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

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Abstract: Astragalus L. is one of the largest angiosperm complex genera that belongs to the family Fabaceae, subfamily Papilionoideae or Faboideae under the subtribe Astragaliinae 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. vogelli. 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

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. (“Gaævæn” 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, \textit{rpoC1}, and \textit{rpoC2} have been used in \textit{Astragalus} \textit{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 \textit{Astragalus} plants, the numerical taxonomy utilized to explain genetic relationships between these species, reconfirming the identification of \textit{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 \textit{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]. (\textit{Astragalus} species arranged alphabetically).

| No. | Studied Taxa                     | Abb. | Section                     | Sites of Collection                                                                 | Collection Date |
|-----|----------------------------------|------|-----------------------------|------------------------------------------------------------------------------------|-----------------|
| 1   | \textit{Astragalus hauarensis} Boiss. | A.hau.1 | Harpilobus Bge.          | The Red Sea road, Kafer Homodyne (a distance 40 km from Safaga to El-Quseir or before 20 km from El-Quseir). | May 2019        |
| 2   | \textit{Astragalus hauarensis} Boiss. | A.hau.2 |                             | The Red Sea road, El-Quseir before 60 km from Marsa-Alam.                             | May 2019        |
| 3   | \textit{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   | \textit{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   | \textit{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   | \textit{Astragalus sieberi} DC. | A.sie.4 |                             | Matrouh road, North Coast, Aleamid direction.                                        | March 2019      |
| 7   | \textit{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   | \textit{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   | \textit{Astragalus spinosus} (Forssk.) Muschl. | A.spi.3 |                             | Matrouh road, North Coast, El-Omeid direction.                                       | March 2019      |
| 10  | \textit{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                        | 1     | Lifespan      |            | Annual  | 0    |
|                          |       |               |            | Perennial | 1    |
| 2                        | 2     | Life form     |            | Herb    | 0    |
|                          |       |               |            | Spiny shrub | 1    |
| 3                        | 3     | Succulence    |            | Succulent | 0    |
|                          |       |               |            | Non-succulent | 1    |
| 4                        | 4     | Habit         |            | Climbing | 0    |
|                          |       |               |            | Not climbing | 1    |
| 5                        | 5     | Conservation  |            | Threatened | 0    |
|                          |       |               |            | Not threatened | 1    |
| 6                        | 6     | Spines        |            | Absent   | 0    |
|                          |       |               |            | Present  | 1    |
| 7                        | 7     | Height (cm)   |            | 5–30     | 0    |
|                          |       |               |            | 40–60    | 1    |
| stem                     |       |               |            |          |      |
| 8                        | 8     | Habit         |            | Erect    | 0    |
|                          |       |               |            | Prostrate | 1    |
| 9                        | 9     | Surface       |            | Smooth   | 0    |
|                          |       |               |            | Rough    | 1    |
| 10                       | 10    | Status        |            | Winged   | 0    |
|                          |       |               |            | Not winged | 1    |
| 11                       | 11    | Spines on internodes | Absent   | 0    |
|                          |       |               |            | Present  | 1    |
| 12                       | 12    | Tipped of lateral branches | Spiny   | 0    |
|                          |       |               |            | Not spiny | 1    |
Table 2. Cont.

| 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    |
| Leaf          | 17            | Length (mm)      | 3 or less | 0    |
|                |               |                  | 7 or less | 1    |
|                | 18            | Shape            | Triangle  | 0    |
|                |               |                  | Lanceolate| 1    |
|                | 19            | Apex             | Acute     | 0    |
|                |               |                  | Acuminate | 1    |
| Petal         | 20            | Leaf midrib      | Turned spines | 0    |
|                |               |                  | Not turned spines | 1    |
| leaf          | 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    |
|                | 24            | shape            | Elliptical-oblong | 0    |
|                |               |                  | Ovate-oblong | 1    |
| Leaf or leaflet | 25         | Margin           | Entire    | 0    |
|                |               |                  | Not entire| 1    |
| Leaf or       | 26            | Status           | Evergreen | 0    |
| leaflet       |               |                  | 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.

| 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                  | ACAATGGCTACCACCTGAC      | Collard and Mackill, [37], Xiong et al., [38] |
|        | SCOT 9      | 18 bp                  | ACAATGGCTACCACCTGCC      |        |
|        | SCOT 10     | 18 bp                  | ACAATGGCTACCACCCAGC      |        |
|        | SCOT 11     | 18 bp                  | ACAATGGCTACCACCTACC     |        |
|        | SCOT 14     | 18 bp                  | ACCATGGCTACCACCGCG      |        |
|        | SCOT 24     | 18 bp                  | CCATGGCTACCACCGAGC      |        |
|        | SCOT 28     | 18 bp                  | CAACAATGGCTACCACCA       |        |
|        | SCOT 32     | 18 bp                  | CAACAATGGCTACCACGC      |        |
|        | SCOT 35     | 18 bp                  | AACCATGGCTACCACCA       |        |
|        | SCOT 46     | 18 bp                  | ACCATGGCTACCACCGCC      |        |
| ITS2   | ITS2 2F     | 20 bp                  | ATGCGATACCTTGTGTGAAT     | Chen et al., [53] |
|        | ITS2 3R     | 21 bp                  | GACGGCTTCTCCAGACTACAAT   | 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 | 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 turned | Present | Adnate   |
| 4   | A.sie.2      | Erect | Smooth | Not winged | Absent | Not turned | Present | Adnate   |
| 5   | A.sie.3      | Erect | Smooth | Not winged | Absent | Not turned | Present | Adnate   |
| 6   | A.sie.4      | Erect | Smooth | Not winged | Absent | Not turned | Present | Adnate   |
| 7   | A.spi.1      | Erect | Smooth | Not winged | Present | Not turned | Present | Adnate   |
| 8   | A.spi.2      | Erect | Smooth | Not winged | Present | Not turned | Present | Adnate   |
| 9   | A.spi.3      | Erect | Smooth | Not winged | Present | Not turned | Present | Adnate   |
| 10  | A.vog.1      | Prostrate | Smooth | Not winged | Absent | Not turned | 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  | Triangle | Acute | 3 or less | Present | Not turned | 10 or less | 1 or more |
| 4   | A.sie.2  | Triangle | Acute | 3 or less | Present | Not turned | 10 or less | 1 or more |
| 5   | A.sie.3  | Triangle | Acute | 3 or less | Present | Not turned | 10 or less | 1 or more |
| 6   | A.sie.4  | Triangle | Acute | 3 or less | Present | Not turned | 10 or less | 1 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 | Acute | 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.

![UPGMA Dendrogram](image)

**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.
### Table 1: Pod and Seed Characters

| No. | Studied taxa | Pod characters | Seed characters |
|-----|--------------|----------------|-----------------|
| 1   | A.hau.1      | Hairy          | Curved          | Absent          |
|     |              |                | 0.5 or less     | Quadratic       |
|     |              |                | Smooth          | Brown           |
| 2   | A.hau.2      | Hairy          | Curved          | Absent          |
|     |              |                | 0.5 or less     | Quadratic       |
|     |              |                | Smooth          | Brown           |
| 3   | A.sie.1      | Glabrous       | Curved          | Long            |
|     |              |                | 0.4 or less     | Quadratic       |
|     |              |                | Irregular       | Brown           |
| 4   | A.sie.2      | Glabrous       | Curved          | Long            |
|     |              |                | 0.4 or less     | Quadratic       |
|     |              |                | Irregular       | Brown           |
| 5   | A.sie.3      | Glabrous       | Curved          | Long            |
|     |              |                | 0.4 or less     | Quadratic       |
|     |              |                | Irregular       | Brown           |
| 6   | A.sie.4      | Glabrous       | Curved          | Long            |
|     |              |                | 0.4 or less     | Quadratic       |
|     |              |                | 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           |

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

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**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.

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

**Figure 3.** Cont.
3.2.2. Amplification of SCoT Region

DNA fragments were amplified by utilizing ten primers that were selected to assess DNA polymorphism for genetic diversity studies. The primers used were SCoT 7, 9, 10, 11, 14, 24, 28, and 32. All primers amplified clear and reproducible bands as shown in Figure 3. (a–h) Gel amplification profiles obtained typically with primers SCoT 7, 9, 10, 11, 14, 24, 28, and 32, 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 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.

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              | 97.67                | Astragalus sieberi RMG1  |
| 4   | Astragalus sieberi 2                               | Astragalus sieberi              | 97.67                | 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 shrubby 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 high 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.

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