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# Taxonomic, DNA Barcoding and Phylogenetic Reassessment of the Egyptian *Ephedra* L. (Ephedraceae)

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## ABSTRACT

This study attempts to carry out a critical revision for the genus *Ephedra* in Egypt based on morphological characters for leaf, stem and flower. DNA sequencing data used for the first time for DNA barcoding of the family Ephedraceae in Egypt. One chloroplast marker-intergenic spacer *trn*-H/*psb*-A and another nuclear Internal Transcribed Spacer (ITS) were sequenced to authenticate the identification and to reconstruct the phylogenetic relationships of the Egyptian *Ephedra* species. The results revealed that, the name of *Ephedra ciliata* Fischer and C. A. Mey., was illegitimate and became a synonym to *Ephedra foliata* Boiss., which reported here as accepted name. The current work recommends using the DNA barcode as a tool for species identification of *Ephedra* species as well as other gymnosperms in Egypt.

**Keywords**: *Ephedra*, ITS, Gymnosperms, Gnetales, Systematics, Phylogenetic, *trn*-H/*psb*-A.

## INTRODUCTION

Family Ephedraceae includes around 68 species in the genus Ephedra L. (Christenhusz and Byng, 2016). In addition to Gnetum and Welwitschia, the genus *Ephedra* comprise Gymnospermous Gnetales group, which characterized by nonflowering seed plants with limited evolutionary history (Ickert-Bond and Wojciechowski, 2004; Rydin *et al*., 2006). Despite the numerous studies in the past century, the systematic position of the Gnetales is still poorly understood and in most morphological analyses a close affinity between Gnetales and higher angiosperms was implied (Mundry and Stützel, 2004).

Species of *Ephedra* are equally distributed in both old and new world (Ickert-Bond, 2003), they are usually xeromorphic dioecious shrubs or under shrubs with green branches, leaves are opposite or whorled; often reduced to membranous sheathes (El Hadidi, 2000). The genus shows high tolerability against extraordinary aridity. This leads to reduction in vegetative growth. Little taxonomic emphasis was given for such xeric Gymnosperms in Egypt. Usually, the vegetative stages of *Ephedra* are vigorous in the field as well as in preserved specimens. Moreover, the rare opportunity for investigator to find plant and/or herbarium specimen with mature sexual phases represent a great challenge for species delimitation. When morphologically-based biosystematics of any group of plants has been so troublesome, the utilization of DNA sequencing data to discriminate between problematic taxa and to infer phylogenies becomes unavoidable choice (Ickert-Bond and Wojciechowski, 2004).

*Ephedra* has been a subject of many phylogenetic studies (Huang *et al*., 2005; Ickert-Bond and Rydin, 2011; Ickert-Bond *et al*., 2009; Ickert-Bond and Wojciechowski, 2004; Long *et al*., 2004; Rydin *et al*., 2010). According to Ickert-Bond and Rydin (2011), the morphological and molecular diversity within *Ephedra* still limited and more phylogenetic investigations is needed to understand the evolution of this genus. Rydin *et al*. (2006) reported that all species of *Ephedra* are very closely similar in gross. Monophyly of the genus was considered, however this assumption has not been investigated thoroughly (Huang *et al*., 2005; Price, 1996; Rydin *et al*., 2002).

The genus *Ephedra* has been studied world-widely regarding the systematics significances by (Anueva-Almanza and Fonseca, 2011; Cutler, 1939; Freitag and Maier-Stolte, 1996; Ickert-Bond, 2003; Price, 1996; Stapf, 1889). Huang *et al*. (2005) used chloroplast marker matK gene and nuclear marker ITS for subgeneric classification of the genus. However, in Egypt, *Ephedra* and other Gymnosperms have never been subjected to taxonomic revision except in the context of floras (Boulos, 1999; El Hadidi, 2000; Täckholm, 1974).

In Egypt, Ephedraceae is one of the two families belonging to subdivision Gymnospermae. The Egyptian Ephedraceae is represented by only *Ephedra* L. (Boulos, 1999). Täckholm (1974) reported four species of genus *Ephedr*a namely: *E. alata* Decne., *E. aphylla* Forssk., *E. ciliata* Fischer and C. A. Mey. and *E. campylopoda* C. A. Mey, while Boulos (1999) added *E. pachyclada* Boiss. as a new record to the flora of Egypt, he consid-ered *E. campylopoda* C. A. Mey. as a synonym to *E. foeminea* Forssk. Boulos (op. cit.) enumerated five taxa of *Ephedra*, viz. *Ephedra alata* Decne., *Ephedra aphyl-la* Forssk., *Ephedra ciliata* Fischer and C. A. Mey., *Ephedra foeminea* Forssk. and *Ephedra pachyclada* Boiss. subsp. *sinaica* (Riedl) Freitag and Maier-Stolte.

The nomenclatural and systematics background of many of the Mediterranean species of *Ephedra* have been particularly complex, and most of names now treated as a synonymy (e.g., *E. alte* C. A. Mey., *E. campylopoda* C. A. Mey. and *E. ciliata* Fischer and C. A. Mey.) have been widely used in the literatures (Price, 1996).

*Ephedra* has been used for treatment of asthma and

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bronchitis. It has been proven to have potent efficacy to relief symptoms of cold and flu such as fever, cough and nasal congestion (Zheng, 1997). *Ephedra alata* has been used by Bedouins in Sinai Peninsula in folk medicine as treatment herb for Central Nervous Disorders (CNS) and several other healing uses (Eissa *et al.*, 2014).

Kress *et al*. (2005) postulated that the DNA barcoding aims to develop a universal database of specific DNA sequences which might be used for unknown species identification and taxonomic delimitations. The first successful attempt of this developing technique was using a DNA sequence of Cytochrome oxidase 1 (CO1) mitochondrial gene especially in animals. In plants, launching this step was challenging, relevant to the selection of standard and universal candidate marker to be used as plant DNA barcode. After several broad screenings of gene regions in the plant genome, three plastids (*rbc*L, *mat*K, and *trn*-H/*psb*-A) and one nuclear (ITS) gene regions have become the standard barcode of choice in most investigations for plants.

Techen *et al*. (2014) recommended the using of two-locus barcode versus a three-locus barcode (*mat*K + *rbc*L + *trn*-H/*psb*-A). They pointed out; the two-locus barcode was preferred to avoid the increased costs of sequencing. The barcode combination *rbc*L + *mat*K was the preferred choice as barcode of medicinal plants.

A search of the literature in SciFinder (a chemical abstracts service database) from 2010 to 2013 resulted in 60 publications. In the literature analyzed in this review, a total of 17 barcode regions (*mat*K, *rbc*L, ITS, ITS2, *trn*-H/ *psb*-A, *atp*F-*atp*H, ycf5, *psb*K-I, *psb*M *trn*D, *rps*16, *cox*I, *nad*1, *trn*L-F, rpoB, *rpo*C1, *atp*F-*atp*H) of medicinal plants were reported to aid in the authentication and identification of medicinal plant materials. Most barcoding regions mentioned in the literature were the ITS region (26 references), *trn*-H/ *psb*-A (21 references), *mat*K (19 references), and *rbc*L (14 references). Further genomic regions used for barcoding were ITS2 (9 references), *rpo*C1 (6 references), *rpo*B (4 references), and *trn*L-F (3 references).

Due to lack of previous taxonomic studies of *Ephedra* in Egypt, this study attempts to carry out a critical revision for the genus *Ephedra* in Egypt based on morphological characters for leaf, stem and flower. Moreover, the present study seeks to test the monophyly of *Ephedra* species in Egypt; provides a preliminary insight of the validity of the traditional taxonomic divisions into sections.

## MATERIALS AND METHODS

### A. Specimen collections

The work was based on collections kept in different Egyptian herbaria: ASTU, CAI, CAIM, as well as SCUI and on photos of type specimens in C, B, BM, G, HUJ, JE, K, MPU and P herbaria. The code of herbarium abbreviation follows Thiers (2017). Photos of the type specimens were seen by the authors indicated by "!". The specimens were identify according to (Täckholm, 1974) and (Boulos, 1999 and 2009). Specimens were exa-mined by Olympus SZ61 stereomicroscope provided with a digital Olympus camera SC100. Table (1) summarizes the available data about the taxa under investigation as reported in the earlier works for the flora of Egypt. The distribution map of *Ephedra* species was created based on the distribution regions that cited in Boulos (1999) Map (1).

### B. DNA Extraction

As *Ephedra* is characterized by minute leaves, it was difficult to obtain reasonable amount of fresh and/or dry leaves for DNA extraction. Instead, the total genomic DNA was extracted from 0.25-1.00 gm of stems grounded in liquid nitrogen. Methods of (Doyle and Doyle, 1987) was implied with modification by adding 2% PVP 40 (polyvinyl pyrrolidone) to the buffer (2% CTAB, 20 mM EDTA, 1.4 mM NaCl and 100 mM Tris-HCl, pH8) to improve the quality of DNA.

### C. PCR amplification and DNA Sequencing

Double-stranded DNA was amplified by the Polymerase Chain Reaction (PCR) using previously published primers sets for the selected DNA markers. Thermal cyclers Veriti™ Dx 96-well Thermal Cycler, 0.2 ml (Applied Biosystems®) was used. The reaction usually carried out in 25µl for bidirectional sequencing using (BioMix®, Bioline, UK) a complete ready to use 2x reaction mix containing an ultra-stable DNA polymerase according to the manufacturer's instructions. To enhance amplification Bovine Serum Albumin (BSA) added to the PCR aliquot prior to the start of the reaction.

### D. Bioinformatics analyses

*a) DNA barcoding*

DNA sequencing data was used for the first time for DNA barcoding of the family Ephedraceae in Egypt. One chloroplast marker-intergenic spacer *trn*-H/*psb*-A and another nuclear Internal Transcribed Spacer (ITS) were sequenced to authenticate the identification and to reconstruct the phylogenetic relationships of the Egyptian *Ephedra* species. Sequences for *trn*-H/*psb*-A and ITS loci from *Ephedra* species were submitted to Gene-Bank database with accession numbers (Table 2). The *trn*-H/*psb*-A and ITS loci of the genus *Ephedra* in Egypt were compared with other sequences of non-Egyptian species belonging to the genus *Ephedra* retrieved from Gene-Bank database. Multiple alignment of the sequences performed with CLUSTAL W2 (www.ebi.ac.uk/Tools/clustalw2) and T-COFFEE (www.ebi.ac.uk/Tools/t-coffee). DNA barcode-based trees were obtained by using Neighbor-Joining and Kimura-2 parameter to evaluate the relationships of species and compare the DNA barcode-based classifications of species with classical taxonomic classifications of species, (Fig. 1). Gene-bank database search via Basic Local Alignment Search Tool (BLAST) was done with the newly generated DNA sequences for both loci, (Table 2).

*b) Phylogenetic analysis*

Bayesian phylogenetic Inferences (BI) were conducted using the Mr Bayes software (ver. 3.2) (Ronquist *et al.*, 2012). Three independent datasets were analyzed. These were made up of two types: single locus datasets (2 datasets) and a concatenated dataset of chloroplast DNA (cp DNA) plus nuclear DNA (nr DNA). The opti-

mal nucleotide substitution model was selected for each alignment via the AIC criterion (Akaike, 1974) using PAUP version 4.0 (Swofford, 2003) and the Mr Modelblock command from Mr Model test (Nylander, 2004). For each matrix, two independent Bayesian analyses were performed to check for convergence (Miller *et al*., 2002), with four chains per analysis and trees sampled every specified number of generations relevant to each dataset. All compatible trees were calculated in Mr Bayes. A plot of negative log .

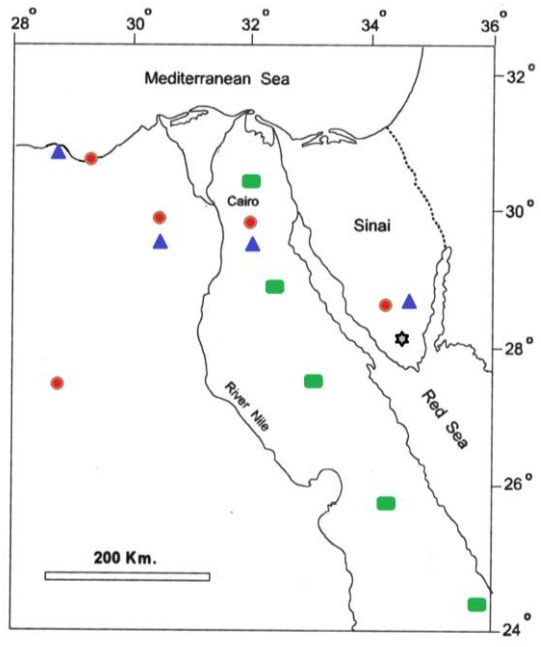
Likelihoods against generation time were done using Markov chain Monte Carlo (MCMC Trace Analysis). Tool Version 1.6.0, 2003 to establish the burn in (Rambaut *et al*., 2013). Trees found before reaching stability were pruned out and the rest used to compile an all compatible tree. All compatible trees were exported to tree Graph2 (ver. 2.0.50-314 beta) software for visualization and editing (Stöver and Müller, 2010). Posterior Probabilities (PP) were used to measure clade support.

### E. Taxonomic classifications of taxa

The most important sectional treatment of the genus *Ephedra* was carried out by Stapf (1889), in which the genus was divided into three major sections based on bracts of female cones: *Alatae*, *Ephedra* and *Asarca*. The Egyptian taxa of *Ephedra* represented only in two sections: *Alatae* and *Ephedra*. Table (1) shows the sectional and tribal classification of Egyptian *Ephedr*a according to classification of Stapf (1889).

**Table (1):** *Ephedra* species reported by the different authors who are concerned with the flora of Egypt, including the current study (+ = present, - = absent, x = recorded as a synonym, 1= Forsskål 1775, 2= Boissier 1867 - 1879, 3= Muschler 1912, 4= Täckholm 1956, 5= Täckholm 1974, 6= El Hadidi & Fayed 1994/1995, 7= El Hadidi 2000, 8= Boulos 2009, 9= Present study 2018).

| Section | Tribe | Taxa | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Alatae | Tropidolepides | ***Ephedra alata* Decne.** | - | + | + | + | + | + | + | + | + |
| Ephedra | Scandentes | ***Ephedra aphylla* Forssk.**<br>= *Ephedra alte* C. A. Mey | X | X | + | X | + | + | + | + | + |
| | | ***Ephedra foliata* Boiss.**<br>= *Ephedra ciliata* Fischer & C. A. Mey.<br>=*Ephedra peduncularis* Boiss. & Hausskn. | - | + | - | + | + | X | + | X | + |
| | | ***Ephedra foemina* Forssk.**<br>= *Ephedra campylopoda* C. A. Mey.<br>=*Ephedra fragilis* Desf. subsp. *campylopoda* (C. A. Mey.) Asch. & Graebn. | X | X | - | - | X | - | - | + | + |
| | Pachyclada | ***Ephedra pachyclada* Boiss.**<br>= *Ephedra sinaica* Riedl. | - | + | - | - | - | + | + | + | + |




**Map (1):** Distribution of *Ephedra* species in Egypt. *Ephedra alata* (●), *Ephedra aphylla* (▲), *Ephedra foliate* (▬), *Ephedra pachyclada* subsp. *sinaica* (✡).

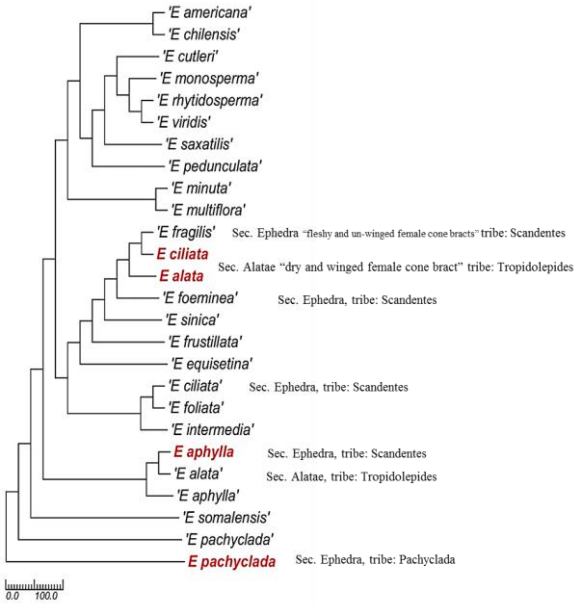



**Figure (1):** Cladogram of Neighbor Joining tree (NJ) of Egyptian *Ephedra* species inferred from combined dataset of sequences of two markers *trn*-H/*psb*-A, and ITS species. Red typos represent the Egyptian species with its corresponding sectional classification.

**Table (2):** Similarity search using BLAST tool and identification status of Egyptian *Ephedr*a species.

| Query (Egyptian species) | Accession number | BLAST Result | Accession number * | Locus | Identification (%similarity) | Identification status |
|---|---|---|---|---|---|---|
| *Ephedra alata* | MG550042 | *Ephedra fragilis* (synonym) | AY849363.1 | *trn*-H/*psb*-A | 95 % | Succeeded |
| *E. aphylla* | MG569946 | *E. aphylla* | GU968569.1 | ITS | 98 % | Succeeded |
| *E. pachyclada* | MG550043 | *E. sinica* (synonym) | GQ463516.1 | *trn*-H/*psb*-A | 95 % | Succeeded |
| *E. pachyclada* | MG569945 | *E. pachyclada* | AY755779.1 | ITS | 99 % | Succeeded |
| *E. ciliata* | MG550041 | *E. foeminea* | KT934791.1 | *trn*-H/*psb*-A | 92 % | Failed |

*****Accession number of non-Egyptian species retrieved from database

## RESULTS

### Systematic treatment

***Ephedra*** L., Sp. Pl. ed. 1: 1040 (1753).

**Type species**: *Ephedra distachya* L. Sp. pl. 2: 1040 (1753).

According to Mabberley (2008) and Christenhusz and Byng (2016), *Ephedra* consists of about 68 species distributed between the old and new world and native to arid and semiarid regions of Europe, N Africa WN America, S America and Asia. Our taxonomic revision of the genus in Egypt revealed the presence of 5 species including one subspecies (representing in 2 sections) as shown in table (1):

### A. Sect. Alatae Stapf

Characterized by having a dry and winged female cone bract.

**1. Tribe Tropidolepides Stapf**

**1. *Ephedra alata*** Decne., Ann. Sci. Nat. Bot., Sér. 2, 2: 239 (1834).

**Type**: Egypt: Sinai, Algdé Arab. Désert de Suez, *Bové, N. 215*, 1 June 1832. Isotype: K (K000076236 and K000076235 photos!), G (G-355819/1), MPU (MPU027036 photo!); Lectotype: P (P00738802). Isolectotype: P (P00738804).

**Distribution**: In Egypt: The Oases of the western desert, the Mediterranean coastal strip and all the deserts of the country including that of Sinai (Map 1). General distribution: North Africa, Palestine, Arabia, Iraq.

**Ecology**: Desert sandy plans.

**Representative specimens**: Suez: Cairo-Suez desert road, 15 March 1974, El-Hadidi *et al*. s.n. (CAI); Kilo 20 on Cairo-Suez road, 10 March 1930, F. W. Oliver s.n. (CAI); Kilo 21 on Cairo-Suez road, 9 June 1971, Saad *et al.* s.n. (CAIM); Wadi Katamiya, 11 March 1960, V. Täckholm *et al*. s.n. (CAI); 30 km, south of Suez, 4 Oct. 1989, El Garf s.n. (CAI); Wadi Araby, between the two Galalas, 4 Feb. 1960, V. Täckholm et al. s.n. (CAI); South Galala, cretaceous foot hills, 6 Feb. 1960, V. Täckholm *et al*. s.n. (CAI). Cairo: Wadi Degla, west Maadi, 15 April 1979, M. Atta *et al*. 115 (CAIM); Wadi Hoff, March 1980, Fayed and el-Naggar s.n. (ASTU). Sinai: Wadi Feiran, 10 May 1956, V. Täckholm s.n. (CAI); Abo Zeinema, 19 Feb. 1969, M. Abdalla 539 (CAIM); Wadi El-Hamammat, January 1962, El-Hadidi s.n. (ASTU); Wadi Fereeh, 24 April 1961, El-Hadidi s.n. (ASTU); Wadi El-Tayeb, 24 April 1961, El-Hadidi s.n. (ASTU); At the entrance of wadi Feiran, 21 Aptil 1961, V. Täckholm *et al*. s.n. (CAI); Wadi Feiran, 16 April 1962, M. Abdalla *et al*. 794 (CAIM); Wadi Hamamet Faroan, near the red sea, 16 May 1956, V. Täckholm s.n. (CAI); Wadi Abu Khodirate, 85 km. west of Zafarana, 13 April 1997, M. Fadel s.n. (CAI).

### B. Sect. Ephedra Stapf

Characterized by having fleshy and un-winged female cone bracts.

**1. Tribe Scandentes Stapf**

**2. *Ephedra aphylla*** Forssk., Fl. Aegypt.–Arab. 170 (1775).

**Type**: Palestine: Jaffa in sepibus, *Bormüller Iter Syriacum 1749*, 12 May 1897. Designated by Freitag and Maier-Stolte (1989). Iso-lectotype: B (B100296982 photo!), Neotype: JE (JE00006800 photo!); Isoneotypes: K (K000459012 photo!), BM (BM000884450 photo!).

**Synonym**: *Ephedra alte* C. A. Mey., Monogr. Ephedra, Mém. Acad. Sci. Pétersb. 5: 75 (1846).

**Distribution**: In Egypt: The Mediterranean coastal strip and all the deserts of the country including that of Sinai (Map 1). General distribution: Northeast Africa, Syria to northern Arabia.

**Ecology**: Calcareous slopes and wadi beds.

**Representative specimens**: Mediterranean coastal strip: Before Mersa Matrouh, on the road, 3 May 1966, V. Täckholm s.n. (CAI); Saniet Hagg Ayyad, wadi El-Habes, before Agiba, 23 March 1974, V. Täckholm s.n. (CAI); Ras El-Hekma, 25 May 1954, Migahid and Shafey s.n. (CAI); Burg El-Arab, Roman Cistern, 9 March 1978, Merxmüller *et al*. s.n. (CAI); 11 March 1978, El-Hadidi and A. Soliman s.n. (CAI); 18 Sept. 1970, Mahdi s.n. (CAI); Mariout, 12 Aug. 1928, M. Hassib s.n. (CAI); 18 March 1931, Oliver s.n. (CAI); 14-17 March 1958, V. Täckholm s.n. (CAI); 30 April 1976, J. Chrtek s.n. (CAI); Alexandria, Vectoria, 25 Aug. 1921, J. Brown s.n. (CAIM). Cairo: Wadi Hoff, 11 April 1978, G. Fahmy s.n. (CAI); Giza, El-Busseili, 23 Sept. 1971, Iman *et al*. (CAI). Sinai: entrance of wadi El-Arbaein, 23 April 1961, Jack *et al*. s.n. (CAI); Deir El-Rahba garden, Saint Catherin, 5 May 1939, M. Drar 356 (CAIM); Wadi El-Arbaein, Saint Catherin, 18 May 1988, M. Kassas s.n. (CAI); Wadi El-Kid, 28 March 2004, 28.34474 N, 34.17169 E, A. Fayed *et al*. s.n. (ASTU); Wadi Allalaqi, 1963, M. Abdalla *et al*. s.n. (CAIM). Gebel Elba, 28 Feb. 1938, Shabetai 5146 (CAIM).

**3. *Ephedra foliata*** Boiss., Diagn. Pl. Orient. 7: 101 (Jul.-Oct. 1846).

**Type**: Iran: Islamic Republic of Gilan, *Aucher-Eloy, P. M. R. 5338*, no date. Isotype: K (K000456219 photo!), P (P00738820 photo!); Iso-lectotype: BM (BM 000884470 photo!).

According to the recent database of World Checklist of Selected Plant families WCSP (2017), *Ephedra ciliata* Fischer and C. A. Mey. was illegitimate and became a synonym to *Ephedra foliata* Boiss. which reported as accepted name.

**Synonyms**: *Ephedra ciliata* Fischer and C. A. Mey., Monogr. *Ephedra*, Mém. Acad. Sci. Pétersb. 4: 100 (Mar. 1846). **nomen nudum**,

*Ephedra peduncularis* Boiss. and Hausskn., Fl. Orient. 5: 716 (1884).

**Distribution**: In Egypt: Desert east of the Nile including that of Sinai, Gebel Elba and the surrounding mountainous regions (Map 1). General distribution: North and East Africa, Arabia, eastwards to India.

**Ecology**: Scrambling on shrubs and trees, rocky slopes.

**Representative specimens**: Sinai: Red sea, Gebel Hamata, 7 Feb. 1961, V. Täckholm *et al*. s.n. (CAI); Farsh Deghymat, 28 32 69 N, 33 54 81 E, Saint Catherin, 18 April 2008, Ahmed EL-Banhawy (SCUI); Wadi Adaib, Saint Catherin, 20 Jan. 1930, M. Hassib s.n. (CAI); Wadi Reem, 28.66806 N, 33.66742 E, 23 April 2004, A. Fayed *et al*. s.n. (ASTU); Wadi Gebal, 28.3228 N, 33.5253 E, 28 April 2004, A. Fayed *et al*. s.n. (ASTU); Wadi Alletehi, 28.09732 N, 34.04545 E, 11 April 2004, A. Fayed *et al*. s.n. (ASTU); Wadi Al Ratam, 28.23901 N, 34.23850 E, 28 March 2004, A. Fayed *et al*. s.n. (ASTU); Gebel Serbal region, wadi Aleyaat, 28.6686 N, 33.65377 E, 22 April 2004, A. Fayed *et al*. s.n. (ASTU). Qena: Gebel Hamra Dom, 9 Feb. 1932, M. Drar s.n. (CAIM). Gebel Elba: 4 Jan. 1933, M. Hassib s.n. (CAI); Sept. 1936, M. Drar s.n. (CAIM); Wadi Santit, 23 Jan. 1962, V. Täckholm *et al*. (CAI).

**4. *Ephedra foeminea*** Forssk., Fl. Aegypt.-Arab. 219 (1775).

**Type**: Turkey: Gökceada, *P. Forsskål 1246*, July 1761. Lectotype: C (C10002224 photo!).

**Synonyms**: *Ephedra campylopoda* C. A. Mey., Monogr. Ephedra, Mém. Acad. Sci. Pétersb. 4: 107 (1846).

*Ephedra fragilis* Desf. subsp. *campylopoda* (C. A. Mey.) Asch. and Graebn., Syn. Mitteleur. Fl. 1: 258 (1897).

**Distribution**: In Egypt: Sinai Peninsula. General distribution: Southern Arabia and Ethiopia.

**Ecology**: Rocky cliffs.

**Representative specimens**: no specimens were seen.

**Notes**: The conservation status of *Ephedra foeminea* is endangered as reported in Plant Red Data Book of Egypt (El-Hadidi *et al*., 1991).

**2. Tribe Pachyclada Stapf**

**5. *Ephedra pachyclada*** Boiss., Fl. Orient. 5: 713 (1884) subsp. ***sinaica*** (Riedl) Freitag and Maier-Stolte, Edinb. J. Bot. 49: 92 (1992).

**Type**: Egypt: South Sinai, 10 km. S. of Nebi Salah, in fissures of flat granite, 1350-1400 m. *A. Danin s.n.*, 4. April 1971. (HUJ).

**Synonym**: *Ephedra sinaica* Riedl, Notes Roy. Bot Gard. Edinb. 38: 291 (1980).

**Distribution**: In Egypt: Sinai Peninsula (Map 1). General distribution: Arabia, extending eastwards to Iran and Pakistan.

**Ecology**: Rocky cliffs and slopes.

**Representative specimens**: Cairo: Giza, 15 Oct. 1963, El-Mahdi s.n. (CAI). Sinai: Wadi El-Kid, 28.34474 N, 34.17164 E, 27 March 2004, A. Fayed *et al*. s.n. (ASTU); Wadi Gebal region, Wadi Al-Talaa Al-Kabera, 28.2345 N, 33.5245 E, 28 March 2004, A. Fayed *et al*. s.n. (ASTU); Ain Al-Tofaha, 28.3254 N, 33.5626 E, 28 March 2004, A. Fayed *et al*. s.n. (ASTU).

**Key to the species of *Ephedra* in Egypt:** (Figures 2-5, Table 3)

1- Twigs flexible; gynodioecious; seed up to 1mm length; usually completely covered by bracts,..…….…… ***E. foeminea***

- Twigs rigid; dioecious; seed up to 7mm length; upper part of seed emerging from bracts ……..……..…...…..…..…… **2**

2. Leaves 10-17 mm; stem surface ciliate; bracts of female cone 6-8 mm length in 2 pair…….…….…….. ***E. foliata***

- Leaves 2-3 mm; stem surface smooth or papillose; bracts of female cone 3-5 mm length in more than 2 pair……..…….. **3**

3. Surface of stem smooth; female cone bracts free, in 5 pairs, dry, marginal winged, 2 seeds; anthers distinctly stipitate ..…. ***E. alata***

- Surface of stem papillose; female cone bracts fused; in 3-4 pairs; fleshy; marginal un-winged, 1 seed, anthers sessile ……**.4**

4. Margins of leaves and bracts ciliate; seed up to 7 mm, 3-4 anthers per one flower; stamens not exceeding 3 mm length... ***E. aphylla***

- Margins of leaves and bracts glabrous, seed up to 5 mm, 5-8 anthers per one flower; stamens exceeding 3 mm length.. ***E. pachyclada*** subsp**. *sinaica***

**DNA barcoding**

In correlation of the Egyptian *Ephedra* species; chloroplast genome *trn*-H/*psb*-A, and the nuclear genome (ITS) experienced the standardized DNA barcoding. By examination, *trn*-H/*psb*-A had a value of divergence (0.33%), while ITS had a much lower divergence value (0.20 %). Although the sequence of ITS was shorter than 800bp, we included them in the investigation considering their high interspecific variability. The current interspecific investigation over all available taxa confirmed variation between the two markers as for the three barcoding criteria: ease of amplification, length of the sequence, and sequence divergence.

The current study managed to produce two newly generated sequences of ITS of *Ephedra aphylla* and *Ephedra pachyclada* from Egypt. On the other hand, the chloroplast intergenic spacer *trn*-H/*psb*-A was sequenced for all accessible taxa except for *Ephedra foeminea*

where no accessible specimen neither discovered nor gathered all through Egypt recently. Genebank database search via BLAST online similarity search inveterate morphology with the available DNA sequences which are then corresponding to an online reference collection (NCBI's GeneBank) supports to authorize identification, retrieved inquires results shown in table (2). Utilizing two markers, DNA barcoding has been flourished to affirm identification of *Ephedra pachyclada* while single DNA barcoding was prevailing to affirm the identification of two taxa *Ephedra alata* and *Ephedra aphylla*. In contrast, single marker DNA barcoding failed to affirm the identification of *Ephedra foliata* (Table 4, Figures 1, 6, 7 and 8).

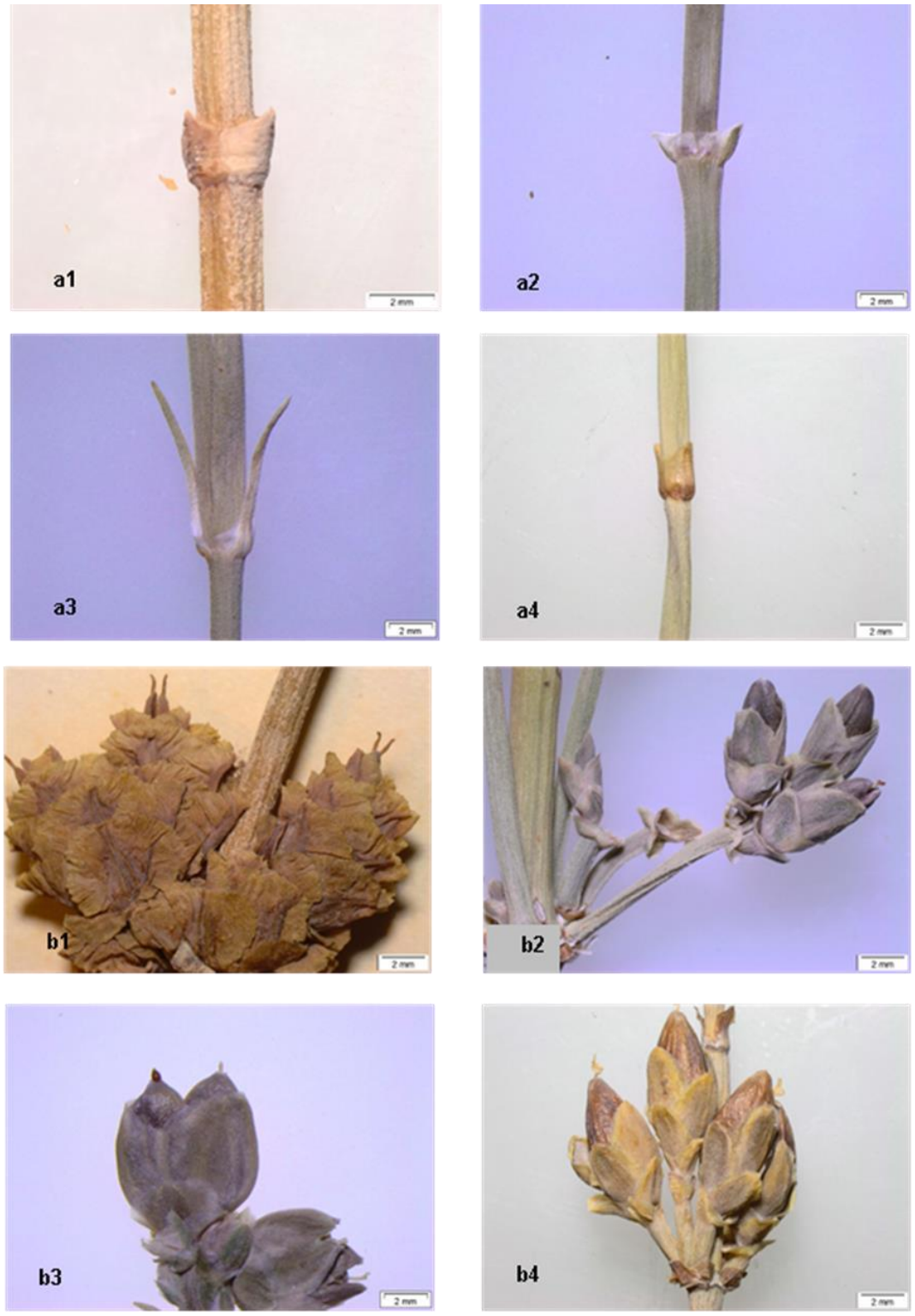



**Figure (2):** Morphology of *Ephedra* species, **a**, vegetative shoot showing leaves arrangement; **b**, female cone: **1**, *E. alata*; **2**, *E. aphylla*; **3**, *E. foliata*; **4**, *E. pachyclada* subsp. *sinaica*.


**Figure (3): a**, an enlarged female strobilus; **b**, seed: **1**, *E. alata*; **2**, *E. aphylla*; **3**, *E. foliata*; **4**, *E. pachyclada* subsp. *sinaica*.


**Figure (4): a**, male cone; **b**, an enlarged male strobilus: **1**, *E. alata*; **2**, *E. aphylla*; **3**, *E. foliata*; **4**, *E. pachyclada* subsp. *Sinaica*

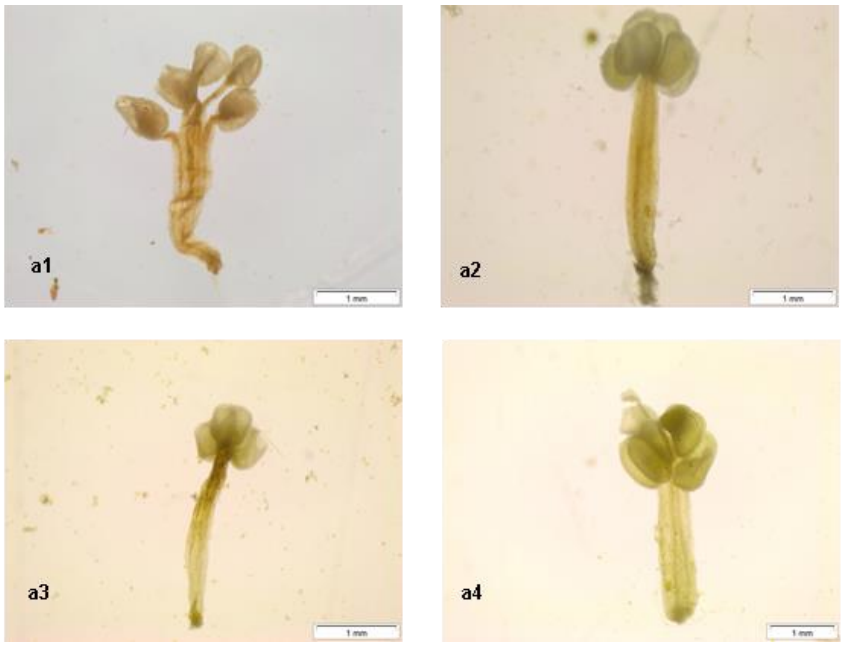



**Figure (5): a**, microsporangia and sporangiophore: **1**, *E. alata*; **2**, *E. aphylla*; **3**, *E. foliata*; **4**, *E. pachyclada* subsp. *sinaica*

**Table (3):** Main differential characters of the *Ephedra* species

| Character | *E. alata* | *E. aphylla* | *E. foliata* | *E. pachyclada* subsp. *sinaica* |
|---|---|---|---|---|
| **Leaf length (mm)** | 2-2.5 | 2-3 | 10-17 | 2-3 |
| **Margins of leaves and bracts** | Ciliate | Ciliate | Ciliate | Glabrous |
| **Stem surface** | Smooth | Papillose | Ciliate | Papillose |
| **Bracts of female cone** | Free | Fused | Fused | Fused |
| **Number of female cone bracts (pairs)** | 5 | 3 | 2 | 4 |
| **Length of innermost female flower bracts (mm)** | 4-5 | 4-5 | 6-8 | 3-5 |
| **Number of ovules per cone** | 2 | 1 | 1-2 | 1 |
| **Length of seed (mm)** | 6-7 | 6-7 | 6-7 | 4-5 |
| **Anthers** | Distinctly stipitate | Sessile | Sessile | Sessile |
| **Number of anthers per one flower** | 4-6 | 3-4 | 3-4 | 5-8 |
| **Length of stamens (mm)** | 2.5-2.8 | 2.5-2.8 | 2.4-2.6 | 3-3.6 |

**Table (4):** PCR success and DNA sequence length of trn-H/psb-A and ITS markers used in DNA barcoding and phylogenetic analysis of Egyptian *Ephedra* species.

| Taxa | *trn*-H/*psb*-A | Length (bp) | ITS | Length (bp) |
|---|---|---|---|---|
| ***Ephedra alata*** | + | 519 | - | × |
| ***E. aphylla*** | - | × | + | 356 |
| ***E. foeminea*** | - | × | - | × |
| ***E. foliata*** | + | 541 | - | × |
| ***E. pachyclada*** | + | 536 | + | 357 |

(+) PCR successful, (-) PCR failed, numbers = length of sequence in base pair (bp), (×) sequence failed.

## Phylogeny

The *trn*-H/*psb*-A region had an aligned length of 541 bp while The ITS region had an aligned length of 357 bp. The combined alignment had an aligned length of 1094 bp (Table 4). By contrast, the *trn*-H/*psb*-A based Bayesian phylogenetic tree contained three internal nodes with a posterior probability (PP) of 1.0 (Fig. 6). The Bayesian 50% majority rule consensus tree for ITS contained one internal node with (PP) of 1.0 (Fig. 7). The combined ITS and *trn*-H/*psb*-A tree contained two internal nodes with a (PP) of 1.0 (Fig. 8).

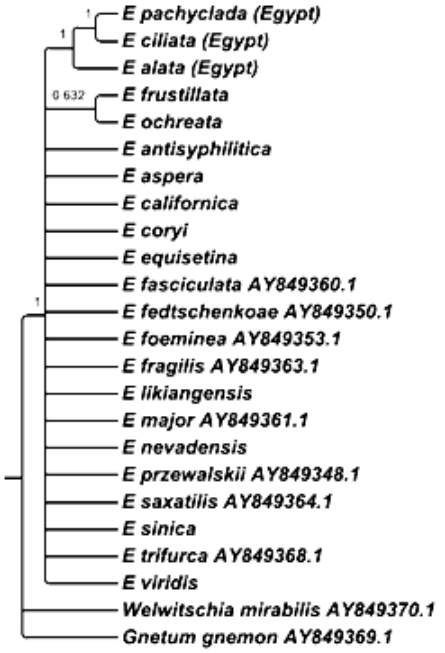



**Figure (6):** Cladogram of Bayesian 50% majority rule consensus of *trn*-H/*psb*-A. of Egyptian *Ephedra* species.

The phylogenetic analysis of the sequence dataset of the *trn*-H/*psb*-A includes 24 *Ephedra* species as well as *Welwitschia mirabilis* and *Gentum gnemon* as outgroup. While the analysis of the ITS region includes 18 *Ephedra* species and one outgroup species. In the analysis of *trn*-H/*psb*-A; the Egyptian *Ephedra* species were represented by three species; *Ephedra pachyclada, Ephedra ciliata* and *Ephedra alata.* While in the analysis of the ITS region they were represented by two species *Ephedra pachyclada* and *Ephedra aphylla*.

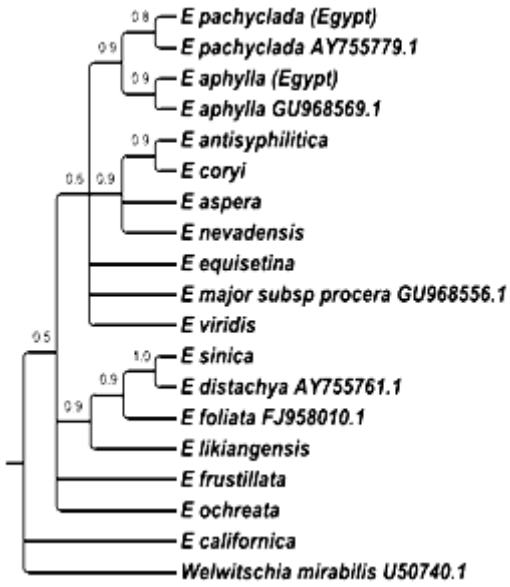



**Figure (7):** Cladogram of Bayesian 50% majority-rule consensus of ITS of Egyptian *Ephedra* species

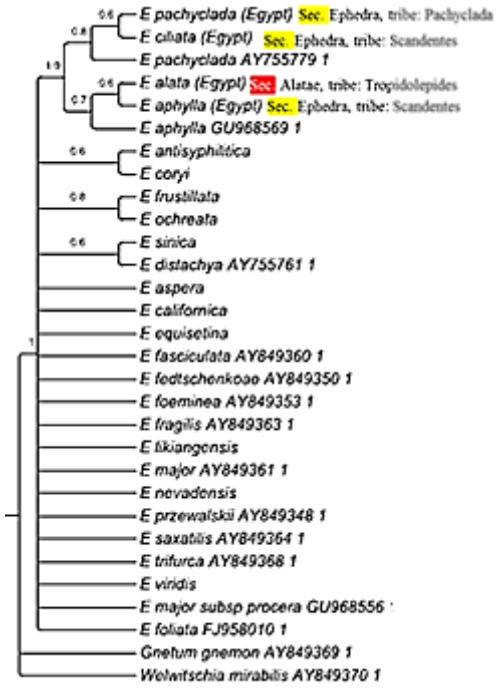



**Figure (8):** Cladogram of Bayesian all compatible tree inferred from combined datasets of *trn*-H/*psb*-A and ITS markers of *Ephedra* species. Number above branches represents posterior probability of the branch.

The nucleotide substation model used was GTR+I+G for the both region. The analysis was run for one million generation and produced a total of 202 trees in two files; each file contained 101 trees of which 76 were sampled. The in group was composed of the 22 *Ephedra* species as well as two out group species in the *trn*-H/*psb*-A analysis and 18 *Ephedra* species and one out group in the ITS-based analysis. Bayesian Inference (BI) of all 50% majority rule consensus phylogenetic trees with accom-panying Posterior Probability (PP) for *trn*-H/*psb*-A and ITS region are presented in (Figures 6 and 7 respectively).

## DISCUSSION

Due to the extremely reduced morphological characters of *Ephedra* and the trivial number of character states, the taxonomy of the genus *Ephedra* L. has always been doubtful and have a partial taxonomical studies (Ickert-Bond *et al*., 2003). The infrageneric relationships between *Ephedra* have been still uncertain and that because the most classification of the genus was based on limited vegetative characters such as leaf length, female cone bracts, number of seeds per female cone and plant habit (Huang *et al*., 2005). Meyer (1846) carried out the first and the earliest classification treatment of the genus. He divided the genus into two sections (Plagiostoma and Discostoma) based the morphology and the number of ovulate strobili at a node. The pioneer world-wide monograph of *Ephedra* is that of Stapf (1889) who divided the genus into three sections based on bract's nature in the ovulate cones namely *Alatae*, *Ephedra* and *Asarca*. Section *Alatae* Stapf is represented in Egypt by only *E. alata* under the tribe Tropidolepides. It characterized by dry, membranous, wavy winged, female cone bracts. *Ephedra alata* (Figures 2:b1; 3:b1; 4:a1) can easily distinguished from other *Ephedra* species by smooth stem surface, free; dry; marginal winged; 5 pairs of female cone bracts, 2 seeds and its anthers are distinctly stipitate (Boulos, 1999; El Hadidi, 2000; Ickert-Bond and Wojciechowski, 2004; Rydin *et al*., 2010; Zohary and Feinbrun-Dothan, 1966).

Section *Ephedra* Stapf (= section Pseudobacatae Stapf) is characterized by fleshy and un-winged female cone bracts. Two tribes (Scandentes and Pachyclada) were recognized in this section within Egyptian *Ephedra*. Tribe Scandentes represented in Egypt by *Ephedra aphylla*, *Ephedra foliata* and *Ephedra foeminea*, while *Ephedra pachyclada* subsp. *sinaica* was included in tribe Pachyclada. According to (El Hadidi, 2000; Freitag and Maier-Stolte, 1989; Freitag and Maier-Stolte, 1992; Hufford, 1996; Ickert-Bond and Wojciechowski, 2004; Price, 1996; Rydin *et al*., 2010; Zohary and Feinbrun-Dothan, 1966), margins of leaves and bracts was glabrous in *Ephedra pachyclada*, while being ciliate in both *Ephedra aphylla* and *Ephedra ciliata* (Figures 2-5, Table 3). Price (1996) reported that the systematical history and nomenclature of the Mediterranean species of *Ephedra* has been particularly complicated, and most of names such as *E. alte*, *E. campylopoda*

and *E. ciliata* placed as a synonym. Our results revealed that *Ephedra ciliata* Fischer and C. A. Mey. is a synonym to *Ephedra foliata* Boiss. Recently, *Ephedra foliata* was used and accepted by many authors: (Dobignard and Chatelain, 2011; Freitag and Maier-Stolte, 2003; Govaerts, 2001; Hedberg *et al*., 2009; Miller and Morris, 2004).

Kress *et al*. (2005) recommended that the *trn*-H/*psb*-A intergenic spacer is the best plastid choice for a DNA barcoding for land plants because it has excellent priming sites, length, and interspecific variation. Moreover, this intergenic spacer does present in non-flowering land plants. In an inquiry of Gene-Bank, we found that the *trn*-H/*psb*-A has been efficiently amplified in angiosperms, gymnosperms, mosses, and liverworts. Our findings on the properties of *trn*-H/*psb*-A agree with (Shaw *et al.*, 2007) in their wide survey of non-coding plastid DNA for phylogenetic purposes. By applying standardized barcode criteria (i.e., length considerations and universality) to the framework of their study, we conclude that *trn*-H/*psb*-A intergenic spacer has greater potential for species-level discrimination than the Internal Transcribed Spacer (ITS) for the Egyptian *Ephedra* species. For the first time in Egypt, we have shown that there are gene sequences suitable for DNA barcoding of non-flowering plants.

In non-flowering plants, to attain the species-level discrimination, it may be necessary to employ analysis of more than one locus. Our combined Bayesian analysis of *trn*-H/*psb*-A and ITS confirmed that the Egyptian *Ephedra* species are polyphyletic. Nevertheless, the monophyly of *Ephedra* is generally unquestionable, and is maintained by other molecular phylogenetic research (Ickert-Bond, 2003), as well as a set of ecological features like xeromorphic characteristic and other morphological characters, including female cone bracts, stem texture and leave margin. Overall, our data do not provide sufficient phylogenetic resolution to draw conclusions concerning the monophyly or non-monophyly of Egyptian *Ephedra*. Despite the recovery of several well-supported lineages in Egyptian *Ephedra* the basal branching relationships among these lineages is not well resolved by *trn*-H/*psb*-A, ITS, or the combined data (Figures 6, 7 and 8). However, it should be noted that *trn*-H/*psb*-A and ITS provide some evidence for the cohesiveness of Egyptian *Ephedra* species.

**Testing classification**

Our sampling of the Egyptian *Ephedra* species has given the prospect to test prevailing morphology-based hypotheses on infraspecific relationships. Our results reinforced the most recent infraspecific classification of the Egyptian *Ephedra* proposed by many authors. Although, the taxonomic investigations upheld the current sectional classification of the genus *Ephedra* in Egypt, the phylogenetic analysis uncovered irrational overlapping between two sections in Egypt. *Ephedra alata* which belongs to section *Alatae* was imbedded within species of the section *Ephedra* (Fig. 8). This overlapping could be explained that the genus *Ephedra* still in its route of speciation. This hypothesis is supported by obscured morphological features of the genus. The delimitation between the abovementioned two closely related sections is based on the morphological character of the female cone (dry or fleshy). Again, the female cone character is rather difficult to be traced in the available samples for the current work. The difficulty of the morphological identification and delimitation between *Ephedra* species in Egypt is still challenge. The current work recommends using the DNA barcode as a tool for species identification of *Ephedra* species as well as other gymnosperms in Egypt. This work will likely require wide sampling and sequencing of supplementary Loci from both the chloroplast and nuclear genome.

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## إعادة تقييم جنس الافيدرا (الفصيلة الافيدرية) في مصر باستخدام كل من الدلائل التصنيفية، الشفرة الوراثية وكذلك العلاقات التطورية

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### الملخص العربي

تم في هذه الدراسة اجراء مراجعة تصنيفية دقيقة لجنس الافيدرا في مصر بالاعتماد على الخصائص المورفولوجية للورقة، الساق وكذلك الزهرة. أيضا تم استخدام تقنية تتابع الشفرة الوراثية DNA sequencing لأول مرة على الفصيلة الافيدرية في مصر. تم استخدام ماركر من البلاستيدات الخضراء (*trn*-H/*psb*-A) واخر من النواة (ITS) لتأكيد تعريف الانواع المصرية من جنس الافيدرا وكذلك لإعادة توثيق العلاقات التطورية بينها. اسفرت النتائج ان اسم *Ephedra ciliata* Fischer and C. A. Mey غير قانوني واصبح مرادف لـ *Ephedra foliata* Boiss. الذي ذكر هنا في هذه الدراسة كاسم صحيح. اكدت النتائج انه يمكن استخدام تقنية الشفرة الوراثية DNA barcode كاداه لتعريف أنواع الافيدرا وكذلك الأنواع الأخرى من معرات البذور في مصر.