43.	Seed shape	Reniform (0), quadrate (1)
44.	Seed colour	Yellow (0), brown (1)
45.	Seed surface	Smooth (0), Irregular (1)

Table 3. RAPD and ISSR primers used for DNA fingerprinting in *Astragalus* L. taxa.

RAPD primers			ISSR primers		
No.	Primer code	Primer base sequence	No.	Primer code	Primer base sequence
1	A14	5′TCT GTG CTGG 3′	1	UBC808	$(AG)_8C$
2	B17	5′AGG GAA CGAG 3′	2	UBC809	$(AG)_8G$
3	OPA01	5′CAG GCC CTTC 3′	3	UBC810	$(GA)_8T$
4	OPB07	5′GCT GAC GCAG 3′	4	UBC812	$(GA)_8A$
5	OPB20	5′GGA CCC TTAC 3′	5	UBC 830	$(TG)_8G$
6	F01	5′ACG GAT CCTG 3′	6	UBC840	$(GA)_8CT$
7	O04	5′AAG TCC GCTC 3′	7	UBC848	$(CA)_8AG$
8	O06	5′CCA CGG GAAG 3′	8	UBC855	$(AC)_8CT$
9	O08	5′CCT CCA GTGT 3′			
10	O16	5′TCG GCG GTTC 3′			

Results and Discussion

RAPD and ISSR fingerprinting analyses

A total of 91 RAPD bands were generated by 10 primers in 14 samples of *Astragalus* taxa investigated. Of these 68 bands are polymorphic and 23 are monomorphic. The polymorphic bands include 12 unique bands that have been revealed by seven primers (Table 4). The highest number of both total bands (20) and polymorphic bands (17) was produced by the primer OPB07. The RAPD fingerprints generated by the primer OPB07 is shown in Fig. 1. The primer A14, on the other hand, produced the highest number of monomorphic and unique bands (Table 4). The least number of bands (4 bands) was generated by two primers, namely O04 and O08; the number of polymorphic bands was 2 for the primer O04 and only 1 for the primer O08 with 50% and 25% polymorphism respectively (Table 4).

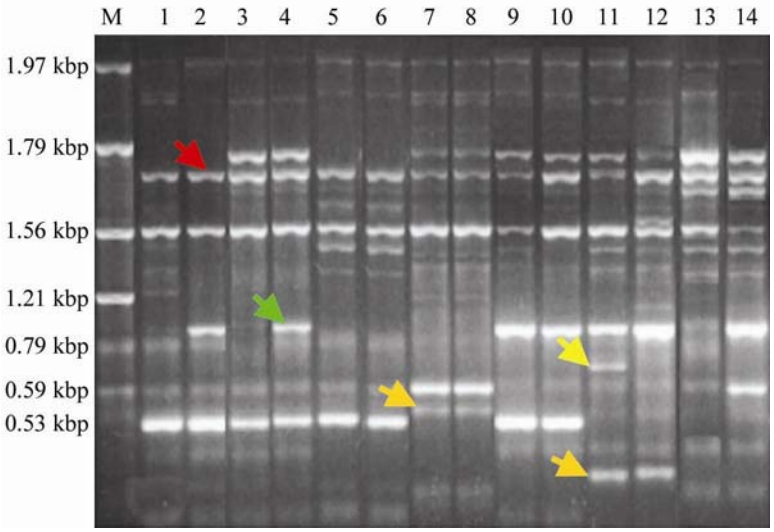

Fig. 1. RAPD fingerprints of the studied 14 samples of *Astragalus* L. as revealed by the primer OPB07. The lane to the left is a molecular size marker. Numbers on lanes 1-14 correspond to the serial numbers of samples as numbered in Table 1. First arrow indicates a monomorphic band, second arrow indicates polymorphic band, other arrows indicate unique band.

The number of amplified bands generated by RAPD markers and their molecular size are given in Table 5. The primer A14 generated the highest number of bands ranging from 11 in *A. stella* and the varieties of *A. tribuloides* to 13 in *A. schimperi* and *A. sinaicus*. OPB07 generated a total of 121 bands ranging from 6 in *A. asterias* to 12 in *A. tribuloides* var. *minutes*. In contrast, the least number of bands were produced by the primer O06 (Table 5).

Table 4. Number and types of amplified RAPD bands generated in the examined 14 samples of *Astragalus* L.

Types of bands	RAPD Primers and number of bands										
	A14	B17	OPA01	OPB07	OPB20	F01	O04	O06	O08	O16	Total
Monomorphic	4	2	3	2	2	3	2	1	3	1	23
Unique	4	1	1	1	2	0	0	1	0	1	11
Polymorphic	10	4	5	17	9	4	2	3	1	4	59
Total bands	18	7	9	20	13	7	4	5	4	6	93
% of polymorphism	77.8	71.4	66.7	90	84.6	57.1	50	80	25	83.3	75.3

Eight ISSR primers produced a total of 37 bands including only 14 polymorphic bands (Table 6; Fig. 2). The number of bands ranged from 3 as revealed by the three primers 809, 848 and 855 to 7 revealed by the primer 810; all of the bands produced by the two primers 809 and 812 were monomorphic. The primer 830 (Fig. 2C) produced a band that in all taxa except the two samples

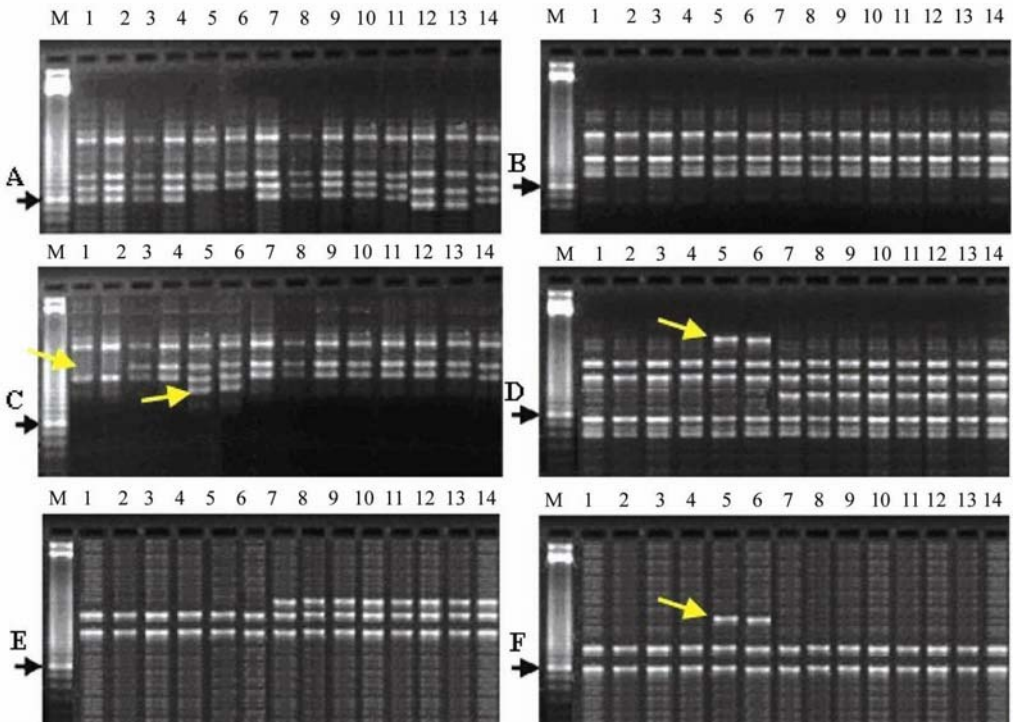

Fig. 2. ISSR fingerprints for 14 samples of *Astragalus* L. as revealed by six ISSR primers; primer codes are as follows: A = Primer UBC810, B = Primer UBC812, C = Primer UBC830, D = Primer UBC840, E = Primer UBC848, F = Primer UBC855 (see Table 4). Short arrows to the lane M indicate 250 bp and long arrows shows bands unique to one species. Number on lanes 1-14 correspond to the serial numbers of *Astragalus* taxa as numbered in Table 1.

Table 5. Number of amplified bands and their molecular size (in bp) produced in the 14 samples of *Astragalus* taxa as revealed by RAPD primers.

No.	Taxon	RAPD Primers, number and size range of bands										
		A14	B17	OPA01	OPB07	OPB20	F01	O04	O06	O08	O16	Total Bands
1.	*Astragalus asterias* 1	12 (530-1900)	2 (700-1700)	6 (500-1500)	6 (530-1970)	8 (400-1300)	5 (500-900)	2 (750-900)	2 (550-750)	4 (590-1200)	4 (1400-1700)	51
2.	*A. asterias* 2	12 (530-1900)	2 (700-1700)	5 (500-1500)	6 (530-1970)	8 (400-1300)	5 (500-900)	2 (750-900)	2 (550-750)	4 (590-1200)	4 (1400-1700)	50
3.	*A. schimperi* 1	13 (530-1900)	2 (700-1700)	5 (500-1500)	8 (530-1970)	7 (350-1250)	5 (500-900)	2 (750-900)	2 (550-750)	4 (590-1200)	4 (1400-1700)	52
4.	*A. schimperi* 2	13 (530-1900)	2 (700-1700)	5 (500-1500)	7 (530-1970)	7 (350-1250)	5 (500-900)	2 (750-900)	2 (550-750)	4 (590-1200)	4 (1400-1700)	52
5.	*A. sinaicus* 1	13 (530-1900)	3 (700-1700)	5 (500-1500)	9 (530-1970)	7 (350-1250)	4 (500-900)	2 (750-900)	2 (550-750)	4 (590-1200)	5 (1400-1700)	54
6.	*A. sinaicus* 2	12 (530-1900)	4 (700-1700)	5 (500-1500)	9 (530-1970)	7 (350-1250)	4 (500-900)	2 (750-900)	3 (550-750)	4 (590-1200)	4 (1400-1700)	54
7.	*A. stella* 1	11 (530-1900)	3 (700-1700)	7 (500-1900)	9 (450-1970)	9 (350-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700)	58
8.	*A. stella* 2	11 (530-1900)	3 (700-1700)	7 (500-1900)	9 (450-1970)	9 (350-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700)	58
9.	*A. tribuloides* 1	13 (530-1900)	3 (700-1700)	4 (500-1900)	8 (550-1970)	10 (450-1700)	5 (500-900)	4 (750-900)	2 (500-750)	4 (590-1200)	3 (1350-1700)	56
10.	*A. tribuloides* 2	13 (530-1900)	3 (700-1700)	4 (500-1900)	8 (550-1970)	9 (450-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3(1350-1700)	56
11.	*A. tribuloides* var. *mareoticus* 1	12 (530-1900)	3 (700-1700)	4 (500-1900)	9 (530-1970)	9 (450-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700))	56
12.	*A. tribuloides* var. *mareoticus* 2	11 (530-1900)	3 (700-1700)	5 (500-1900)	9 (530-1970)	10 (450-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700)	57
13.	*A. tribuloides* var. *minutus* 1	11 (530-1900)	3 (700-1700)	5 (500-1900)	12 (480-1970)	9 (450-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700)	59
14.	*A. tribuloides* var. *minutus* 2	11 (530-1900)	3 (700-1700)	5 (500-1900)	12 (480-1970)	9 (450-1700)	5 (500-900)	4 (750-900)	3 (500-750)	4 (590-1200)	3 (1350-1700)	59

of *A. asterias* (lanes 1&2); the same primer, produced a band in the profile *A. sinaicus* (lanes 5 & 6) that were absent in the profile of other taxa. The two samples of the same species are also clearly distinguished by two bands in profile of primer 840 (Fig. 2D). In the profile of primer 848 (Fig. 2E), one band was evident in the ISSR profile of the two samples of *A. stella* (lanes 7-8) and the six samples representing the three varieties of *A. tribuloides* (lanes 9-14) and was absent from the profile of the taxa representing *A. asterias*, *A. schimperi* and *A. sinaicus* (lanes 1-6). In the profile of primer 855 (Fig. 2F), it is apparent that the ISSR profiling clearly differentiated *A. sinaicus* (lanes 5 & 6) by the presence of two bands that are absent in all other taxa. A glimpse on the ISSR profiling in all samples indicates that *A. asterias* (lanes 1-2) is characterized by the absence of one band in the profile of primer 830 (Fig. 2C) and *A. sinaicus* (lanes 5-6) is distinguished by presence of three unique bands in profile of primers 830, 840 and 855.

Table 6. Number and type of amplified bands generated by the eight primers in *Astragalus* section Sesamei.

Types of bands	ISSR Primers and number of bands								Total
	808	809	810	812	830	840	848	855	
Monomorphic	4	3	2	4	2	4	2	2	23
Unique	0	0	0	0	0	0	0	0	0
Polymorphic	1	0	5	0	4	2	1	1	14
Total bands	5	3	7	4	6	6	3	3	37
% of polymorphism	20	0	71.4	0	66.7	33.3	33.3	33.3	37.8

Relationship among Astragalus taxa based on morphological variation:

The 14 samples of *Astragalus* are clearly divided into two groups in the UPGMA tree (Fig. 3), one comprising the taxa of *A. sinaicus*, *A. asterias* and *A. schimperi* and the other is comprised of taxa representing *A. stella* and the six samples representing *A. tribuloides* and its two varieties *A. tribuloides* var. *mareoticus* and *A. tribuloides* var. *minutus*. In the former group, the two samples of *A. sinaicus* are clearly delimited from the four samples representing *A. asterias* and *A. schimperi*. In the other group, the two samples representing *A. stella* are delimited from the other six samples representing *A. tribuloides*, *A. tribuloides* var. *mareoticus* and *A. tribuloides* var. *minutus*. The level of distance that separates the taxa of *A. tribuloides* exceeds the levels that separate the taxa representing *A. asterias* and *A. schimperi* (Fig. 3).

Relationship among Astragalus taxa based on RAPD and ISSR polymorphism:

The analyses of RAPD and ISSR data show that the two samples representing *A. sinaicus* are clearly delimited from the other taxa (Fig. 4). The other 12 samples are divided into two subgroups; one comprised of four samples representing the two species *A. asterias* and *A. schimperi*. The second subgroup includes the two samples representing *A. stella* and the six samples representing *A. tribuloides*. In this subgroup the two samples of the former species are clearly separated from the six samples representing *A. tribuloides* at the distance of 4.80. The two samples representing *A. tribuloides* var. *mareoticus* are separated from the four samples representing *A. tribuloides* and *A. tribuloides* var. *minutus* at a distance of 3.70. The separation of the two samples representing *A. sinaicus* is clearly associated with the presence of three ISSR bands that are confined to material of this species and absent in the other taxa (Fig. 2C, D & F).

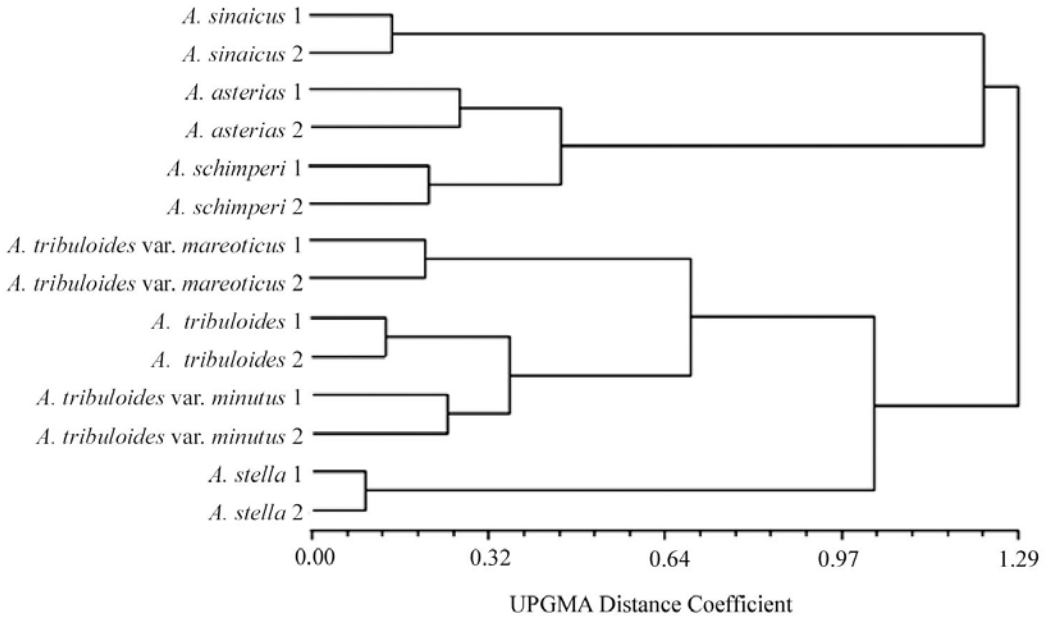

Fig. 3. UPGMA tree illustrating the relationships among *Astragalus* taxa based on morphological characters.

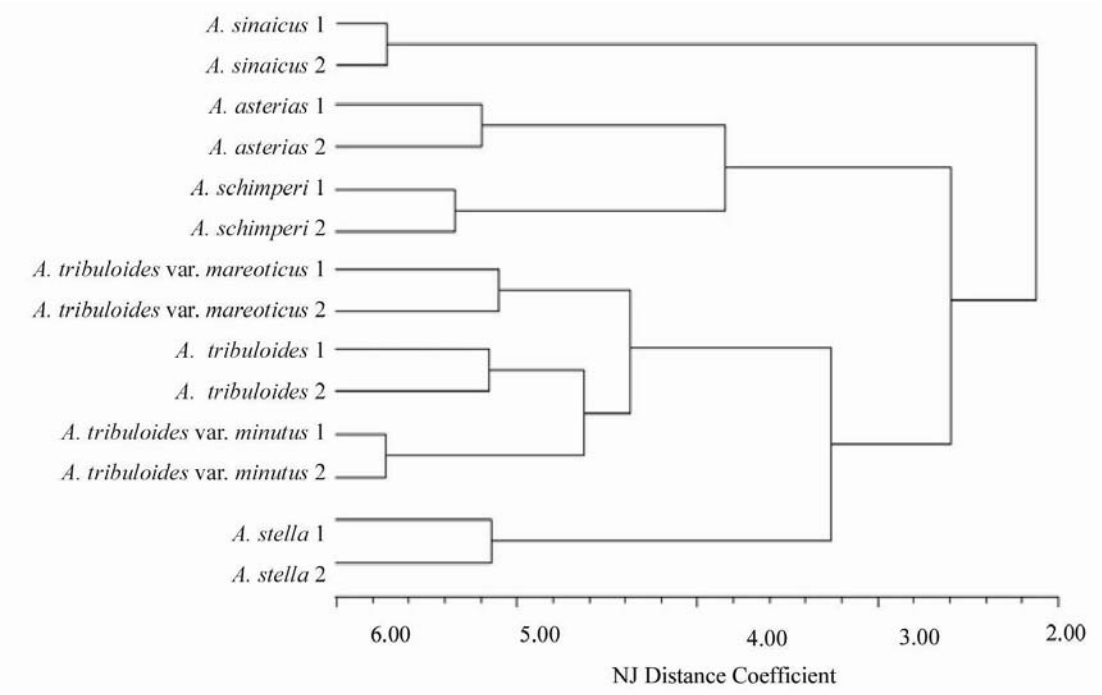

Fig. 4. Neighbour joining tree illustrating the relationships among *Astragalus* taxa based on RAPD and ISSR markers.

Relationship among Astragalus taxa based on morphological variation and molecular polymorphism:

Relationships among *Astragalus* taxa studied based on morphological variation and molecular polymorphism is shown in the UPGMA tree (Fig. 5). In this tree, the two samples representing *A. sinaicus* are clearly delimited from the other taxa. The other 12 taxa are clearly divided into two groups at a distance of 1.15, one comprising the four taxa of *A. asterias* and *A. schimperi* and the other is comprised of the two samples representing *A. stella* and the six samples representing *A. tribuloides*. It is noted that the two samples of *A. schimperi*, in the first group, are delimited at a relatively high distance of 0.81 indicating considerable morphological variation among material of this species from Egypt and Saudi Arabia. In the other group, the two samples representing *A. stella* are delimited from the other six samples representing *A. tribuloides, A. tribuloides* var. *mareoticus*, *A. tribuloides* var. *minutus* at a distance of 1.15 on the distance scale. The two samples of *A. stella* are also distinguished from each other at a distance of 0.70 indicating considerable variation among material of this species from Egypt and Saudi Arabia.

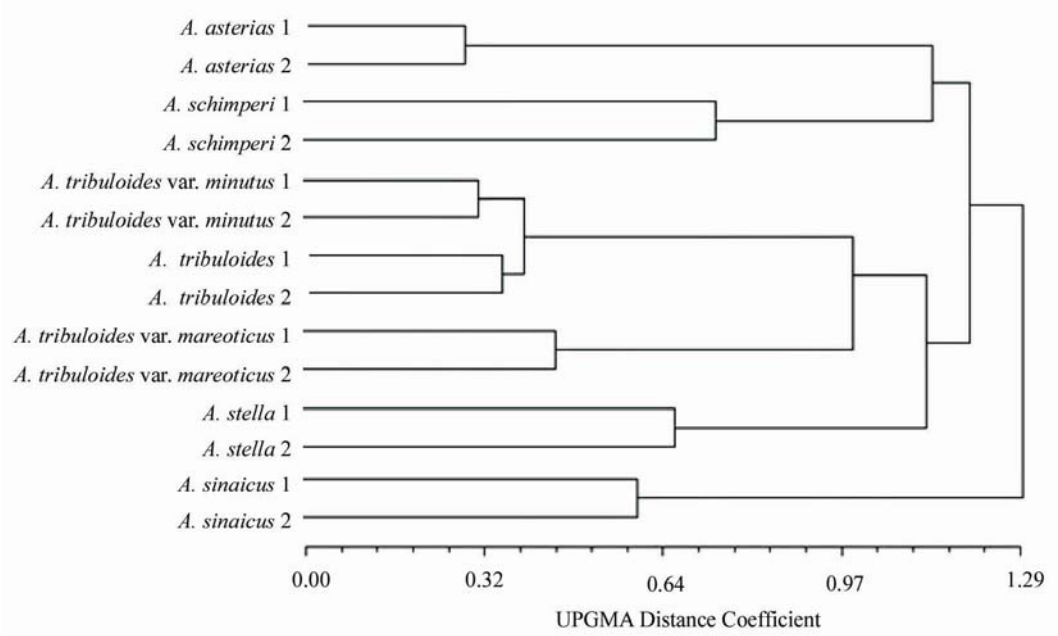

Fig. 5. UPGMA tree showing the relationships among *Astragalus* taxa based on morphological characters and molecular markers.

Podlech (1991) suggested that *A. sinaicus* is not existent in Egypt and assumed that the type may be from Greece and was erroneously attributed to Sinai by Boissier (1872) and may be considered as *A. tribuloides* which is a multiform species. This view was contradicted by Sharawy (2001) based on evidence derived from morphological and anatomical characters. *Astragalus sinaicus* was also clearly distinguished from the species of section Sesamei including *A. stella*, based on cytological evidence, as it has longer chromosomes and more symmetric karyotype compared to the other species (Badr and Sharawy, 2007). The analysis of morphological variation in the present study delimited *A. sinaicus* in a major group that also includes *A. asterias* and *A. schimperi* but remained distinguished as a separate identity. The distinction of this species is also clearly reflected in the relationship based of the analysis of ISSR and RAPD fingerprinting polymorphism.

The grouping of *A. asterias* and *A. schimperi* based on morphological variation and molecular polymorphism is congruent with their grouping together based on the analysis of seed protein electrophoretic profile (Al-Nowaihi *et al*., 2002). However, evidence from seed protein electrophoretic analysis also indicated the grouping of *A. asterias* with *A. tribuloides* (Al-Nowaihi *et al*., 2002) that is not supported by molecular evidences expressed by the analysis of morphological variation and molecular polymorphism which is correlated with similarities between these two species in spermoderm characteristics (Sharawy, 2001). *A. asterias* possesses sessile leaves and fruits with double indumentum (Sharawy *et al.,* 2003) that distinguish it from other species in section Sesamei, which can also be distinguished based on pollen characters (Saad and Taia, 1988).

Gazer (1993) divided the species of section Sesamei into four groups; i.e. *Astragalus asterias* group, *A. schimperi* group, *A. sinaicus* group and *A. stella* group; the latter group also comprised *A. tribuloides*. In the present investigation, the recognition of *A. sinaicus* and *A. stella* as distinct groups is supported by the relationships as expressed in the UPGMA trees based on morphological and molecular evidences. Both morphological criteria and molecular markers indicated considerable distance between the two samples of *A. stella* and the six samples of *A. tribuloides* and its two varieties, i.e. *A. tribuloides* var. *mareoticus* and *A. tribuloides* var. *minutus*. The distance levels among these varieties confirm the observations by Boissier (1872) and Podlech (1986) that *A. tribuloides* is a multiform species. In the present investigation *A. tribuloides* var. *mareoticus* is clearly distinct from *A. tribuloides* and *A. tribuloides* var. *minutus*.

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(Manuscript received on 4 January 2013; revised on 8 May 2014)