agriculture

MDPI

Article

Biosystematic Study on Some Egyptian Species of *Astragalus* L. (Fabaceae)

Monier M. Abd El-Ghani [1], Ashraf S. A. El-Sayed [2], Ahmed Moubarak [3], Rabab Rashad [3], Hala Nosier [4] and Adel Khattab [1,*]

[1] Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt; elghani@yahoo.com
[2] Department of Botany and Microbiology, Faculty of Science, Zagazig University, Al Sharqiya Governorate 44519, Egypt; ash.elsayed@gmail.com
[3] Department of Botany and Microbiology, Faculty of Science, Benha University, Benha 13518, Egypt; Ahmedmoub@yahoo.com (A.M.); Rababhendawy@yahoo.com (R.R.)
[4] Department of Botany, Faculty of Science, Ain Shams University, Cairo 11566, Egypt; nosairhr@gmail.com
* Correspondence: adelkhattab01@gmail.com or kadel@sci.cu.edu.eg

Abstract: *Astragalus* L. is one of the largest angiosperm complex genera that belongs to the family Fabaceae, subfamily Papilionoideae or Faboideae under the subtribe *Astragalinae* of the tribe Galegeae. The current study includes the whole plant morphology, DNA barcode (ITS2), and molecular marker (SCoT). Ten taxa representing four species of *Astragalus* were collected from different localities in Egypt during the period from February 2018 to May 2019. Morphologically, identification and classification of collected *Astragalus* plants occurred by utilizing the light microscope, regarding the taxonomic revisions of the reference collected *Astragalus* specimens in other Egyptian Herbaria. For molecular validation, ten SCoT primers were used in this study, producing a unique banding pattern to differentiate between ten samples of *Astragalus* taxa which generated 212 DNA fragments with an average of 12.2 bands per 10 *Astragalus* samples, with 8 to 37 fragments per primer. The 212 fragments amplified were distributed as 2 monomorphic bands, 27 polymorphic without unique bands, 183 unique bands (210 Polymorphic with unique bands), and ITS2 gene sequence was showed as the optimal barcode for identifying *Astragalus* L. using BLAST searched on NCBI database, and afterward, analyzing the chromatogram for ITS region, 10 samples have been identified as two samples representing *A. hauarensis*, four samples representing *A. sieberi*, three samples representing *A. spinosus* and one sample representing *A. vogelii*. Based on the ITS barcode, *A. hauarensis* RMG1, *A. hauarensis* RMG2, *A. sieberi* RMG1, *A. sieberi* RMG2, *A. sieberi* RMG3, *A. sieberi* RMG4, *A. spinosus* RMG1, *A. spinosus* RMG2, *A. spinosus* RMG3, *A. vogelii* RMG were deposited into GenBank with accession # MT367587.1, MT367591.1, MT367593.1, MT367585.1, MT367586.1, MT367588.1, MT160347.1, MT367590.1, MT367589.1, MT367592.1, respectively. These results indicated the efficiency of SCoT markers and ITS2 region in identifying and determining genetic relationships between *Astragalus* species.

Keywords: morphology; DNA extraction; SCoT polymorphism; ITS2 region; phylogeny

Citation: Abd El-Ghani, M.M.; El-Sayed, A.S.A.; Moubarak, A.; Rashad, R.; Nosier, H.; Khattab, A. Biosystematic Study on Some Egyptian Species of *Astragalus* L. (Fabaceae). *Agriculture* **2021**, *11*, 125. https://doi.org/10.3390/agriculture11020125

Academic Editors: Robert Jarret and Maarten Van Zonneveld
Received: 31 December 2020
Accepted: 22 January 2021
Published: 4 February 2021

1. Introduction

The family Leguminosae (Fabaceae) is the third-largest family of angiosperms (flowering plants) after the Orchidaceae and the Asteraceae or Compositae, with 727 genera and 19,325 species [1,2], comprising annual and perennial herbs, shrubs, and trees [3]. This family distributed in cold mountainous regions in Europe, Asia, and North America, also has very economic importance [4] which is the main sources of gums, dyes, fuel, timber, medicinals, and pulses [5]. It includes the five largest genera: *Astragalus* (over 2400 species), *Acacia* (over 950 species), *Indigofera* (about 700 species), *Crotalaria* (about 700 species), and *Mimosa* (about 500 species), that comprise around a quarter of all legume species and

radiated extensively in disturbed habitats. About 19,000 known bean species reach 7% of flowering plant species [6,7]. Fabaceae contains three subfamilies Caesalpinioideae DC, Mimosoideae DC and Faboideae Rudd (Papilionoideae). The Papilionoideae divided into 10 tribes involving Galegeae that includes *Astragalus* L. [8,9].

This family is the most common family widespread in tropical rainforests and dry forests in the Americas and Africa [10]. Fabaceae shows spectacular morphological characters and life history diversity, from giant rain forest trees and woody to desert shrubs, ephemeral herbs, herbaceous twining climbers, aquatics, and fire-adapted savanna species [11].

Astragalus L. ("Gævæn" in Persian language) is probably the largest and most abundant genus of vascular plants on Earth, comprising nearly 2500–3000 annual and perennial species and more than 250 taxonomic sections in the world [12–15] distributed in all continents, mainly around the Northern Hemisphere, Western North America, South America, Central Asia, tropical East Africa and not found in Australia [16]. The giant genus is extensively founded in the temperate and arid continental regions of the world and is chiefly located in south western Asia (ca. 1000–1500 spp.), the Sino-Himalayan regions (ca. 500 spp.), western North America (ca. 400–450 spp.), found along the Andes in South America (ca. 100 spp.), and also in Africa found on mountains [17,18]. It is also in the territory of Iran, with more than 60% endemism [19]. However, *Astragalus* L., presumed the center of origin and biodiversity is Eurasia, particularly in the drier mountainous parts of south western (SW) and south central (SC) Asia, there may be contained more than 800 species (belongs to more than 60 sections) and the Himalaya plateau [12,20]. Its species are situated in many sections. These sections are established depending on morphological characters such as stem length, stipule connection, leaf stipules shape, pod texture, flower color, and seed characters. Species of *Astragalus* growing in North Africa are Mediterranean or Arabian Saharan plants. They are represented by over 50 species determined in several sections, and 15 species which are found in the Sahara of Algeria. It is also diverse in southern Europe and Mediterranean climatic regions along the Pacific coasts of North and South America [12].

In Egypt, *Astragalus* L. is represented by 37 species [21], about 32 species [22]. The genus *Astragalus* is represented by eight species in Qatar [23] and in Saudi Arabia about 25–26 species [24]. Alshammari and Sharawy [25] registered 6 species in the Hema Faid region. Recently, Llewellyn et al. [26] registered 7 species of *Astragalus* in the Aja Mountains. The number of known (published and valid) *Astragalus* species in Iran is around 840 up to today [27].

The traditional identification of organisms depended on several common morphological characters that primarily required collecting the whole plant in vegetative, flowering, and fruiting stages at the same season of the study, and the experienced taxonomists only can identify these plants. On the other hand, these characters are often evolved from the same ancestor's species, and also these features are strongly affected by factors either environmental or developmental during the plant growth [28], and these procedures are challenging, consume a long time, and might not identify these organisms at species level. Moreover, if the taxa have the same phenotyping or are collected in the immature stage, even an experienced taxonomist can cause misidentification [29]. Because of the problems faced in taxonomy between the species of genus *Astragalus* complex and to establish our morphological observations, these problems must be investigated at the molecular level to confirm their taxonomic identity, the relationship among them, showed an obvious separation among them in the phylogenetic tree and used for analyzed the genetic variability. The DNA based markers could serve as the best taxonomic tools in such cases to control the limitations of morphological markers. There are unlimited numbers of molecular markers which are devoid of any environmental or developmental effects and show a high level of polymorphism [30]. DNA barcoding is an extensively applicable molecular method utilized to identify *Astragalus* plants [31]. The ITS2 sequence has been utilized for identification to

the species level [32]. Hebert et al., [33,34] who stated that DNA barcoding is an extensively used molecular marker technology.

In recent years, several molecular markers such as RFLP, RAPD, *rpo*C1, and *rpo*C2 have been used in *Astragalus* L. for phylogenetic studies as mentioned by Kazempour Osaloo et al., [35,36] and Wojciechowski, [20]. Among all the different marker systems, a new marker type appeared namely Start Codon Targeted (SCoT) polymorphism, used because it is a novel DNA marker, technically simple, highly polymorphic, requiring very little and not necessarily high-quality DNA; it is a simple interpretation of results and new gene-targeted marker technique based on the translation start codon [37,38].

The objectives of the current work were to study morphological characters of collecting *Astragalus* plants, the numerical taxonomy utilized to explain genetic relationships between these species, reconfirming the identification of *Astragalus* plants on molecular characterization by using DNA barcode ITS2 and determining variation among taxa by using SCoT polymorphism.

2. Materials and Methods

2.1. Taxonomy Study

2.1.1. Taxon Sampling and Collection of Plant Specimens

A total of 10 fresh and healthy samples of *Astragalus* were collected from different locations in Egypt during the period from February 2018 to May 2019. Detailed information of each sample is showed in Table 1.

Table 1. The studied taxa, collection details, and their sectional delimitation according to Podlech [39]. (*Astragalus* species arranged alphabetically).

No.	Studied Taxa	Abb.	Section	Sites of Collection	Collection Date
1	*Astragalus hauarensis* Boiss.	A.hau.1	Harpilobus Bge.	The Red Sea road, Kafer Homodyne (a distance 40 km from Safage to El-Quseir or before 20 km from El-Quseir).	May 2019
2	*Astragalus hauarensis Boiss.*	A.hau.2		The Red Sea road, El-Quseir before 60 km from Marsa-Alam.	May 2019
3	*Astragalus sieberi* DC.	A.sie.1	Chronopus Bge.	Matrouh road (before about 8 Km from El-Alamein or before about 52km from El-Dabaa Gate.	May 2018
4	*Astragalus sieberi* DC.	A.sie.2		Alexandria El-Alamein desert road, courage village at a distance 25 km after El-Alamein Gate.	March 2019
5	*Astragalus sieberi* DC.	A.sie.3		Wadi El-Natron El-Alamein desert before 65 km from the entrance to the El-Alamein.	March 2019
6	*Astragalus sieberi* DC.	A.sie.4		Matrouh road, North Coast, Aleamid direction.	March 2019
7	*Astragalus spinosus* (Forssk.) Muschl.	A.spi.1	Poterium Bge.	Matrouh road, North Coast, El-Alamein before El-Dabaa axis or after 40 km from El-Hammam.	May 2018
8	*Astragalus spinosus* (Forssk.) Muschl.	A.spi.2		Alexandria El-Alamein desert road, courage village at a distance 25 km after El-Alamein Gate.	March 2019
9	*Astragalus spinosus* (Forssk.) Muschl.	A.spi.3		Matrouh road, North Coast, El-Omeid direction.	March 2019
10	*Astragalus vogelii* (Webb) Bornm.	A.vog.1	Herpocaulos Bge.	The Red Sea road, Kafr Homodyne (a distance 40 km from safaga to El-Quseir or before 20 km from El-Quseir).	May 2019

2.1.2. Taxonomical Studies

The identification and taxonomy of all samples were carried out by the aid following relevant literature in floras of Egypt [21,22,38,39]. Moreover, the identification of plant materials was confirmed by matching them with images of type and non-type material on various websites (e.g., www.aluka.org; www.tropicos.org, http://coldb.mnhn.fr).

2.1.3. Morphological Studies

According to Täckholm, [21], and Boulos, [22,40,41], the Morphological Characters descriptions as habit, stem, rachis, leaves, pod, and seed were recorded in Table 2. For construction, a data matrix of computation that required recorded morphological characters for each specimen was coded as a double matrix expresses as absent (0) and present (1). The matrix was analyzed by PAST, version 3.18 software [42]. Distance estimates were performed by unweighted pair-group method analysis using arithmetic averages (UPGMA).

Table 2. The qualitative and quantitative investigations of the 35 Morphological characters and their codes for numerical analysis of all studied taxa.

Morphological Characters				
Organ	**Serial Number**	**Characters**	**State**	**Code**
Whole plant	1	Lifespan	Annual	0
			Perennial	1
	2	Life form	Herb	0
			Spiny shrub	1
	3	Succulence	Succulent	0
			Non-succulent	1
	4	Habit	Climbing	0
			Not climbing	1
	5	Conservation	Threatened	0
			Not threatened	1
	6	Spines	Absent	0
			Present	1
	7	Height (cm)	5–30	0
			40–60	1
stem	8	Habit	Erect	0
			Prostrate	1
	9	Surface	Smooth	0
			Rough	1
	10	Status	Winged	0
			Not winged	1
	11	Spines on internodes	Absent	0
			Present	1
	12	Tipped of lateral branches	Spiny	0
			Not spiny	1

Table 2. *Cont.*

Morphological Characters				
Organ	**Serial Number**	**Characters**	**State**	**Code**
Rachis	13	Detection	Absent	0
			Present	1
	14	Spines	Turned	0
			Not turned	1
Leaf stipules	15	Detection	Absent	0
			Present	1
	16	Adnation	Free	0
			Adnate	1
	17	Length (mm)	3 or less	0
			7 or less	1
	18	Shape	Triangle	0
			Lanceolate	1
	19	Apex	Acute	0
			Acuminate	1
leaf	20	Leaf midrib	Turned spines	0
			Not turned spines	1
	21	Length (cm)	6 or less	0
			10 or less	1
	22	Width (cm)	1 or more	0
			2 or more	1
Petal	23	Color	White	0
			Not white	1
Leaf or leaflet	24	shape	Elliptical-oblong	0
			Ovate-oblong	1
	25	Margin	Entire	0
			Not entire	1
	26	Status	Evergreen	0
			Deciduous	1
	27	Upper surface	Hairy	0
			Glabrous	1
	28	Lower surface	Hairy	0
			Glabrous	1
Pod	29	Texture	Hairy	0
			Glabrous	1
	30	Curvature	Curved	0
			Not curved	1
	31	Pedicle	Absent	0
			Stipitate	1
			Long	2

Table 2. *Cont.*

Morphological Characters				
Organ	**Serial Number**	**Characters**	**State**	**Code**
Seed	32	Length (cm)	0.4 or less	0
			0.5 or more	1
	33	Shape	Reniform	0
			Quadrate	1
	34	Surface	Smooth	0
			Irregular	1
	35	Color	Brown	0
			Yellow	1

2.2. Molecular Study

2.2.1. Genomic DNA Extraction, PCR and Sequencing

The total genomic DNA of *Astragalus* L. was extracted from fresh young leaves of plants with CTAB (Cetyl trimethyl ammonium bromide) lysis buffer [43–52]. Brief description to CTAB method (three-five sentences). The quality of extracted genomic DNA was checked by 1.5% agarose gel with ethidium bromide. The isolated DNA was stored at −20 °C for further analyses. Universal ITS2 and SCoT primers used are presented in Table 3. General PCR reaction, the total volume of ITS2 amplification was 20 µL, made up 10 µL of 2× PCR master mixture, 1 µL forward ITS2 primer, 1 µL reverse ITS2 primer (10 pmol), 1 µL genomic DNA, and 7 µL sterile distilled water, while the total volume of SCoT amplification was 20 µL, made up 10 µL 2× PCR master mixture, 0.5 µL for each primer separately, 2 µL genomic DNA, and 7.5 µl sterile distilled water. The PCR program consisted of 3 min at 94 °C for Initial denaturation followed by 37 cycles of 1 min at 94 °C for Denaturation, 30 s at 56 °C for Annealing ITS2 primers, 30 s at 50 °C for Annealing SCoT primers and 1 min at 72 °C for each extension step, followed by a final extension of 10 min at 72 °C. Amplicons were visualized by electrophoresis on 1.5% agarose gels. For ITS2 purified PCR products were sequenced in one direction using an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, Foster City, CA, USA).

Table 3. The universal primers of SCoT, ITS2 and their sequencing region.

Region.	Primer Name	Base Pair Primers (bp)	Primer Sequence (5′-3′)	Source
SCOT	SCOT 7	18 bp	ACAATGGCTACCACTGAC	Collard and Mackill, [37], Xiong et al., [38]
	SCOT 9	18 bp	ACAATGGCTACCACTGCC	
	SCOT 10	18 bp	ACAATGGCTACCACCAGC	
	SCOT 11	18 bp	ACAATGGCTACCACTACC	
	SCOT 14	18 bp	ACCATGGCTACCAGCGCG	
	SCOT 24	18 bp	CCATGGCTACCACCGCAG	
	SCOT 28	18 bp	CAACAATGGCTACCACCA	
	SCOT 32	18 bp	CAACAATGGCTACCACGC	
	SCOT 35	18 bp	AACCATGGCTACCACCAC	
	SCOT 46	18 bp	ACCATGGCTACCACCGCC	
ITS2	ITS2 2F	20 bp	ATGCGATACTTGGTGTGAAT	Chen et al., [53]
	ITS2 3R	21 bp	GACGCTTCTCCAGACTACAAT	Gao et al., [54]

2.2.2. Clustering Analysis for SCoT Analysis

The obtained visual PCR products with all the primers were scored. To minimize errors only clear, reproducible, and intense bands were scored. The marker bands were delimited by their molecular weights based on size standard. Amplified bands were recorded as (1)

to signify presence and absence of a band was recorded as (0) to form a binary matrix for all the samples. Data analysis was performed using the NTSYS-pc software version 2.1 [55]. Jaccard's similarity coefficients were used to generate a dendrogram using Unweighted Pair Group Method with Arithmetic Average (UPGMA) [56] and relationships between the samples were represented in the dendrogram.

3. Results

3.1. Morphological Data Analysis

Description of Morphological Characters is the first basic tool, and classified *Astragalus* species are shown in Table 4.

Table 4. Description of morphological characters of all studied taxa after revision.

No.	Studied Taxa	Lifespan	Life Form	Succulence	Habit	Conservation	Spines	Height (cm)
1	A.hau.1	Annual	Herb	Succulent	Not climbing	Not threatened	Absent	8–30
2	A.hau.2	Annual	Herb	Succulent	Not climbing	Not threatened	Absent	9–30
3	A.sie.1	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	18–40
4	A.sie.2	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	19–40
5	A.sie.3	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	22–40
6	A.sie.4	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	20–40
7	A.spi.1	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	20–60
8	A.spi.2	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	22–60
9	A.spi.3	Perennial	Spiny shrub	Non-succulent	Not climbing	Not threatened	Present	24–60
10	A.vog.1	Annual	Herb	Succulent	Not climbing	Not threatened	Absent	10–40

No.	Studied Taxa	Stem Characters					Leaf Stipule Characters	
		Habit	Surface	Status	Spines on Internodes	Tipped of Lateral Branches	Detection	Adnation
1	A.hau.1	Prostrate	Smooth	Not winged	Absent	Not winged	Present	Free
2	A.hau.2	Prostrate	Smooth	Not winged	Absent	Not winged	Present	Free
3	A.sie.1	Erect	Smooth	Not winged	Absent	Not winged	Present	Adnate
4	A.sie.2	Erect	Smooth	Not winged	Absent	Not winged	Present	Adnate
5	A.sie.3	Erect	Smooth	Not winged	Absent	Not winged	Present	Adnate
6	A.sie.4	Erect	Smooth	Not winged	Absent	Not winged	Present	Adnate
7	A.spi.1	Erect	Smooth	Not winged	Present	Not winged	Present	Adnate
8	A.spi.2	Erect	Smooth	Not winged	Present	Not winged	Present	Adnate
9	A.spi.3	Erect	Smooth	Not winged	Present	Not winged	Present	Adnate
10	A.vog.1	Prostrate	Smooth	Not winged	Absent	Not winged	Present	Free

No.	Studied Taxa	Leaf Stipules Characters			Rachis		Leaf Characters	
		Shape	Apex	Length (mm)	Detection	Spines	Length (cm)	width (cm)
1	A.hau.1	Triangle	Acute	3 or less	Present	Not turned	10 or less	1 or more
2	A.hau.2	Triangle	Acute	3 or less	Present	Not turned	10 or less	1 or more
3	A.sie.1	Lanceolate	Acuminate	7 or less	Present	Turned	10 or less	2 or more
4	A.sie.2	Lanceolate	Acuminate	7 or less	Present	Turned	10 or less	2 or more
5	A.sie.3	Lanceolate	Acuminate	7 or less	Present	Turned	10 or less	2 or more
6	A.sie.4	Lanceolate	Acuminate	7 or less	Present	Turned	10 or less	2 or more
7	A.spi.1	Triangle	Acute	5 or less	Present	Turned	5 or less	2 or more
8	A.spi.2	Triangle	Acute	5 or less	Present	Turned	5 or less	2 or more
9	A.spi.3	Triangle	Acute	5 or less	Present	Turned	5 or less	2 or more
10	A.vog.1	Triangle	Acuminate	3 or less	Present	Not turned	5 or less	1 or more

No.	Studied Taxa	Petal Color	Leaf Midrib Spines	Leaf or Leaflet Characters				
				Shape	Upper Surface	Lower Surface	Margin	Status
1	A.hau.1	White	Not turned	Elliptical	Hairy	Hairy	Entire	Evergreen
2	A.hau.2	White	Not turned	Elliptical	Hairy	Hairy	Entire	Evergreen
3	A.sie.1	Not white	Turned	Ovate	Glabrous	Hairy	Entire	Deciduous
4	A.sie.2	Not white	Turned	Ovate	Glabrous	Hairy	Entire	Deciduous
5	A.sie.3	Not white	Turned	Ovate	Glabrous	Hairy	Entire	Deciduous
6	A.sie.4	White	Turned	Ovate	Glabrous	Hairy	Entire	Deciduous
7	A.spi.1	White	Not turned	Ovate	Glabrous	Hairy	Entire	Deciduous
8	A.spi.2	White	Not turned	Ovate	Glabrous	Hairy	Entire	Deciduous
9	A.spi.3	White	Not turned	Ovate	Hairy	Hairy	Entire	Deciduous
10	A.vog.1	Not white	Not turned	Ovate	Hairy	Hairy	Entire	Deciduous

Table 4. *Cont.*

No.	Studied Taxa	Pod Characters			Seed Characters			
		Texture	Curvature	Pedicle	Length (cm)	Shape	Surface	Color
1	A.hau.1	Hairy	Curved	Absent	0.5 or less	Quadrate	Smooth	Brown
2	A.hau.2	Hairy	Curved	Absent	0.5 or less	Quadrate	Smooth	Brown
3	A.sie.1	Glabrous	Curved	Long	0.4 or less	Quadrate	Irregular	Brown
4	A.sie.2	Glabrous	Curved	Long	0.4 or less	Quadrate	Irregular	Brown
5	A.sie.3	Glabrous	Curved	Long	0.4 or less	Quadrate	Irregular	Brown
6	A.sie.4	Glabrous	Curved	Long	0.4 or less	Quadrate	Irregular	Brown
7	A.spi.1	Hairy	Not curved	Stipitate	0.4 or less	Reniform	Smooth	Brown
8	A.spi.2	Hairy	Not curved	Stipitate	0.4 or less	Reniform	Smooth	Brown
9	A.spi.3	Hairy	Not curved	Stipitate	0.4 or less	Reniform	Smooth	Brown
10	A.vog.1	Hairy	Not curved	Long	0.5 or less	Reniform	Irregular	Brown

The UPGMA dendrogram clustering algorithm generated from 35 morphological characters (Figure 1) displays that all studied taxa are divided into two major clusters and have an average taxonomic distance of about 4.9.

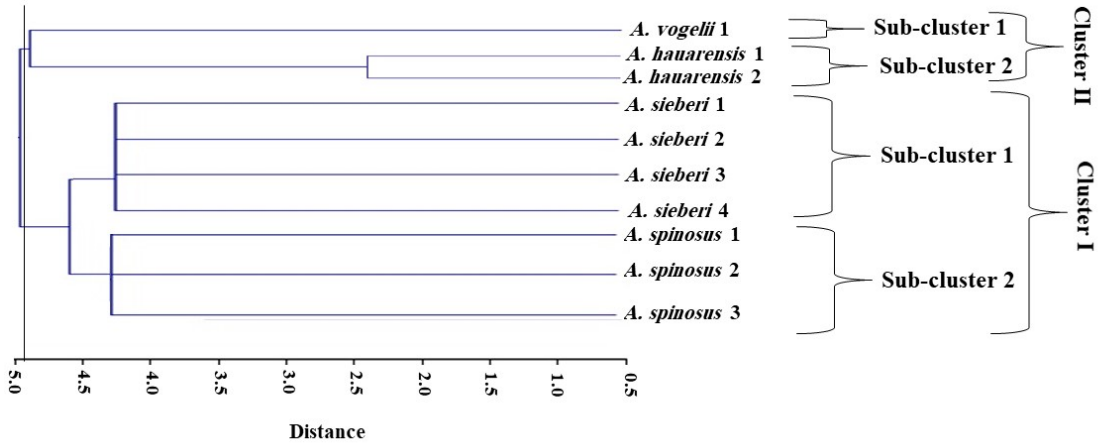

Figure 1. UPGMA dendrogram matrix illustrating the relationships and average taxonomic distance between *Astragalus* species based on 35 morphological characters.

At the 4.6 level, the first cluster (I) of two species is also delimited as two different taxa but appears to form one group. The first cluster (I) divided into two sub-clusters: sub-cluster 1 included the four samples representing *A. sieberi,* which separated at a distance level of about 4.21; while sub-cluster 2 comprised the three samples representing *A. spinosus* and separated at a distance close to 4.22 level.

At the 4.8 level, the second cluster (II) of two species is also delimited as two different taxa. The second cluster (II) divided into two sub-clusters: sub-cluster 1 included one sample representing *A. vogelii* and separated at a distance level of about 4.8; while sub-cluster 2 comprised the two samples representing *A. hauarensis* which separated at a distance level of about 2.4.

3.2. Molecular Data Analysis

3.2.1. DNA Extraction

The genomic DNA of plant samples was extracted by CTAB reagent and electrophoresed on agarose gel electrophoresis (Figure 2) of the tested 10 samples of 4 *Astragalus* species for PCR reaction in two regions both in the nucleus.

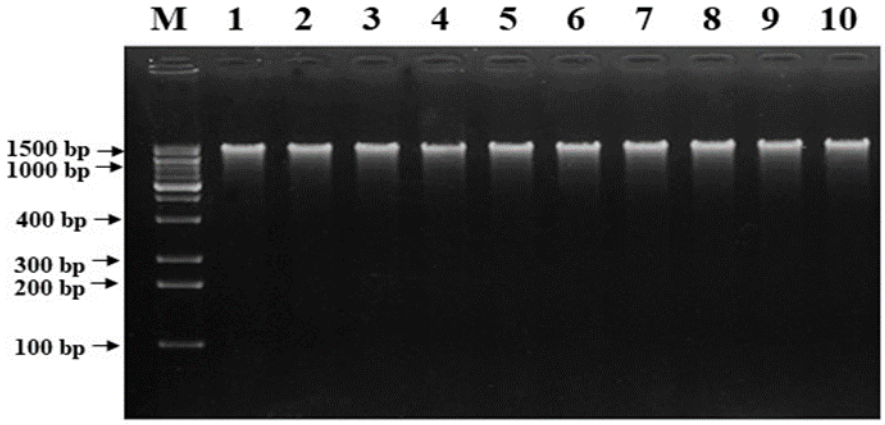

Figure 2. Agarose gel electrophoresis showing DNA extraction for 10 samples.

3.2.2. Amplification of SCoT Region

DNA fragments were amplified by utilizing ten primers that were selected to assess the difference among the samples. Under the same amplification conditions, all amplifications were found to be reproducible when repeated at different times. SCoT results were distinguished among each of the ten species of *Astragalus*. All primers which gave reproducible, clear, and intense bands were selected for analyzing all the ten samples. From 1 to 10 reproducible amplified fragments were observed; size varied in the range from 100 bp to 2000 bp as shown in Figures 3a–h and 4a,b.

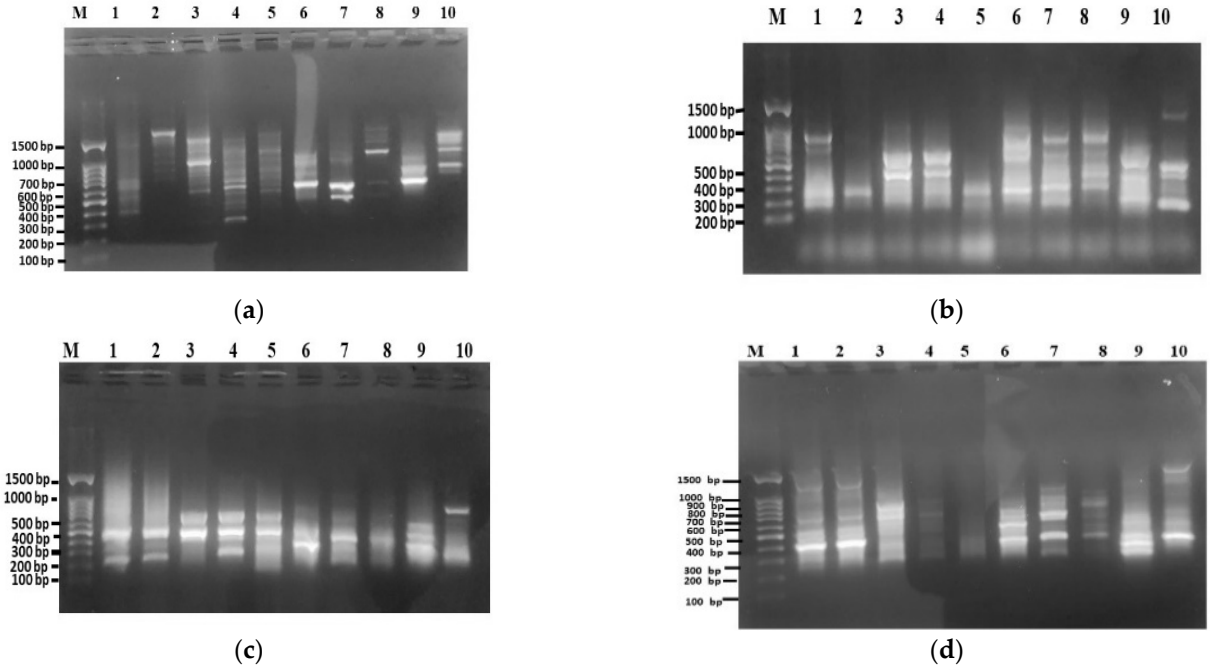

Figure 3. *Cont*.

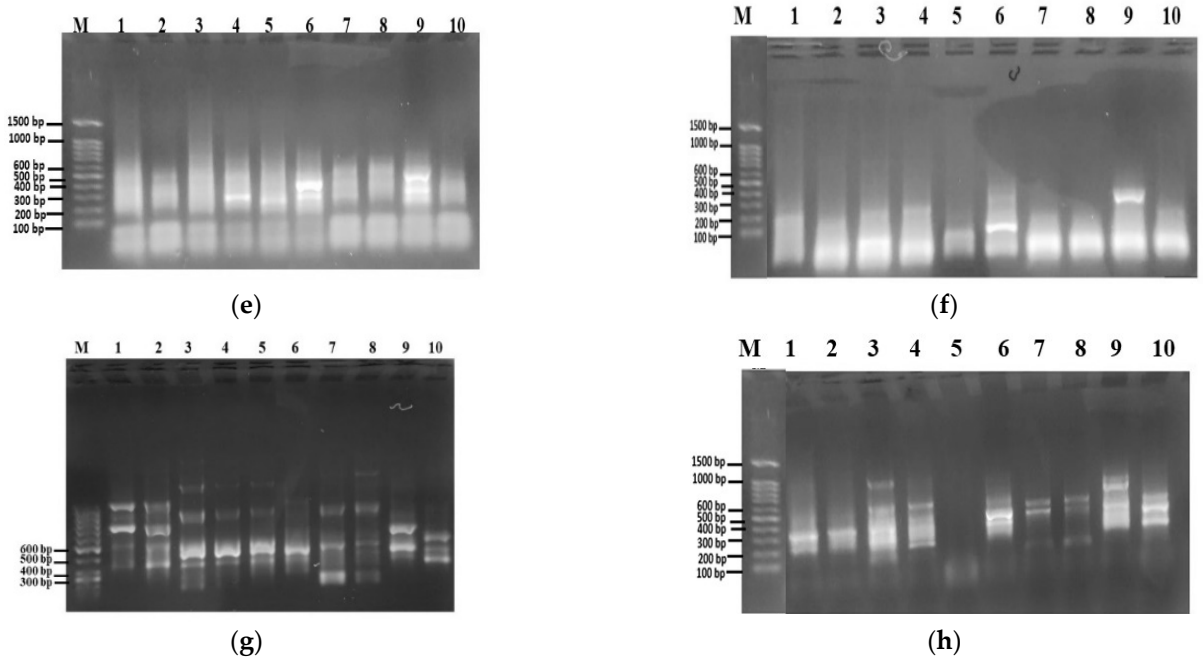

Figure 3. (**a**–**h**) Gel amplification profiles obtained typically with primers SCoT 7, 9, 10, 11, 14, 24, 28, and 32, respectively.

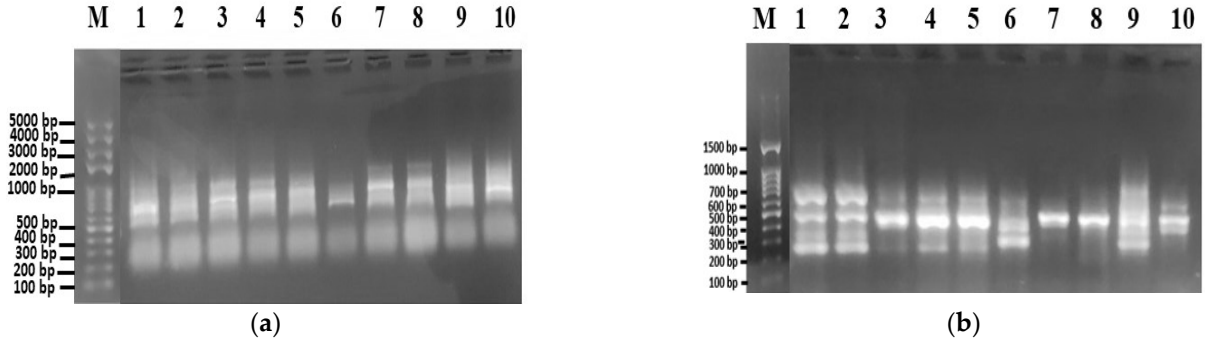

Figure 4. (**a**,**b**) Gel amplification profiles obtained typically with primers SCoT 35, 46, respectively.

3.2.3. Amplification of ITS Region Using Universal ITS2 Primers

The PCR conditions were optimized for ITS2 primers. The amplification reaction was done utilizing the universal primers of ITS region (ITS2) on 10 extracted DNA samples (Figure 5), the PCR amplicons of ITS2 regions for all samples given band size (500 bp–600 bp) on agarose gel.

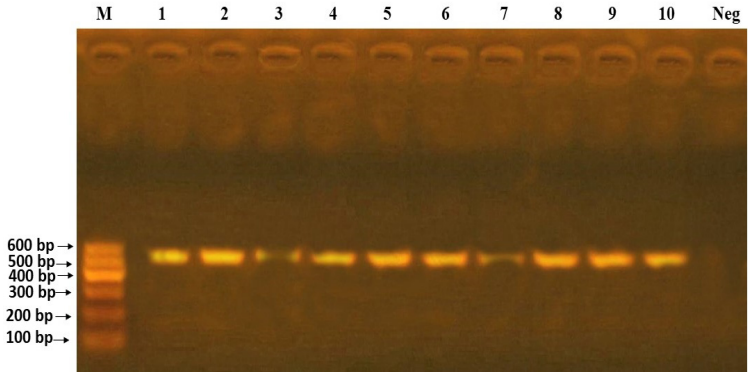

Figure 5. Agarose gel electrophoresis using ITS2 primers of the PCR product.

3.2.4. SCoT Polymorphism in Astragalus

All primers amplified clear and reproducible bands as dictated in Table 5. A total of 10 SCoT primers used in this investigation could produce specific bands to differentiate between 10 samples of *Astragalus* taxa and chosen for shown diversity studies which generated 212 DNA fragments with an average of 12.2 bands from 10 *Astragalus* samples, with 8 to 37 fragments per primer. The 212 fragments amplified were distributed as 2 monomorphic bands, 27 polymorphic without unique bands, and 183 unique bands (210 Polymorphic with unique bands). The primer SCoT 7 produced the highest number of 34 unique bands, SCoT 9, 11, 28, 35, 32, 46, 10, 14 produced 26, 21, 20, 17, 16, 15, 14, 13 bands respectively, whereas the primer SCoT 11 generated the lowest number of 7 unique bands.

Table 5. Data of SCoT primers and the extent of polymorphism. It included Monomorphic bands (M. morph.), Polymorphic (without unique bands) (P. morph. Without unique bands), Polymorphic (with unique bands) (P. morph. With unique bands), Total number of bands (TNB), Polymorphism ratio (%) (P. morphism ratio (%)), Mean of band frequency (MBF).

SI No.	Primer ID.	M. Morph. Bands	P. Morph. (without Unique Bands)	Unique Bands	P. Morph. (with Unique Bands)	TNB	P. Morphism Ratio (%)	MBF
1	Scot 7	0	3	34	37	37	100	0.11
2	Scot 9	0	2	26	28	28	100	0.11
3	Scot 10	0	3	14	17	17	100	0.12
4	Scot 11	0	5	21	26	26	100	0.13
5	Scot 14	0	0	13	13	13	100	0.10
6	Scot 24	1	0	7	7	8	87.5	0.21
7	Scot 28	0	6	20	26	26	100	0.12
8	Scot 32	0	3	16	19	19	100	0.12
9	Scot 35	0	2	17	19	19	100	0.11
10	Scot 46	1	3	15	18	19	94.7	0.17
	Total	2	27	183	210	212	-	-

The primers SCoT 7, 9, 11, and 28 gave the highest amplified number of DNA fragments of 37, 28, 26, and 26, respectively. The least number of DNA fragments showed in the primer SCoT 24 and 14 with 13 and 8 per primer. The values of the polymorphism ratio of each primer ranged from 87% to 100%. Eight primers, including SCoT 7, 9, 10, 11, 14, 28, 32, 35 had produced the same polymorphism ratio value 100%. The primer SCoT 46 and 24 had amplified 94.7% and 87.5% polymorphism respectively.

Generally, the MBF values of these 10 SCoT primers ranged from 0.10 to 0.21. SCoT 24 had the highest MBF value of 0.21, while SCoT 14 was the lowest MBF value of 0.10. A total of 10 SCoT primers had the ability to effectively differentiate between 10 *Astragalus* samples.

3.2.5. Clustering Analysis

The UPGMA dendrogram clustering algorithm was produced from 10 SCoT primers; at the genetic similarity coefficient of 2.7, the dendrograms of 10 samples were divided into two major clusters I, II (Figure 6) among the *Astragalus* species, with short index length of 0.9 to 9.6, and some clusters divided further into sub-clusters; additionally, some sub-clusters subdivided into groups. Two samples *A. hauarensis*, four samples *A. sieberi*, three samples *A. spinosus*, and one sample *A. vogelii were assigned* into cluster II and cluster I, respectively.

The SCoT analysis proposed that there was a clear genetic similarity between species. For example, the smallest similarity value (0.9) proposed the heigh variance among *A. sieberi, A. spinosus*, and *A. hauarensis*, and the maximum similarity value (9.6) was found between *A. sieberi, A. spinosus, A. hauarensis*, and *A. vogelii.* This showed that all four species of *Astragalus (A. sieberi, A. spinosus*, and *A. hauarensis)* were distinguished depending on a dendrogram constructed by using Jaccard's UPGMA. These results determined the efficiency of SCoT markers in identifying polymorphism between *A. vogelii,*

A. sieberi, *A. spinosus*, and *A. hauarensis* and successfully determined genetic relationships between species.

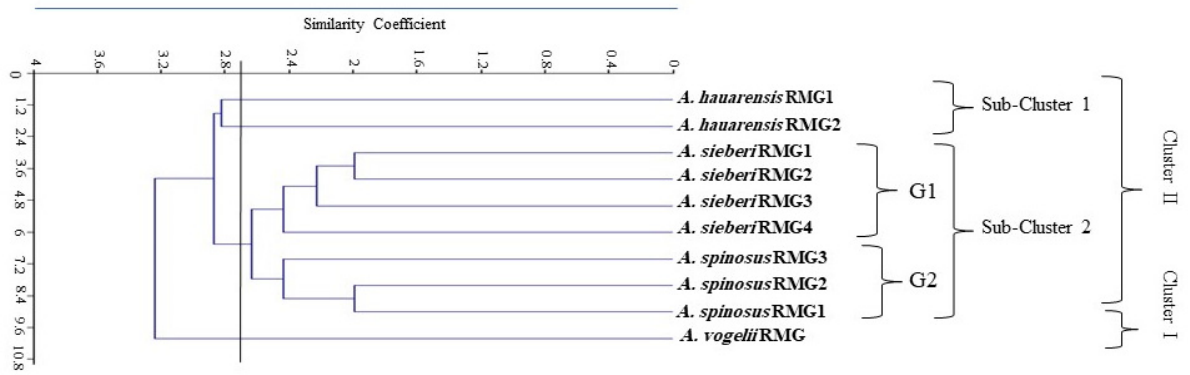

Figure 6. UPGMA dendrogram matrix illustrating the relationship between *Astragalus* specimens based on SCoT marker.

3.2.6. Molecular Identification of Specimens by Using ITS2 Gene Sequence

ITS2 gene sequence was used to identify plants by using BLAST searched on NCBI database. Afterwards, analyzing the chromatogram for ITS region, 10 samples have been identified as two samples representing *A. hauarensis*, four samples representing *A. sieberi*, three samples representing *A. spinosus,* and one sample representing *A. vogelii*.

3.2.7. Sequences Submission

Based on the ITS barcode, *A. hauarensis* RMG1, *A. hauarensis* RMG2, *A. sieberi* RMG1, *A. sieberi* RMG2, *A. sieberi* RMG3, *A. sieberi* RMG4, *A. spinosus* RMG1, *A. spinosus* RMG2, *A. spinosus* RMG3, *A. vogelii* RMG were deposited into GenBank with accession # MT367587.1, MT367591.1, MT367593.1, MT367585.1, MT367586.1, MT367588.1, MT160347.1, MT367590.1, MT367589.1, MT367592.1, respectively (Table 6).

Table 6. The GenBank accession number of the submitted ITS2 region.

NO.	Studied Taxa	Region	Length/b	Accession Number
1	*Astragalus hauarensis* RMG1	ITS2	434	MT367587.1
2	*Astragalus hauarensis* RMG2		431	MT367591.1
3	*Astragalus sieberi* RMG1		426	MT367593.1
4	*Astragalus sieberi* RMG2		433	MT367585.1
5	*Astragalus sieberi* RMG3		427	MT367586.1
6	*Astragalus sieberi* RMG4		445	MT367588.1
7	*Astragalus spinosus* RMG1		421	MT160347.1
8	*Astragalus spinosus* RMG2		428	MT367590.1
9	*Astragalus spinosus* RMG3		443	MT367589.1
10	*Astragalus vogelii* RMG		420	MT367592.1

3.2.8. Comparison between Results of Morphological Identification and ITS2 Identification

From Table 7, it is clear the BLAST results of ITS2 sequence were near to the morphological identification of chosen genus *Astragalus*. Finally, results from sequences and constructed phylogenetic of the sequence ensure the morphological identification.

Table 7. BLAST Database search match for similarities using ITS2 gene sequence.

NO.	Morphologically Identification after Reinvestigation	BLAST Search Match Identified as	BLAST Similarity (%)	Sequence Submission
1	*Astragalus hauarensis* 1	*Astragalus hauarensis*	97.51	*Astragalus hauarensis* RMG1
2	*Astragalus hauarensis* 2	*Astragalus hauarensis*	96.60	*Astragalus hauarensis* RMG2
3	*Astragalus sieberi* 1	*Astragalus sieberi*	96.76	*Astragalus sieberi* RMG1
4	*Astragalus sieberi* 2	*Astragalus sieberi*	96.76	*Astragalus sieberi* RMG2
5	*Astragalus sieberi* 3	*Astragalus sieberi*	97.70	*Astragalus sieberi* RMG3
6	*Astragalus sieberi* 4	*Astragalus sieberi*	99.31	*Astragalus sieberi* RMG4
7	*Astragalus spinosus* 1	*Astragalus spinosus*	89.28	*Astragalus spinosus* RMG1
8	*Astragalus spinosus* 2	*Astragalus spinosus*	94.66	*Astragalus spinosus* RMG2
9	*Astragalus spinosus* 3	*Astragalus spinosus*	96.70	*Astragalus spinosus* RMG3
10	*Astragalus vogelii*	*Astragalus vogelii*	95.30	*Astragalus vogelii* RMG

4. Discussion

Traditional identification methods, such as morphological characters and microscopic methods, are restricted by the deficiency of clear criteria for character selection, lacking the uniform standard and credible data or coding and thus, mainly based on subjective assessments that these methods easily caused misidentification [29].

Therefore, this study aimed to use DNA barcode and molecular marker with fresh health specimens to identify and find the phylogenetic relationships between closely related taxa and their effect on their morphological established identification. In total, 4 species (10 samples) of *Astragalus* were collected from different localities in Egypt. They comprised 2 annual herbaceous species and 2 perennial spiny species, and this classification is in agreement with Täckholm, [21], and Boulos, [22,40,41]. Morphological characters, numerical taxonomy, and genetic diversity are of great significance for taxonomic studies.

This study explains the output of the UPGMA dendrogram clustering algorithm using 35 morphological characters that indicated a strong relationship between ten samples and categorized the ten samples in two clusters. *Astragalus sieberi* of section Chronopus Bge. and *A. spinosus* of section Poterium Bge. separated together in one cluster I and appeared to form one group. The delimitation of these taxa was characterized by perennial, erect stem, and shrubly habit with persistent spines. *A. sieberi* and *A. spinosus* were grouped as described by Ahmed and Mohamed, [57], and Sharawy, [58], depending on the morphological and anatomical characters. *A. sieberi* and *A. spinosus* were separated into two sub-clusters. This result is in agreement with Badr and Sharawy, [59]. *A. vogelii* and *A. hauarensis* were separated together in a distinct cluster II. The delimitation of these taxa were characterized by present and free leaf stipules, prostrate, smooth, and not winged stem. *A. vogelii* separated in one sub-cluster 1 lonely and *A. hauarensis* separated in one sub-cluster 2 depended on vegetative morphological and anatomical characters in agreement with Sharawy, [58]. *A. vogelii* and *A. hauarensis* were delimited as two entities (sub-cluster 1 and sub-cluster 2, respectively), being featured from all other taxa. The assignment of these taxa to different sections is in agreement with their delimitation according to Podlech, [39]. Moreover, *A. vogelii* is clearly distinguished from all other species as confirmed by all the analyses.

The Plant Working Group (CBOL), [60] pointed out that ideal plant DNA barcode must have enough conserved regions for universal primer design, high efficiency of PCR amplification and sufficient variability to be utilized for identification of species as mentioned by Hebert et al., [33], and Cowan et al., [61]. All sequences from ITS2 are blasted on NCBI website BLAST nucleotide tool to ascertain that the species belong to *Astragalus* can be summarized as follows: *Astragalus hauarensis* 1 was 97.51% identical to *Astragalus hauarensis*, *Astragalus hauarensis* 2 was 96.60% identical to *Astragalus hauarensis*, *Astragalus sieberi* 1 was 96.76% identical to *Astragalus sieberi, Astragalus sieberi* 2 was 96.76% identical to *Astragalus sieberi*, *Astragalus sieberi* 3 was 97.70% identical to *Astragalus sieberi*, *Astragalus sieberi* 4 was 99.31% identical to *Astragalus sieberi*, *Astragalus spinosus* 1 was 89.28% identical

to *Astragalus spinosus*, *Astragalus spinosus* 2 was 94.66% identical to *Astragalus spinosus*, *Astragalus spinosus* 3 was 96.70% identical to *Astragalus spinosus*, and *Astragalus vogelii* was 95.30% identical to *Astragalus vogelii*. The phylogenetic trees proved *Astragalus* species are monophyletic genera and also indicated by previous studies Wojciechowski et al., [62] and Kazempour Osaloo et al., [35,36].

SCoT markers are highly reproducible due to the use of longer primers and indicated the powerful nature of these SCoT markers. SCoT is a novel marker system and preferentially reveals polymorphisms because the primers were designed to amplify from the short-conserved region surrounding the ATG translation start codon as reported by Xiong et al., [38], Collard and Mackill, [37], and Mulpuri et al., [63].

In the current study, ten SCoT primers generated 212 DNA fragments with an average of 12.2 bands from ten *Astragalus* samples, with 8 to 37 fragments per primer. The 212 fragments amplified were distributed as 2 monomorphic bands, 27 polymorphic without unique bands, and 183 unique bands (210 Polymorphic with unique bands). Besides, the results of SCoT analysis proposed that there was a clear genetic similarity between species. For example, the smallest similarity value (0.9) proposed the heigh variance among *A. sieberi*, *A. spinosus,* and *A. hauarensis,* and the maximum similarity value (9.6) was found between *A. sieberi*, *A. spinosus*, *A. hauarensis,* and *A. vogelii*. These results showed a certain connection with the geographical origin and genetically related species between four species of *Astragalus* (*A. sieberi*, *A. spinosus*, and *A. hauarensis*) and were distinguished depending on a dendrogram constructed through using Jaccard's UPGMA. SCoT markers successfully evaluated the genetic relationships and revealed a high percentage of polymorphism between the *Astragalus* species included in this study.

5. Conclusions

(I) To do morphological studies, taxa should be collected in flowering seasons; (II) awareness of the degree of ITS2 region and SCoT primers sequence divergence between *Astragalus* species was useful to demonstrate the phylogenetic relationship, especially at the generic level; (III) sequence divergence was higher within the species *Astragalus* that resulted when the ITS2 region was analyzed; (IV) phylogenetic analysis using MEGA 0.7 separation at the section level is very clear for the genus *Astragalus*; (V) SCoT markers were efficient in identifying polymorphism and successfully determined genetic relationships between *Astragalus* species.

Author Contributions: M.M.A.E.-G. designed and proposed the work, A.S.A.E.-S., A.M., H.N., and A.K. supervised this work, R.R. did the actual experimental work. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All datasets generated or analyzed during this study are included in the manuscript.

Acknowledgments: Our great appreciations to Marwa M. El-Demerdash, for checking the bioinformatic analyses of the plants ITS sequences at the Enzymology and Fungal Biotechnology Lab, Botany and Microbiology Department, Faculty of Science, Zagazig University.

Conflicts of Interest: The authors declare there are no conflict of interest.

Ethics Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Mabberley, D.J. *The Plant-Book: A Potable Dictionary of the Vascular Plants*, 2nd ed.; Cambridge University Press: Cambridge, UK, 1997; p. 858.
2. Haerinasab, M.; Rahiminejad, M. A taxonomic revision of the genus *Trifolium* L. sect. *Fragifera* Koch (Fabaceae) in Iran. *Iran. J. Bot.* **2012**, *18*, 22–30.

3. Ceter, T.; Karaman, S.; Aytac, Z.; Baser, B. Pollen morphology of the genus *Oxytropis* in Turkey. *Bangladesh J. Bot.* **2013**, *42*, 167–174. [CrossRef]
4. Sheikh, N.; Desai, T.; Patel, R. Pharmacognostic Evaluation of *Melilotus officinalis* Linn. *Pharmacognjournal* **2016**, *8*, 239–242. [CrossRef]
5. Wang, L.; Grusak, M. Structure and development of *Medicago truncatula* pod wall and seed coat. *Ann. Bot.* **2005**, *95*, 737–747. [CrossRef]
6. Magallón, S.A.; Sanderson, M.J. Absolute diversification rates in angiosperm clades. *Evolution* **2001**, *55*, 1762–1780. [CrossRef]
7. Judd, W.S.; Campbell, C.S.; Kellogg, E.A.; Stevens, P.F.; Donoghue, M.J. Plant systematics: A phylogenetic approach. *Sinauer Axxoc.* **2002**, *2*, 287–292.
8. Rendle, A.B. *Classification of Flowering Plants*, 2nd ed.; Institute of Terrestrial Ecology: Cambridge, UK, 1952; pp. 348–370.
9. Willis, J.C. *A Dictionary of the Flowering Plants and Ferns*, 7th ed.; Cambridge University Press: London, UK, 1967; pp. 371–375.
10. Burnham, R.J.; Johnson, K.R. South American paleobotany and the origins of neotropical rainforests. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **2004**, *359*, 1595–1610. [CrossRef]
11. Lewis, G.P.; Schrire, B.D.; Mackinder, B.A.; Lock, M. *Legumes of the World*; Royal Botanic Gardens: Kew, India, 2005; pp. 475–481.
12. Maassoumi, A.A. *Astragalus L. in the Old World, Check-List*; Research Institute of Forests and Rangeland: Tehran, Iran, 1998; p. 618.
13. Maassoumi, A.A. The genus *Astragalus* in Iran. *Tehran* **2005**, *5*, 785.
14. Taeb, F.; Zarre, S.; Podlech, D.; Tillich, H.; Kazempour Osaloo, S.; Maassoumai, A.A. A contribution to the phylogeny of annual species of *Astragalus* (Fabaceae) in the Old-World using hair micromorphology and other morphological characters. *Feddes Repert.* **2007**, *118*, 206–227. [CrossRef]
15. IPNI. *The International Plant Names Index*; Royal Botanic Gardens: Kew, India, 2019.
16. Lock, M.; Schrire, B.D. Galegeae. In *Legumes of the World*; Lewis, G.P., Schrire, B.D., Mackinder, B.A., Lock, M., Eds.; Royal Botanic Gardens: Kew, India, 2005; pp. 475–481.
17. Sanderson, M.J.; Wojciechowski, M.F. Improved bootstrap confidence limits in large-scale phylogenies with an example from Neo-Astragalus (Leguminosae). *Syst. Biol.* **2000**, *49*, 671–685. [CrossRef]
18. Podlech, D. The genus *Astragalus* L. (Fabaceae) in Europe with exclusion of the former Soviet Union. *Feddes Repert.* **2008**, *119*, 310–387. [CrossRef]
19. Bidarlord, M.; Ghahremaninejad, F.; Maassoumi, A.A. A new species of the genus *Astragalus* (Leguminosae) from Northwest Iran. *Phytotaxa* **2016**, *252*, 280–284. [CrossRef]
20. Wojciechowski, M.F. *Astragalus* (Fabaceae): A molecular phylogenetic perspective. *Brittonia* **2005**, *57*, 382–396. [CrossRef]
21. Taeckholm, V. *Students' flora of Egypt*, 2nd ed.; Cairo University: Cairo, Egypt, 1974; pp. 261–271.
22. Boulos, L. Flora of Egypt. In *Azollacae-Oxalidacae*; Al-Hadara Publishing: Cairo, Egypt, 1999; Volume 1, pp. 320–336.
23. Norton, J.; Abdul Majid, S.; Allan, D.G.; Alsafran, M.; Boer, B.; Richer, R. *An Illustrated Checklist of the Flora of Qatar*; Browndown Publications: Gosport, UK, 2009.
24. Chaudhary, S.A. *Flora of the Kingdom of Saudi Arabia*; Ministry of Agriculture: Riyadh, Saudi Arabia, 2000.
25. Alshammari, A.M.; Sharawy, S.M. Wild plants diversity of the Hema Faid Region (Ha'il Province, Saudi Arabia). *Asian J. Plant Sci.* **2010**, *9*, 447–454. [CrossRef]
26. Llewellyn, O.A.; Hall, M.; Miller, A.G.; Al-Abbasi, T.M.; Al-Wetaid, A.H.; Al-Harbi, R.J.; Al-Shammari, K.F. Important plant areas in the Arabian Peninsula: 4. Jabal Aja. *Edinb. J. Bot.* **2011**, *68*, 199–224. [CrossRef]
27. Ghahremaninejad, F.; Joharchi, M.R. 840th species of genus *Astragalus* (Fabaceae) for the flora of Iran from Khorassan Province as a new record: A. globiceps Bunge. *J. Plant Res.* **2020**, *32*, 906–910.
28. Cai, Z.H.; Li, P.; Dong, T.T.X.; Tsim, K.W.K. Molecular diversity of 5S-rRNA spacer domain in *Fritillaria* species revealed by PCR analysis. *Planta Medica* **1999**, *65*, 360–364. [CrossRef]
29. Rindi, F.; Guiry, M.D.; López-Bautista, J.M. Distribution, morphology, and phylogeny of Klebsormidium (Klebsormidiales, Charophyceae) in urban environments in Europe. *J. Phycol.* **2008**, *44*, 1529–1540. [CrossRef]
30. Dangi, R.S.; Lagu, M.D.; Chaudhary, L.B.; Ranjekar, P.K.; Gupta, V.S. Assessment of genetic diversity in *Trigonella foenum-graecum* and *Trigonella caerulea* using ISSR and RAPD marker. *BMC Plant Biol.* **2004**, *4*, 13. [CrossRef]
31. Yip, P.Y.; Kwan, H.S. Molecular identification of *Astragalus membranaceus* at the species and locality levels. *J. Ethnopharmacol.* **2004**, *106*, 222–229. [CrossRef]
32. Paul, B. ITS1 region of the rDNA of *Pythium megacarpum* sp. Nov., its taxonomy, and its comparison with related species. *FEMS Microbiol. Lett.* **2000**, *186*, 229–233. [CrossRef] [PubMed]
33. Hebert, P.D.; Cywinska, A.; Ball, S.L. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B Biol. Sci.* **2003**, *270*, 313–321. [CrossRef] [PubMed]
34. Hebert, P.D.; Penton, E.H.; Burns, J.M.; Janzen, D.H.; Hallwachs, W. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14812–14817. [CrossRef] [PubMed]
35. Kazempour Osaloo, S.; Maassoumi, A.A.; Murakani, N. Molecular systematics of the genus *Astragalus* L. (Fabaceae): Phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene ndhF sequences. *Plant Syst. Evol.* **2003**, *242*, 1–32. [CrossRef]
36. Kazempour Osaloo, S.; Maassoumi, A.A.; Murakami, N. Molecular systematics of the old world *Astragalus* (Fabaceae) as inferred from nrDNA ITS sequence data. *Brittonia* **2005**, *57*, 367–381. [CrossRef]

37. Collard, B.C.Y.; Mackill, D.J. Start Codon Targeted (SCoT) polymorphism: A simple, novel DNA marker technique for generating gene-targeted markers in plants. *Mol. Biol. Rep.* **2009**, *27*, 86–93. [CrossRef]
38. Xiong, F.Q.; Tang, R.H.; Chen, Z.L.; Pan, L.H.; Zhuang, W.J. SCoT: A novel gene-targeted marker technique based on the translation start codon. *Mol. Plant Breed* **2009**, *7*, 635–638.
39. Podlech, D. The systematics of annual species of the genus *Astragalus* L. (Leguminosae). *Flora et. Veg. Mundi* **1991**, *9*, 1–18.
40. Boulos, L. Flora of Egypt. In *Geraniaceae-Boraginaceae*; Al-Hadara Publishing: Cairo, Egypt, 2000; Volume 2, p. 352.
41. Boulos, L. Flora of Egypt. In *Verbenaceae Compositae*; Al Hadara Publishing: Cairo, Egypt, 2002; Volume 3, p. 373.
42. Hammer, Ø.; Harper, D.A.T.; Ryan, P.D. PAST: Paleontological statistics. *Cessadoem* **2001**, *25*, 2009. Available online: www.uv.es/~{}pardomv/pe/2001_1/past/pastprog/past.pdf (accessed on 20 June 2001).
43. Padmalatha, K.; Prasad, M.N.V. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *Afr. J. Biotechnol.* **2006**, *5*, 230–234.
44. El-Sayed, A.S.A.; Mohamed, N.M.; Safan, S.; Yassin, M.A.; Shaban, L.; Shindia, A.; Ali, G.S.; Sitohy, M. Restoring the biosynthetic machinery of taxol of *Aspergillus terreus via* cocultivation with the endophytic microbiome of *Podocarpus gracilior* Pilger. *Sci. Rep.* **2019**, *9*, 11534. [CrossRef]
45. El-Sayed, A.S.A.; Ali, G.A. *Aspergillus flavipes* is a novel efficient biocontrol agent of Phytophthora parasitica. *Biol. Control* **2020**, *140*, 104072. [CrossRef]
46. El-Sayed, A.S.A.; Yassin, M.A.; Ali, G.S. Transcriptional and proteomic profiling of *Aspergillus flavipes* in response to sulfur starvation. *PLoS ONE* **2015**, *10*, e0144304. [CrossRef]
47. El-Sayed, A.S.A.; Safan, S.; Mohamed, N.Z.; Shaban, L.; Ali, G.S.; Sitohy, M.Z. Induction of Taxol biosynthesis by *Aspergillus terreus*, endophyte of Podocarpus gracilior Pilger, upon intimate interaction with the plant endogenous microbes. *Process Biochem.* **2018**, *71*, 31–40. [CrossRef]
48. El-Sayed, A.S.A.; Akbar, A.; Iqrar, I.; Ali, R.; Norman, D.; Brennan, M.; Ali, G.S. A Glucanolytic *Pseudomonas* sp. associated with Smilax bona-nox L. displays strong activity against Phytophthora parasitica. *Microbiol. Res.* **2018**, *207*, 140–152. [CrossRef] [PubMed]
49. El-Sayed, A.S.A.; Ruff, L.E.; Ghany, S.E.A.; Ali, G.S.; Esener, S. Molecular and Spectroscopic Characterization of *Aspergillus flavipes* and *Pseudomonas putida* L-Methionine γ-Lyase in Vitro. *Appl. Biochem. Biotechnol.* **2017**, *181*, 1513–1532. [CrossRef]
50. El-Sayed, M.T.; El-Sayed, A.S.A. Biocidal Activity of Metal Nanoparticles Synthesized by *Fusarium solani* against Multidrug-Resistant Bacteria and Mycotoxigenic Fungi. *J. Microbiol. Biotechnol.* **2020**, *30*, 226–236. [CrossRef]
51. El-Sayed, A.S.A.; Shindia, A.A.; Zeid, A.A.A.; Yassin, A.M.; Sitohy, M.Z.; Sitohy, B. *Aspergillus nidulans* thermostable arginine deiminase-Dextran conjugates with enhanced molecular stability, proteolytic resistance, pharmacokinetic properties and anticancer activity. *Enzym. Microbiol. Technol.* **2019**, *131*, 109432. [CrossRef]
52. El-Sayed, A.S.A.; George, N.M.; Bolbol, A.A.; Mohamed, M.S. Purification and biochemical characterization of *Aspergillus terreus* ornithine decarboxylase: Curcumin is a potent enzyme inhibitor. *Molecules* **2019**, *24*, 2756. [CrossRef] [PubMed]
53. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* **2010**, *5*, e8613. [CrossRef]
54. Gao, T.; Yao, H.; Ma, X.Y.; Zhu, Y.J.; Song, J.Y. Identification of *Astragalus* plants in China using the region ITS2. *World Sci. Technol. Mod. Tradit. Chin. Med. Mater. Med.* **2010**, *12*, 222–227.
55. Rohlf, F.J. NTSYS-pc. In *Numerical Taxonomy and Multivariate Analysis System, Version 2.10*; Exeter Software: New York, NY, USA, 2002.
56. Sneath, P.H.A.; Sokal, R.R. *Numerical Taxonomy*; W. H. Freeman and Company: San Francisco, CA, USA, 1973.
57. Ahmed, K.; Mohamed, A. Comparative anatomical studies on some species of *Astragalus* growing in Egypt. *Ann. Agric. Sci.* **1988**, *33*, 745–760.
58. Sharawy, S.M. Taxonomic Studies on Interspecific and Intraspecific Relationships in the Genus *Astragalus* in Egypt. Ph.D. Thesis, Ain Shams University Cairo, Cairo, Egypt, 2001.
59. Badr, A.; Sharawy, S.M. Karyotype analysis and systematic relationships in the Egyptian *Astragalus* L. (Fabaceae). *Int. J. Bot.* **2007**, *3*, 147–159. [CrossRef]
60. CBOL (Consortium of Barcode of Life). A DNA barcode for land plants. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12794–12797. [CrossRef]
61. Cowan, R.S.; Chase, M.W.; Kress, W.J.; Savolainen, V. 300,000 species to identify problems, progress, and prospects in DNA barcoding of land plants. *Taxon* **2006**, *55*, 611–616. [CrossRef]
62. Wojciechowski, M.F.; Sanderson, M.J.; Baldwin, B.G.; Donoghue, M.J. Monophyly of aneuploid *Astragalus* (Fabaceae): Evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *Am. J. Bot.* **1993**, *80*, 711–722. [CrossRef]
63. Mulpuri, S.; Muddanuru, T.; Francis, G. Start codon targeted (SCoT) polymorphism in toxic and non-toxic accessions of *Jatropha curcas* L. and development of a codominant SCAR marker. *Plant Sci.* **2013**, *207*, 117–127. [CrossRef]