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Original article

Phylogenetic relationships and DNA barcoding of nine endangered medicinal plant species endemic to Saint Katherine protectorate

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ABSTRACT

A high degree of endemism has been recorded for several plant groups collectively in Saint Katherine Protectorate (SKP) in the Sinai Peninsula. Nine endangered endemic plant species in SKP were selected to test the variable abilities of three different DNA barcodes; Riboluse-1,5- Biphosphate Carboxylase/ Oxygenase Large subunit (*rbcL*), Internal Transcribed Spacer (*ITS*), and the two regions of the plastid gene (*ycf1*) as well as Start Codon Targeted (SCoT) Polymorphism to find the phylogenetic relationships among them. The three barcodes were generally more capable of finding the genetic relationships among the plant species under study, new barcodes were introduced to the National Centre for Biotechnology Information (NCBI) for the first time through our work. The barcode sequences were efficient in finding the genetic relationships between the nine species. However, SCoT polymorphism could only cluster plant species belonging to the same genus together in one group, but it could not cluster plant species belonging to the same families except for some primers solely. *RbcL* was the most easily amplified and identified barcode in eight out of the nine species at the species level and the ninth barcode to the genus level. *ITS* identified all the species to the genus level. Finally, *ycf1* identified six out of the eight species, but it could not identify two of the eight species to the genus level.

1. Introduction

In recent years, Sinai has received great scientific interest, both as an amazing phytogeographical province and as a new growing axis of Egypt (Zahran and Willis, 2009). The recent progress of the socioeconomics and tourism industry in Sinai is focused on the evaluation of its natural resources such as soil, water, animals, and plant wildlife (Kaky and Gilbert, 2020). Studies evaluating these natural resources and tracking changes taking place are urgently needed, particularly on the ecological and molecular levels, on the endemic medicinal plants (Ayyad et al., 2000; El-Barougy et al., 2020; Fakhry et al., 2019). In southern Sinai, there are over 61 rare plant species, some of which are endangered or threatened near-endemics and endemics (Moustafa and Klopatek, 1995). In the Tackholm Egyptian flora (Tackholm, 1974), the databases were reviewed and reported that about 34 of the southern Sinai plants are endemic (Khedr et al., 2002). Such endemic endangered plant species find themselves in vulnerable ecosystems due to high environmental pressure accompanied by global warming, particularly an increasing evapotranspiration rate and harsh climatic conditions at high altitudes (Khan et al., 2012). Consequently, these factors possibly will bring these species under

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environmental pressure until extinction (Al-Qurainy et al., 2018). Therefore, the conservation of genetic diversity is important, as genetic diversity provides continuing evolutionary potential against changing environmental conditions (Dobson, 1998). Medicinal plants play important roles on the therapeutic and economic levels for residents of SKP, The nine target species in this study are collected from their natural habitats for many purposes; for home use, trade and folk medicine by traditional healers and for scientific research, in many cases these plants are collected for grazing and used for fuel wood (Aghakhani Kaaji and Kharazian, 2019; el-Negoumy et al., 1986; El-Wahab et al., 2004; Hashim et al., 2020; IUCN, 2015; Khafaga et al., 2011; Mokni et al., 2019; Moustafa et al., 2015; Omar, 2014; Pieroni et al., 2006; Shaltout et al., 2015; Uritu et al., 2018; Zahran and Willis, 2009). Potential applications in medicine, pharmacy, and in other industrial uses of the nine studied species are mentioned in table S1.

Based on the red list categories of the International Union for Conservation of Nature (IUCN), endemic species found at high conserving hotspots should be documented and inventoried periodically to evaluate their population status and distribution (Ayyad et al., 2000; Baillie and Butcher, 2012). However, botanical records are generally not complete in many biodiversity hotspots, possibly because the taxonomic duty is thwarted by the low discriminatory practice of morphological descriptors for species that are closely related (Francisco-Ortega et al., 2007).

DNA barcoding and genomics approaches are now being applied in many of biodiversity studies, including species identification (Hollingsworth, 2008; Hosein et al., 2017; Savolainen and Karhu, 2000), detection of new taxa (Bell et al., 2012), DNA barcoding for big taxonomic groups (Ojeda et al., 2014), addressing detached taxonomic problems (Feau et al., 2011), conservation of species (Yesson et al., 2011), enabling studies on ecology of plant communities via building phylogenetic trees (Joly et al., 2014).

DNA barcode consists of standard short sequence of DNA, which is unique for every species on our planet in principle and can be easily generated. Numerous plastid sequences were considered, validated and recommended for plant species as ideal barcode loci (Ford et al., 2009; Kress et al., 2005; Pennisi, 2007). The two chloroplast genes segments *rbcL* and maturase k (*matK*) are a pair of these recommended sequences in 2009 by the Consortium for the Barcode of Life (CBOL) Plant Working Group as core barcode (CBOL Plant Working Group, 2009). Given the universality of *rbcL* gene, it has been proposed as a barcode fragment (Hollingsworth et al., 2016). Currently, *rbcL* genes have been commonly used in family and subclass phylogenetic analysis among different seed plant groups (Chase et al., 2007). However, variation in *rbcL* sequence mainly exists at the above-species level, and variation is rarely found at the species level, resulting in low capabilities in species discrimination. Moreover, *rbcL* seems to be more suitable for barcoding lower plants than for seed plants (Chase et al., 2007). *ITS* barcode is a ribosomal DNA in the nuclear genome, that is widely distributed in all photosynthetic eukaryotes (except ferns). *ITS* fragment includes *ITS1*, *ITS2* and 5.8 S, with great differences among the three sequences (Chase et al., 2005). *ITS* barcode characterized by the following advantages: 1) high rate of species identification due to its highly repetition in nuclear genome (Kress and Erickson, 2007); 2) accurately helps in reconstruction of phylogenetic relationships between plant species specifically in lower taxonomic order (Baldwin et al., 1995); 3) there is a large amount of data from *ITS* barcode have been recorded in GenBank (Hollingsworth et al., 2016). *Ycf1* is the second largest gene in chloroplast genome, that encodes a protein of about 1800 amino acids and crucial for viability of plant species (Dong et al., 2015; Kikuchi et al., 2013). Since *ycf1* is too long and likewise variable for the design of universal primers, attention has been paid to DNA barcoding purposes at low taxonomic levels, however its high variability indicates its potency in molecular systemic of higher plants (Dong et al., 2012).

Start Codon Targeted (SCoT) polymorphisms are predominant and reproducible new markers developed on the basis of the short-conserved region flanking the translation start codon (ATG) in plant genes (Bhattacharyya et al., 2013; Collard and Mackill, 2008), which makes it advantageous to other molecular marker techniques. Also, they are considered to be more efficient and reliable, as they exhibit a relatively high annealing temperature (Shahlaei et al., 2014). This technique is designed for most plant research laboratories with standard kits by conventional gel electrophoresis with agarose gels and stains (Zhang et al., 2015). Additionally, because of the lower levels of recombination between gene/traits and SCoT polymorphism, compared with random markers such as Simple Sequence Repeats (SSRs), Random Amplified Polymorphic DNA (RAPD), and Inter-Sequence Simple Repeats (ISSRs), SCoT polymorphisms have a direct use in marker-aided breeding programs (Mulpuri et al., 2013). SCoT markers have been effectively used in the identification of cultivars to evaluate genetic diversity in numerous species including sugarcane, rice, grape, mango, potato, garbanzo and cacti (Abouseadaa et al., 2020; Amirmoradi et al., 2012; Cabo et al., 2014).

In this study, we aimed to identify and classify the nine endemic vascular plant species in SKP using the three DNA barcodes (*rbcL, ITS*, and *ycf1*) for the first time concerning most of them. Moreover, to evaluate the potential and difference between the three barcodes. And finally, to construct phylogenetic trees for assessment the genetic relationships among these nine species using the three barcodes as well as SCoT polymorphism technique. These species are extremely rare, endangered, or critically endangered under the criteria of the IUCN. The results obtained provide a scientific basis for the molecular identification, controlled collection, study the evolutionary relationships and future conservation of these endemic species.

2. Materials and methods

2.1. Plant materials and collection

Nine endangered, endemic, angiosperm plant species were selected from their high-altitude natural habitats in the SKP mountainous region (Fig. 1). The protectorate is located between 28° 30′ to 28° 35′ N and 33° 55′ to 34° 30′ E, and the plateau elevation is between 1,300 to 2,600 m above sea level (Moustafa and Zaghloul, 1996; Moustafa, 2017). The most important consideration for plant species collection was the fact that all collected plants are endemic, endangered, or critically endangered according to the red data book of IUCN (IUCN, 2012), the number of collected individuals per each species was restricted (Almost one individual per species).

Species identification and assignment were independently confirmed prior to the molecular studies and was based on an assessment of morphological descriptors developed by the herbarium section of the Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

The scientific names of the targeted endemic species with their families, life forms, distribution, and their IUCN status are shown in Table 1. The photos of species in their natural habitats are shown in Fig. 2. Plant samples were collected during the spring season of 2019. Immediately after collection, the fresh leaves from the plant samples were placed in silica gel beads for further lab analysis.

2.2. DNA extraction

Genomic DNA was extracted from 100 mg silica-gel dried leaf tissue for each plant species by using a DNeasy Plant Mini Kit (QIA-

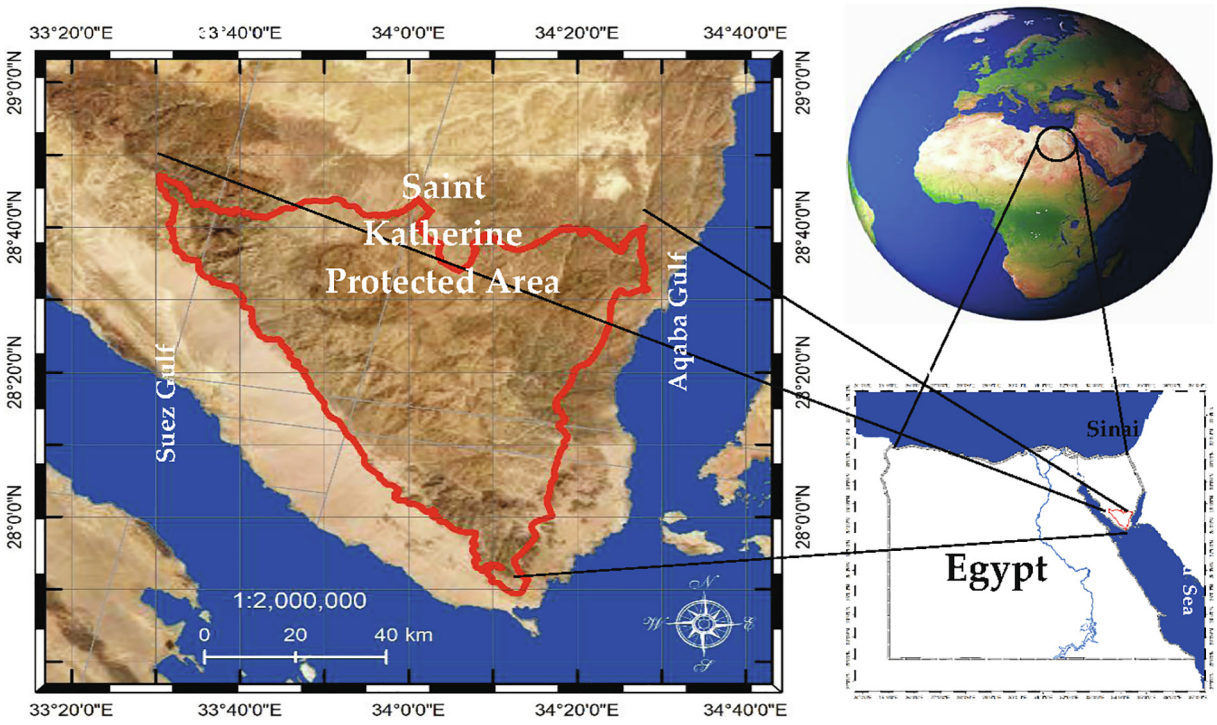

Fig. 1. Map of study area for Saint Katherine protected area, Sinai, Egypt.

Table 1
List of the studied endemic species with their families, Arabic names, life forms, chorology, and their IUCN status.

Family	Plant species	Arabic name	Life form	Distribution	*IUCN status
Solanaceae	*Hyoscyamus boveanus* (Dunal) Asch. & Schweinf	السكران	Chamaephyte	Endemic to Egypt	Endangered
Caryophyllaceae	*Bufonia multiceps* Decne.	العدمة	Hemi-cryptophyte	Endemic to SKP	(very rare) Endangered
	Silene leucophylla Boiss.	————	Hemi-cryptophyte	Endemic to SKP	Endangered
	Silene schimperiana Boiss.	لصيق	Hemi-cryptophyte	Endemic to SKP	Endangered
Lamiaceae	*Nepeta septemcrenata* Benth.	زيتية	Chamaephyte	Endemic to SKP (near endemic)	Endangered
	Phlomis aurea Decene.	عورور	Chamaephyte	Endemic to SKP	Endangered
Polygalaceae	*Polygala sinaica* var. *sinaica* Botsch.	هيكل - صر	Chamaephyte	Endemic to Sinai	Endangered
Primulaceae	*Primula boveana* Decne. ex Duby	خس الجبل - لباخ	Hemi-cryptophyte	Endemic to SKP	Critically endangered
Rosaceae	*Rosa arabica* Crep.	الورد البلدي	Nano-phanerophyte	Endemic to SKP	Critically endangered

GEN, Santa Clarita, CA, USA) according to the manufacturer's protocol. The concentration of the extracted DNA was measured using a Nanodrop Spectrophotometer (Thermo Fisher Scientific Inc.). DNA concentrations were then adjusted to 10 ng/μL in all samples for subsequent SCoT polymorphism and DNA barcoding PCR amplification.

2.3. Scot PCR analysis

SCoT polymorphism PCR-based amplification was performed using nineteen SCoT primers as developed by (Collard and Mackill, 2008). The primers were synthesized by Macrogen (Seoul, Republic of Korea) in 10 nmol stock concentration, obtained lyophilized, then rehydrated using sterile water to become 100 μM final concentration, and finally stored at −20 °C. The nineteen primers were screened against the nine plant species. Only ten of them gave prominent and reproducible bands with the nine samples, so that continued to the final analyses. PCR was performed on reaction mixtures of 25 μL, 25 ng of genomic DNA, 2x MyTaq red Mix 50 Reactions (BIOLINE, Lot No: MTRX-516204), 2.0 μL of each primer (2.5 μM), and distilled deionized water. The PCR amplification protocol was adjusted as follows: 94 °C for 5 min as an initial denaturation cycle, followed by 40 cycles (94 °C for 1 min, 50 °C for 1 min, then 72 °C for 90 s) with a final extension at 72 °C for 7 min. PCR amplification products were resolved by electrophoresis in 2% agarose gel containing ethidium bromide (0.5 μg/mL) in 1X TBE buffer. A 100-bp-plus DNA ladder was used as a molecular size standard. The PCR products were visualized using UV light and photographed using a Gel Doc™ XR + System with Image Lab™ Software (Bio-Rad).

2.4. DNA barcodes PCR analysis

PCR amplification of the three barcode loci; two plastid loci the (*rbcL* and *ycf1)* barcodes and one nucleur locus for the rDNA (*ITS),* were performed in a Thermal Cycler TC-TE BOE 8,089,602 (BOECO, Germany) using three primer pairs, shown in Table 2. PCR reactions were performed in a 50-μL volume containing approximately 50 ng of genomic DNA, 2X MyTaq red Mix 50 Reactions (BIOLINE, Lot No: MTRX-516204), 1.0 μL of each primer (2.5 μM), and distilled deionized water. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and 72 °C for 10 min. The desired PCR products were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 μg/mL) in 1X TBE buffer. A 100-bp-plus DNA ladder was used as a molecular size standard.

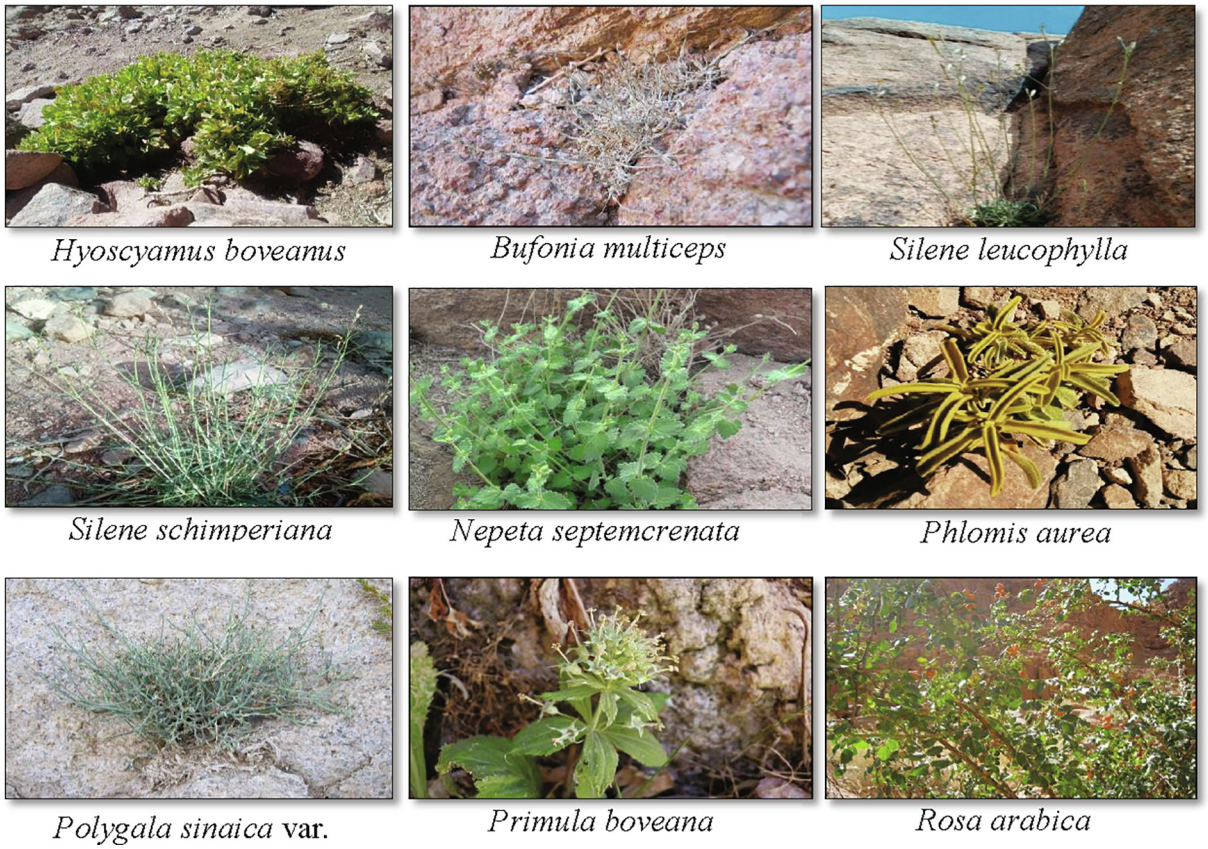

Fig. 2. Photos and scientific names of nine species grown in their natural habitats at the mountainous area of SKP.

Table 2
PCR amplification of the three primer pairs; *rbcL*, *ycf1*, and *ITS* regions.

Primer name	Primer Sequence	Tm (°C)	Reference
rbcLa_F	5′-ATGTCACCACAAACAGAGACTAAAGC-3′	64.7	(CBOL, 2009)
rbcLa_R	5′-GTAAAATCAAGTCCACCRCG-3′	56.4	
ycf1b_F	5′-TCTCGACGAAAATCAGATTGTTGTGAAT-3′	64.3	(Dong et al.,2015)
ycf1b_R	5′-ATACATGTCAAAGTGATGGAAAA-3′	60.4	
ITS18_F	5′-GTCCACTGAACCTTATCATTTAGAGG-3′	64.7	(Rohwer et al., 2019)
ITS26_R	5′-GCCGTTACTAAGGGAATCCTTGTTAG-3′	66.3	

The PCR products were visualized using UV light and photographed. Amplicons of the appropriate size were purified from the gel using Gene JET Gel Extraction Kit, Thermo Scientific Catalog number: K0691. The purified PCR products were directly sequenced by Macrogen lab (Seoul, South Korea).

The obtained sequences were submitted to the GenBank and were given the following accession numbers: MT333246, MT333247, MT333248, MT333249, MT333250, MT333251, MT333252, MT333253, MT333254, MT333255, MT333256, MT333257, MT333258, MT333259, MT333260, MT333261, MT333262, MT333263, and MT333264.

2.5. Data analysis

For SCoT polymorphism data analysis, the amplified bands were scored using the free software PyElph1.4 (https://pyelph.software.informer.com/1.4). Only clear bands were scored, while faint bands were neglected. The bands were scored (1) for presence or (0) for absence to create a binary dataset. The capacity of SCoT primers to discriminate between the nine genotypes were determined by calculating the values of polymorphic information content (PIC), Resolving power (Rp) and Marker Index (MI). PIC value for each primer, which reflects the degree of detecting polymorphism, was calculated according to (McGregor et al., 2000) using the formula: PIC = $1-\sum (Pi)^2$, where Pi is the proportion of samples carrying the i-th allele of a particular locus. Rp value was calculated according to (Powell et al., 1996) using the following equation; Rp = Σ IB, where IB represents the band informativeness, was measured from the following equation, IB = $1 - (2 \times | 0.5 - p |)$, where P is the frequency of accessions that ports bands (Prevost and Wilkinson, 1999). The number of amplified polymorphic bands was divided by the total number of amplified bands by the same primer or primer combination to calculate the percentage of polymorphism. Jaccard's coefficient was used to estimate genetic similarity matrix (Jaccard, 1908). Past 4.03 free software (https://past.en.lo4d.com/windows) was used to construct the dendrogram and the principal component analysis (PCA). The dendrogram was created by using cluster analysis and the un-weighted pair group method of the arithmetic averages (UPGMA). The Principal component analysis (PCA) was performed using a D centre module (Jaccard, 1908).

The resulting DNA barcodes sequences were analysed using different software and online tools, but first the 3′ and 5′ peripheral noisy parts of each sequence were trimmed, and then primers were removed. The local CLUSTAL W (Thompson et al., 1994) free software (http://www.clustal.org/clustal2) was used for the sequence

alignment of barcode sequences. An ITOL online website (https://itol.embl.de/) was used for phylogenetic tree construction (Letunic and Bork, 2007). Principal component analysis (PCA) and heat map analysis were conducted using R software provided by the online free tool ClustVis 2.0 (https://biit.cs.ut.ee/clustvis). BLASTn was used for aligning DNA barcode sequences against sequences in the National Centre of Biotechnology Information (NCBI) database using default parameters (Altschul et al., 1997).

3. Results

3.1. Molecular phylogeny based on SCoT marker analysis

Nineteen SCoT primers were used in the SCoT marker analysis. After three trials, only ten of them were reproducible and yielded scorable bands as shown in Table 3 and Fig. 3. The total number of amplification products was 197, out of which 196 were polymorphic with a polymorphism percentage of 99.5%.

The number of scorable bands per primer ranged from 12 for primer SCoT 20 to 26 for primer SCoT 33, and the polymorphism percentage ranged from 94.7% for primer SCoT 23 to 100% for primers Scot 1, 6, 11, 12, 14, 15, 16, 20, and 33 (Table 3).

The average polymorphism information content (PIC) of the ten used SCoT primers was 0.81 (Table 3). The PIC ranged from 0.75 to 0.87; the highest PIC values were for primers SCoT 6 and SCoT 15, and the lowest PIC values were for primers SCoT 20 and SC

The Rp value ranged from 7.1 to 14.7 (Table 3); where the highest RP values were for primers SCoT 11 and SCoT 33, and the lowest RP values were for the primers SCoT 20 and SCoT 6, with an average of 11.9 for the ten Primers.

The SCoT analysis-based dendrogram obtained using the UPGMA method according to Jaccard's coefficient divided the nine species into two main clusters, as shown in Fig. 4A.

The first cluster has only sample 9 (Primula boveana). The second group is further subclustered to contain the remaining species. Samples 6 and 7 Silene leucophylla and Silene schimperiana were separated together in one group. The results obtained from PCA (Fig. 4B) were in harmony with dendrogram analysis, and both **showed comparable groups.** According to Jaccard's similarity matrix (Table 4), the highest similarity (80.6%) was found between *Silene leucophylla* and *Silene schimperiana* that belong to the same genus followed by (47.2%) for *Nepeta septemcrenata* and *Phlomis aurea* that belong to the same family.

3.2. Plant identification and genetic relationships using DNA barcodes

3.2.1. PCR amplification and identification of the samples

The three barcode primers *rbcL*, *ITS*, and *ycf1* produced expected product sizes of approx. 600 bp, 900 bp, and 750 bp, respectively, except for *Polygala sinaica* with the ycf1 barcode, which did not give the expected result even after many trials with different conditions. The BLASTn tool provided by the NCBI was used to identify most of the sequences according to the species or genus level, on the basis of the availability of corresponding sequences in the database. Species identification according to the *rbcL* barcode revealed that the *rbcL* gene was successful in identifying eight out of **the** nine studied plant species at the species level except for ***Rosa arabica***, (Fig. 4A) with a query coverage of 98%-100% and an identity percentage of 94.47%-99.81% (Table 5A). *Rosa arabica* was only identified to the genus *Rosa*, due to absence of its *rbcL* sequence, and as mentioned before, the *rbcL* sequence published for *Rosa arabica* from our work is the first on databases. For the *ITS* barcode, all the plant species were identified to the genus level only (Fig. 5B) with a query coverage of 100% and an identity percentage of 89.74%-99.03% (Table 5B). The *ycf1* barcode was able to identify six plant species at the genus level (Fig. 5C), with a query coverage of 90%-100% and an identity percentage of 92.65%-99.36% (Table 5C), but it was unable to identify two species (*Phlomis aurea* and *Bufonia multiceps*) neither to the species nor to the genus levels.

Heat maps are based on the similarity matrices generated using the barcodes sequences of the studied plants and previously published barcodes sequences. It reflects how the barcode sequences show possible diversity within the species and genus. Heat map analysis was used to visually differentiate the structure of studied plant samples according to the color intensity. As the color intensity increases, the similarity increases. The analysis revealed the high capacity of *ycf1* followed by *ITS* to differentiate the studied samples according to species and genus (Fig. 1S (A, B and C).

PCA is also based on the similarity generated by sequences alignment. It can explain the diversity by collecting the most similar samples in one cluster, which could be helpful in finding the closest samples according to sequence similarity. Similar results have been concluded using PCA analysis, where *ycf1* and *ITS* overload *rbcL* DNA barcode (Fig. 2S (A, B and C)

3.3. Phylogenetic relationships based on plant DNA barcodes analysis

The barcode sequences obtained from the plant species under study were subjected to multiple alignment with each other. Multiple alignment revealed considerable difference between different samples at the genus level (Fig. 6 A-C). With respect to *rbcL*, *ITS*, and *ycf1* genes, sample 4 (*Bufonia multiceps*) was the most different among other species per the evolutionary basis. The three barcodes were all successful in combining samples 6 and 7 (*Silene leucophylla* and *Silene schimperiana*) in one cluster, which belong to the same genus. Samples 6, 7, and 4 (*Silene leucophylla*, *Silene schimperiana* and *Bufonia multiceps*) all belong to family Caryophyl-

Table 3
Primers codes, sequences, total and polymorphic bands produced, percentage of polymorphism, PIC and RP values.

Primer code	Sequence	Totalbands	Polymorphicbands	Percentage of Polymorphism	PIC	RP
SCoT 1	CAACAATGGCTACCACCA	20	20	100%	0.8	13
SCoT 6	CAACAATGGCTACCACGG	14	14	100%	0.87	8.7
SCoT 11	AAGCAATGGCTACCACCA	25	25	100%	0.83	14.7
SCoT 12	ACGACATGGCGACCAACG	18	18	100%	0.77	10.9
SCoT 14	ACGACATGGCGACCACGC	18	18	100%	0.79	11.8
SCoT 15	ACGACATGGCGACCGCGA	23	23	100%	0.85	12.7
SCoT 16	ACCATGGCTACCACCGAC	22	22	100%	0.80	13.3
SCoT 20	ACCATGGCTACCACCGCG	12	12	100%	0.75	7.1
SCoT 23	CACCATGGCTACCACCAG	19	18	94.7%	0.79	11.6
SCoT 33	CCATGGCTACCACCGCAG	26	26	100%	0.83	14.7
Total		**197**	**196**			
Average				**99.5%**	**0.81**	**11.9**

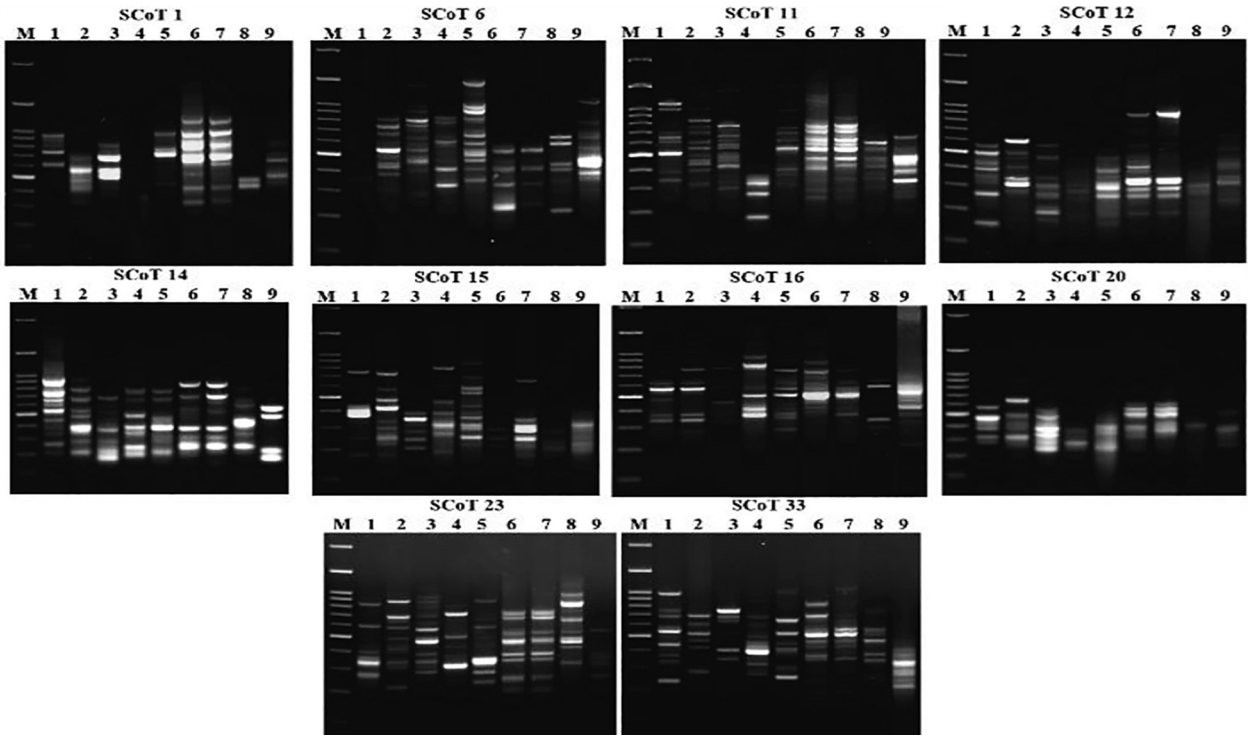

Fig. 3. SCoT–PCR product on 2% agarose gel using the ten SCoT primers. M: is the DNA size marker (100 bp plus). Lanes from 1 to 9 (left to right) 1. *Hyoscyamus boveanus*, 2. *Phlomis aurea*, 3. *Nepeta septemcrenata*, 4. *Bufonia multiceps*, 5. *Polygala sinaica*, 6. *Silene leucophylla*, 7. *Silene schimperiana*, 8. *Rosa arabica*, 9. *Primula boveana*.

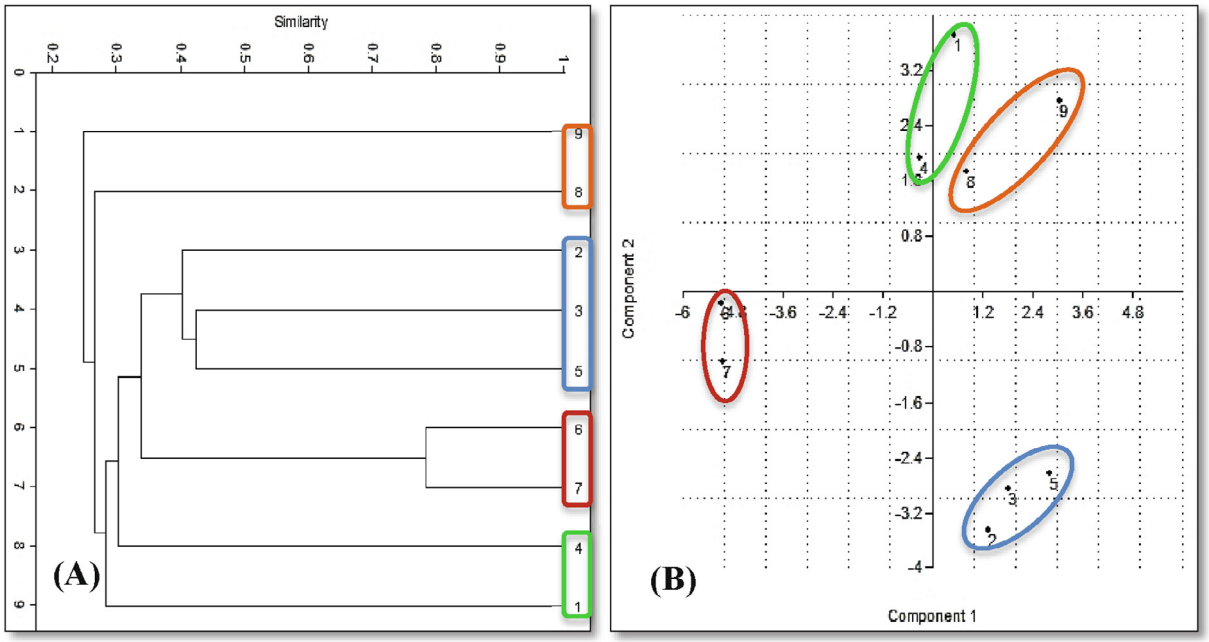

Fig. 4. (A) Dendrogram of UPGMA cluster analysis based on Jaccard's similarity coefficient of SCoT analysis of the nine plant species. (B) Principal Component Analysis (PCA) of the SCoT data of the nine species showing the two-dimensional (PC1 and PC2) plot. 1. *Hyoscyamus boveanus*, 2. *Phlomis aurea*, 3. *Nepeta septemcrenata*, 4. *Bufonia multiceps*, 5. *Polygala sinaica*, 6. *Silene leucophylla*, 7. *Silene schimperiana*, 8. *Rosa arabica*, 9. *Primula boveana*.

laceae. However, only *rbcL* and *ycf1* combined samples 2 and 3 *(Phlomis aurea* and *Nepeta septemcrenata) which belonged to family Labiatae, together in one cluster.*

The *rbcL* available sequences for the studied species that were previously published on the NCBI database, were used to construct a phylogenetic tree to compare it with the tree obtained from the

Table 4
Jaccard's similarity matrix based on the SCoT analysis of the nine plant species.

	H. boveanus	*P. aurea*	*N. septemcrenata*	*B. multiceps*	*P. sinaica*	*S. leucophylla*	*S. schimperiana*	*R. arabica*	*P. boveana*
H. boveanus	100%								
P. aurea	27.9%	100%							
N. septemcrenata	26.6%	47.2%	100%						
B. multiceps	12.9%	23.3%	19.6%	100%					
P. sinaica	24.8%	29.9%	28.7%	21.1%	100%				
S. leucophylla	24.2%	23.6%	22.5%	40.2%	22.5%	100%			
S. schimperiana	19.8%	24.1%	25.7%	45.9%	23.1%	80.6%	100%		
R. Arabica	24.7%	20.7%	23.7%	20.4%	25%	23.1%	22.7%	100%	
P. boveana	25.8%	25.9%	28.9%	22.6%	27.2%	20.9%	21.5%	19.1%	100%

Table 5
BLASTn results for the barcodes sequences against NCBI databases. Query; the barcode sequence obtained in the current study, Subject; NCBI published sequences, Subject ID; accession number of the NCBI published sequences.

(A) *rbcL*

Query	Subject species	Subject ID	Bit score	Query cover	E-value	Identity %
H. boveanus	*H. boveanus*	MF668605.1	939	100%	0.0	98.51%
Phlomis aurea	*Phlomis aurea*	KY794564.1	931	99%	0.0	98.31%
N. septemcrenata	*N. septemcrenata*	KY794558.1	974	99%	0.0	99.08%
Bufonia multiceps	*Bufonia multiceps*	KX709610.1	806	100%	0.0	96.89%
Polygala sinaica	*Polygala sinaica*	KY656729.1	896	98%	0.0	98.25%
Silene leucophylla	*Silene leucophylla*	MK055336.1	948	98%	0.0	99.81%
Silene schimperiana	*Silene schimperiana*	MF668591.1	865	100%	0.0	99.58%
Rosa arabica	*Rosa canina*	FN689381.1	843	100%	0.0	94.42%
Primula boveana	*Primula boveana*	KY656738.1	826	100%	0.0	97.15%

(B) *ITS*

Query	Subject species	Subject ID	Bit score	Query cover	E-value	Identity %
H. boveanus	*H. bornmulleri*	KU295790.1	795	100%	0.0	89.74%
P. aurea	*P. lychnitis*	AY792771.1	730	100%	0.0	89.82%
N. septemcrenata	*N. crassifolia*	AJ515307.1	198	100%	4e-47	93.08%
Bufonia multiceps	*Bufonia parviflora*	JN589044.1	891	100%	0.0	91.38%
Polygala sinaica	*Polygala erioptera*	KF805107.1	1271	100%	0.0	98.21%
Silene leucophylla	*Silene flavescens*	KX757520.1	1288	100%	0.0	99.03%
Silene schimperiana	*Silene armena*	KX757619.1	1218	100%	0.0	98.41%
Rosa arabica	*Rosa canina*	FM164946.1	1112	100%	0.0	95.31%
Primula boveana	*Primula verticillata*	AY680732.1	1242	100%	0.0	97.52%

(C) *Ycf1*

Query	Subject species	Subject ID	Bit score	Query cover	E-value	Identity %
H. boveanus	*Hyoscyamus niger*	KF248009.1	695	98%	0	96.02%
Phlomis aurea	*Colquhounia coccinea*	MN165115.1	1112	99%	0	94.01%
N. septemcrenata	*Nepeta cataria*	JF289048.1	797	100%	0	98.25%
Bufonia multiceps	*Psammosilene tunicoides*	NC_045947.1	418	100%	2.16E-112	94.30%
Silene leucophylla	*Silene littorea*	MN365983.1	893	98%	0	98.051%
Silene schimperiana	*Silene littorea*	MN365983.1	1086	90%	0	96.77%
Rosa arabica	*Rosa luciae*	MN689791.1	1411	100%	0	99.36%
Primula boveana	*Primula knuthiana*	NC_039350.1	547	100%	2.64E-151	92.65%

rbcL sequences of our own specimens. Both phylogenetic trees (Fig. 6.A and Fig. 7) were the same.

4. Discussion

To the best of our knowledge, this is the first work to examine the ability of DNA barcodes and SCoT polymorphism in investigating the genetic relationships between the selected species. In addition, DNA barcoding involves the production of PCR amplicons from particular regions to sequence them and these sequence data are used to identify or "barcode" that organism to make a distinction from other species (Lebonah et al., 2014), one *rbcL* sequence for *Rosa arabica*, and all *ITS* and *ycf1* sequences were novel barcodes for these species to the NCBI database.

SCoT molecular marker was able to differentiate between the nine species; it was also able to combine samples 6 and 7 that belonged to the same genus in one group but was unable to combine different species that belonged to the same family together in one group. Only different SCoT primers were able to combine different species that belonged to the same family together in one group; for example, SCoT primers 1, 12, and 23 combined samples 4, 6, and 7 that belong to the same family together in one group, and SCoT primers 6 and 16 combined samples 2 and 3 that belong to the same family together in one group. (Shahlaei et al., 2014) used ISSR and SCoT molecular markers to study the genetic diversity in *Lycopersicum esculentum*, and they estimated that the mean PIC values to be 0.142 and 0.088 for SCoT and ISSR respectively, and the mean RP values to be 1.88 and 1.55 for both markers respectively.

DNA barcoding has been proposed as a powerful tool not only for identifying and confirming species but also for finding genetic relationships among the species. The three barcodes we used were all reproducible and produced the expected band size. Only *ycf1* with sample 5 (*Polygala sinaica*) could not produce the expected

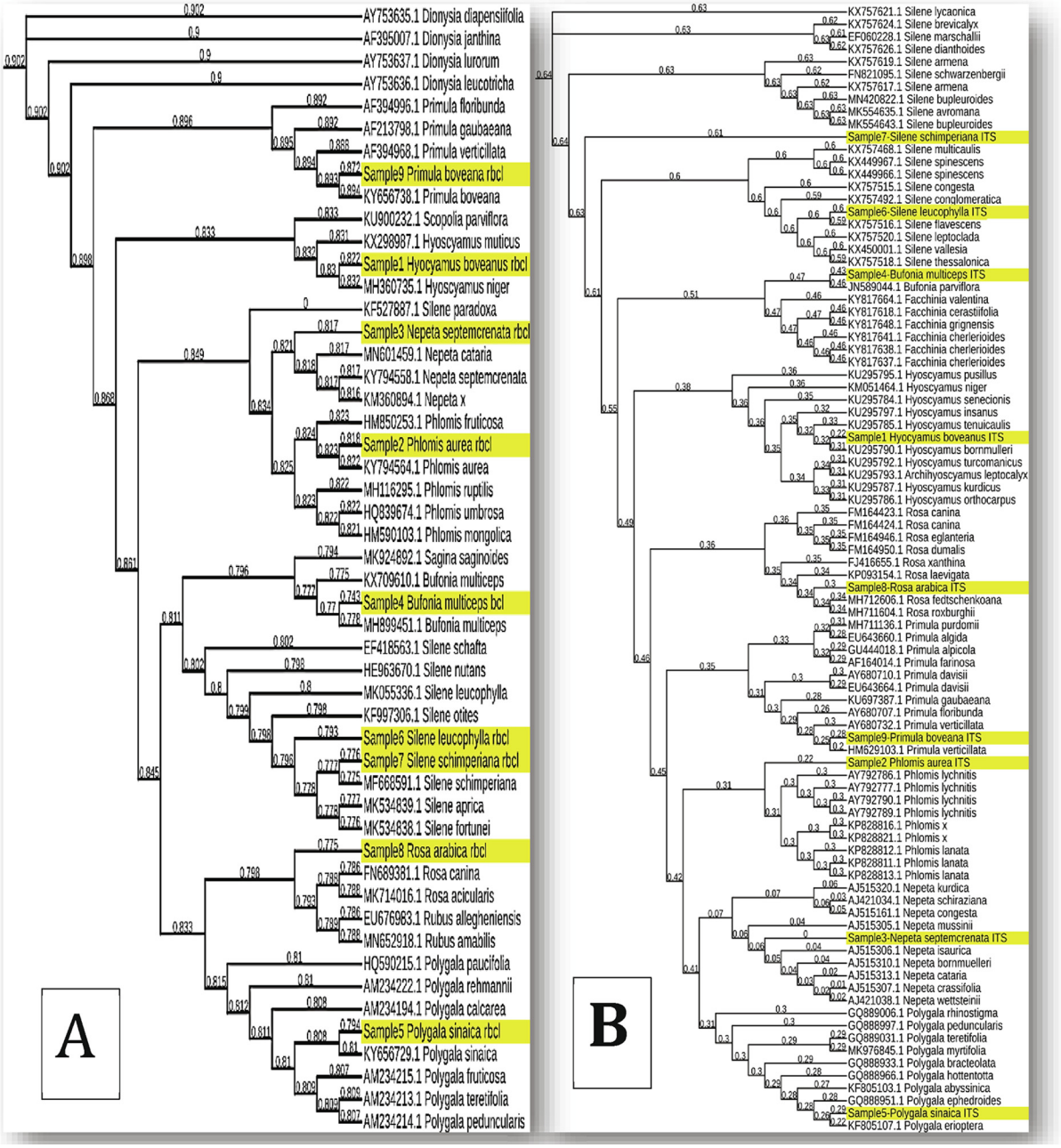

Fig. 5. Neighbor-joining Phylogenetic tree for the nine plant species based on the three barcodes multiple sequence alignments on the NCBI databases. (A) *rbcL*, (B) *ITS* and (C) *ycf1*.

band with different trials and conditions, emphasizing that the same DNA sample was used with the other successful barcodes. The use of the *rbcL* barcode, although all *rbcL* sequence for eight of the nine selected species are already published, was to standardize the consistency of our protocol and sequencing efficiency. As due to the scarcity and endangered nature of the selected species, we couldn't obtain more than one individual for each species to obey the rules stated by the organization of the nature protection sector in the Egyptian Environmental Affairs Agency (EEAA). The phylogenetic relationship between the 8 species and *Rosa canina* as *Rosa arabica* wasn't published before according to the *rbcL* sequences available on the NCBI database was shown in the dendrogram (Fig. 7), and it was exactly similar to that obtained from the *rbcL* sequences of our samples (Fig. 6 A), which ensures that our sequences are correct and the sequence efficiency was fine, and could be relied on with the rest of the sequences. Also, the success of the obtained sequences to correlate the specimens to either the species or genus level proves their success.

The produced barcodes identified most of the plant species under study, especially *rbcL* that identified 8 out of 9 species to the species level and the ninth species (*Rosa arabica*) to the genus level. The *ITS* barcode identified all the nine species to the genus level, but the *ycf1* barcode failed totally to identify two species (*Phlomis aurea* and *Bufonia multiceps*). This failure is due to the absence of available sequences for those species in the NCBI databases. DNA barcoding was more successful than SCoT in finding the

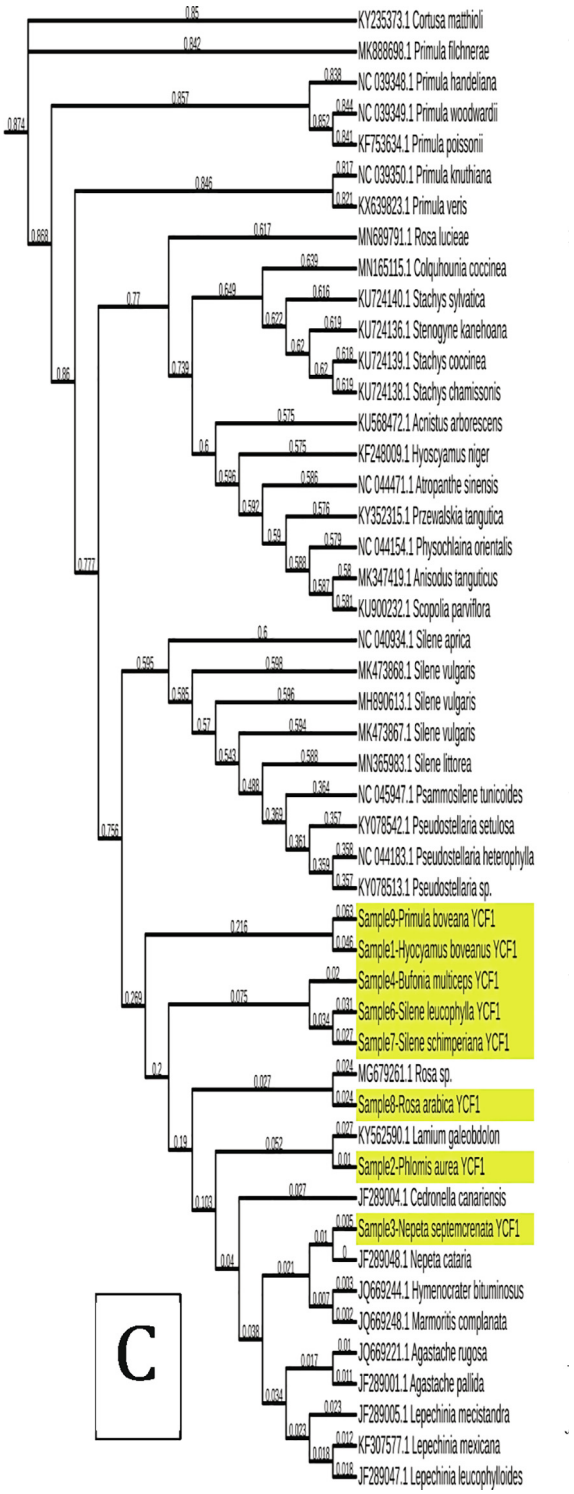

Fig. 5 (*continued*)

genetic relationships among different plant species, although both techniques could combine different species belonging to the same genus. However, DNA barcoding exceeded SCoT in combining different species belonging to the same family together in the same group, except for *ITS* with regard to samples 2 and 3 (*Phlomis aurea* and *Nepeta septemcrenata)*.

The *rbcl* and *ITS* barcodes were used because of the recommendation of the Consortium for the Barcode of Life (CBOL Plant Working Group, 2009) that the chloroplast genes *rbcL* and matK are the core barcodes of plant species. In addition, the intergenic sequence *trnH-psbA* along with the nuclear gene *ITS* act as supplement barcodes (CBOL Plant Working Group, 2009). The *rbcL* barcode was favored in our study to *matK* because of its widespread availability, easy amplification, and comparability (Hollingsworth et al., 2016). In addition, the results obtained with the *rbcL* gene results confirm its efficiency in phylogenetic analysis within the family and subclass of angiosperms (Fazekas et al., 2008). The barcode could combine different species belonging to the same families together in one group. In contrary to (Gonzalez et al., 2009; Kress and Erickson, 2007; Newmaster et al., 2006) *rbcL* sequences were able to find variation at the species level and did not result in poor abilities of species discrimination as they proposed, but it was able to differentiate between samples 6 and 7 and identify them solely as *Silene leucophylla* and *Silene schimperiana* and was able to group them together. So, our study strongly recommends *rbcL* as a core plant barcode.

ITS identified all the plant species to the genus level (100%), but this result may be due to sequence unavailability on the databases for the species under study. *ITS* was previously recommended as a core DNA barcode for identifying land plant species (Chase et al., 2007; Kang et al., 2017). *Ycf1* showed the least amplification percentage of 88.9%, compared with both *rbcL* and *ITS* (100%). In addition, *ycf1* had the least identification ability, which was due to the unavailability of sequences in the databases. However, *ycf1* produced a phylogenetic tree similar to that produced by *rbcL*, and its combined samples 4, 6 and 7 that belonged to the same family together in the same group. In addition, *ycf1* also combined samples 2 and 3 belonging to the same family together in one group. The least amplification percentage of the *ycf1* gene may be attributed to the absence of *ycf1* in some taxa (Dong et al., 2015).

5. Conclusions

In conclusion, in this study, 19 new barcodes sequences were introduced for the first time to the NCBI databases. The efficacy of DNA barcoding to identify different plant species is great and powerful. It will add greatly to taxonomic and evolutionary studies that would be carried on these wild plant species. Also, DNA barcodes sequences were able to efficiently cluster the studied species into the appropriate groups. SCoT molecular marker was unable to cluster plant species belonging to the same family together, it was only able to cluster plant species belonging to the same genus. DNA barcodes are the most powerful tool for plant molecular identification, genetic diversity study, and would help and support in genetic conservation programs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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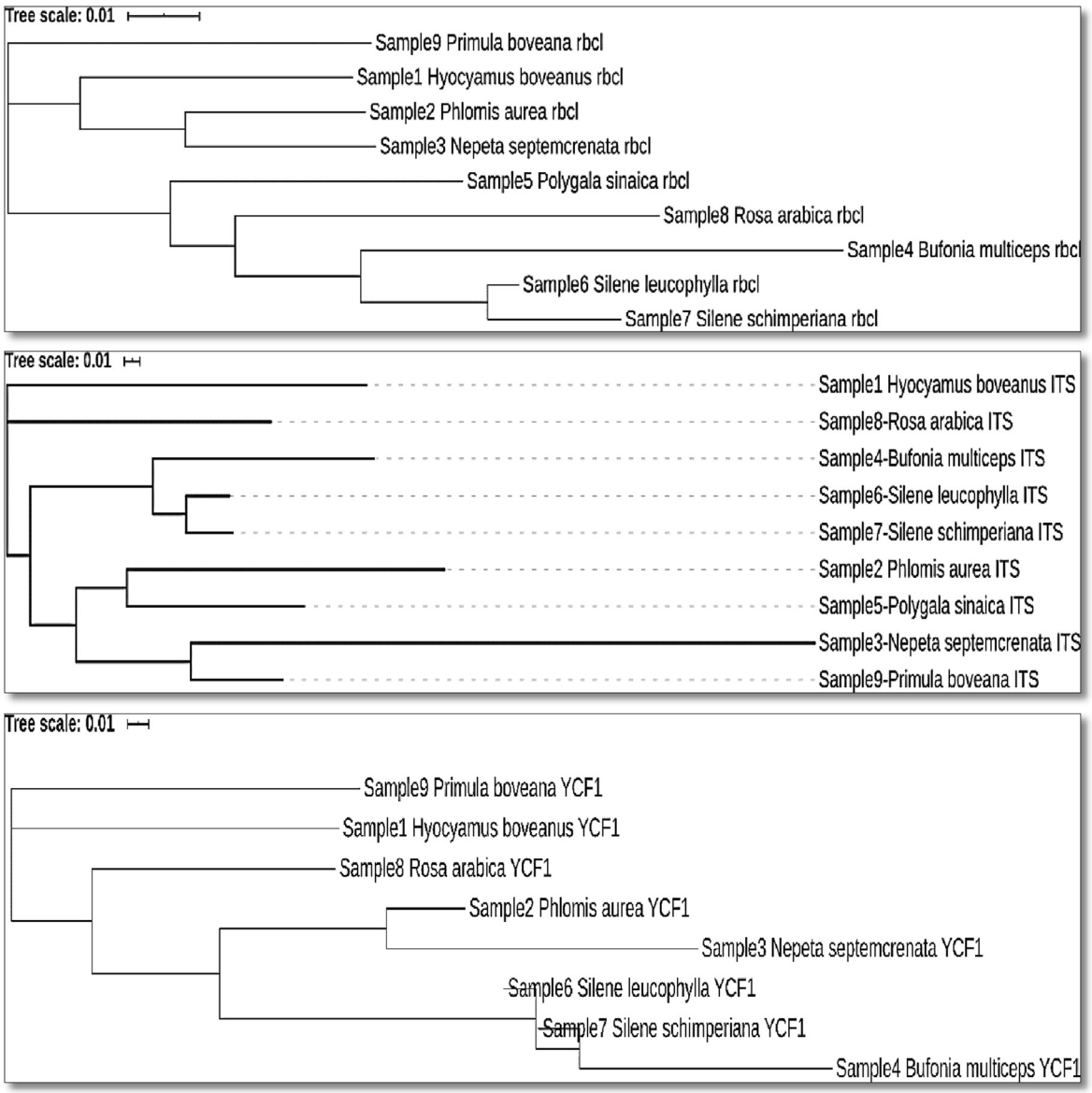

Fig. 6. (A-C) Phylogenetic tree for the nine plant species based on DNA barcodes sequences alignments (A*) rbcL*, (B) *ITS* and (C) *ycf1*.

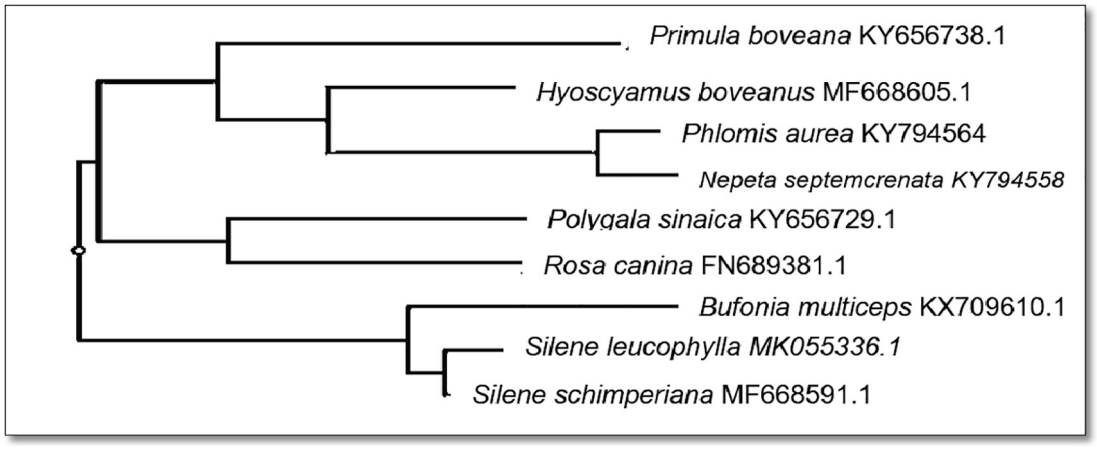

Fig. 7. Phylogenetic tree for the nine plant species based on the *rbcL* sequences available on the NCBI databases.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.12.043.

References

Abouseadaa, H.H., Atia, M.A.M., Younis, I.Y., Issa, M.Y., Ashour, H.A., Saleh, I., Osman, G.H., Arif, I.A., Mohsen, E., 2020. Gene-targeted molecular phylogeny, phytochemical profiling, and antioxidant activity of nine species belonging to family Cactaceae. Saudi J. Biolog. Sci. 27, 1649–1658. https://doi.org/10.1016/j.sjbs.2020.03.007.

Aghakhani Kaaji, F., Kharazian, N., 2019. Flavonoid Diversity and Morphological Variations Among Seven Phlomis Species in Zagros. Iran. Iran J Sci Technol Trans Sci 43, 415–431. https://doi.org/10.1007/s40995-017-0344-4.

Al-Qurainy, F., Nadeem, M., Khan, S., Alansi, S., Tarroum, M., Al-Ameri, A.A., Gaafar, A.-R.-Z., Alshameri, A., 2018. Rapid plant regeneration, validation of genetic integrity by ISSR markers and conservation of Reseda pentagyna an endemic plant growing in Saudi Arabia. Saudi J. Biolog. Sci. 25, 111–116. https://doi.org/10.1016/j.sjbs.2017.07.003.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25, 3389–3402.

Amirmoradi, B., Talebi, R., Karami, E., 2012. Comparison of genetic variation and differentiation among annual Cicer species using start codon targeted (SCoT) polymorphism, DAMD-PCR, and ISSR markers. Plant Syst. Evol. 298, 1679–1688.

Ayyad, M.A., Fakhry, A.M., Moustafa, A.-R.-A., 2000. Plant biodiversity in the Saint Catherine area of the Sinai peninsula. Egypt. Biodiv. Conservat. 9, 265–281. https://doi.org/10.1023/A:1008973906522.

Baillie, J., Butcher, E.R., 2012. Priceless or worthless? : the world's most threatened species.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J., 1995. The its Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. Ann. Mo. Bot. Gard. 82, 247–277. https://doi.org/10.2307/2399880.

Bell, D., Long, D.G., Forrest, A.D., Hollingsworth, M.L., Blom, H.H., Hollingsworth, P. M., 2012. DNA barcoding of European Herbertus (Marchantiopsida, Herbertaceae) and the discovery and description of a new species: DNA BARCODING HERBERTUS. Mol. Ecol. Resour. 12, 36–47. https://doi.org/10.1111/j.1755-0998.2011.03053.x.

Bhattacharyya, P., Kumaria, S., Kumar, S., Tandon, P., 2013. Start Codon Targeted (SCoT) marker reveals genetic diversity of Dendrobium nobile Lindl., an endangered medicinal orchid species. Gene 529, 21–26. https://doi.org/10.1016/j.gene.2013.07.096.

Cabo, S., Ferreira, L., Carvalho, A., Martins-Lopes, P., Martín, A., Lima-Brito, J.E., 2014. Potential of Start Codon Targeted (SCoT) markers for DNA fingerprinting of newly synthesized tritordeums and their respective parents. J. Appl. Genet. 55, 307–312. https://doi.org/10.1007/s13353-014-0211-3.

CBOL Plant Working Group, 2009. A DNA barcode for land plants. Proc. Natl. Acad. Sci. U.S.A. 106, 12794–12797. https://doi.org/10.1073/pnas.0905845106

Chase, M.W., Cowan, R.S., Hollingsworth, P.M., van den Berg, C., Madriñán, S., Petersen, G., Seberg, O., Jørgsensen, T., Cameron, K.M., Carine, M., Pedersen, N., Hedderson, T.A.J., Conrad, F., Salazar, G.A., Richardson, J.E., Hollingsworth, M.L., Barraclough, T.G., Kelly, L., Wilkinson, M., 2007. A Proposal for a Standardised Protocol to Barcode All Land Plants. Taxon 56, 295–299.

Chase, M.W., Salamin, N., Wilkinson, M., Dunwell, J.M., Kesanakurthi, R.P., Haider, N., Haidar, N., Savolainen, V., 2005. Land plants and DNA barcodes: short-term and long-term goals. Philos. Trans. R Soc. Lond. B Biol. Sci. 360, 1889–1895. https://doi.org/10.1098/rstb.2005.1720.

Collard, B.C.Y., Mackill, D.J., 2008. Start Codon Targeted (SCoT) Polymorphism: A Simple, Novel DNA Marker Technique for Generating Gene-Targeted Markers in Plants. Plant Mol. Biol. Rep. 27, 86. https://doi.org/10.1007/s11105-008-0060-5.

Dobson A. P., 1998. Conservation and Biodiversity: A Scientific American Library Volume, Andrew P. Dobson. 1996. Freeman, New York. 264 pages. ISBN: 0-7167-5057-0. $32.95. Bulletin of Science, Technology & Society 18, 297–298. https://doi.org/10.1177/027046769801800429

Dong, W., Liu, J., Yu, J., Wang, L., Zhou, S., 2012. Highly Variable Chloroplast Markers for Evaluating Plant Phylogeny at Low Taxonomic Levels and for DNA Barcoding. PLoS ONE 7,. https://doi.org/10.1371/journal.pone.0035071 e35071.

Dong, W., Xu, C., Li, C., Sun, J., Zuo, Y., Shi, S., Cheng, T., Guo, J., Zhou, S., 2015. ycf1, the most promising plastid DNA barcode of land plants. Sci Rep 5, 8348. https://doi.org/10.1038/srep08348.

el-Negoumy, S.I., Abdalla, M., Saleh, N., 1986. Flavonoids of Phlomis aurea and P. floccosa. https://doi.org/10.1016/0031-9422(86)88052-9

El-Barougy, R.F., Elgamal, I., Rohr, R.P., Probert, A.F., Khedr, A.A., Bacher, S., 2020. Functional similarity and dissimilarity facilitate alien plant invasiveness along biotic and abiotic gradients in an arid protected area. Biol. Invasions 22, 1997–2016. https://doi.org/10.1007/s10530-020-02235-3.

El-Wahab, R.A., Zaghloul, M.S., Moustafa, A.A., 2004. Conservation of Medicinal plants in St. Catherine Protectorate, South Sinai, Egypt. Proceedings of 1st International Conference on Strategy of Egyptian Herbaria, Giza, Egypt, 231-251 231–251.

Fakhry, A.M., El-Keblawy, A., Shabana, H.A., Gamal, I.E., Shalouf, A., 2019. Microhabitats Affect Population Size and Plant Vigor of Three Critically Endangered Endemic Plants in Southern Sinai Mountains. Egypt. Land 8, 86. https://doi.org/10.3390/land8060086.

Fazekas, A.J., Burgess, K.S., Kesanakurti, P.R., Graham, S.W., Newmaster, S.G., Husband, B.C., Percy, D.M., Hajibabaei, M., Barrett, S.C.H., 2008. Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well. PLoS ONE 3. https://doi.org/10.1371/journal.pone.0002802.

Feau, N., Vialle, A., Allaire, M., Maier, W., Hamelin, R.C., 2011. DNA barcoding in the rust genus *Chrysomyxa* and its implications for the phylogeny of the genus. Mycologia 103, 1250–1266. https://doi.org/10.3852/10-426.

Ford, C.S., Ayres, K.L., Toomey, N., Haider, N., Stahl, J.V.A., Kelly, L.J., Wikström, N., Hollingsworth, P.M., Duff, R.J., Hoot, S.B., Cowan, R.S., Chase, M.W., Wilkinson, M.J., 2009. Selection of candidate coding DNA barcoding regions for use on land plants. Bot. J. Linn. Soc. 159, 1–11. https://doi.org/10.1111/j.1095-8339.2008.00938.x.

Francisco-Ortega, J., Santiago-Valentín, E., Acevedo-Rodríguez, P., Lewis, C., Pipoly, J., Meerow, A.W., Maunder, M., 2007. Seed plant genera endemic to the Caribbean Island biodiversity hotspot: A review and a molecular phylogenetic perspective. Bot. Rev 73, 183–234. https://doi.org/10.1663/0006-8101(2007)73[183:SPGETT]2.0.CO;2.

Gonzalez, M.A., Baraloto, C., Engel, J., Mori, S.A., Pétronelli, P., Riéra, B., Roger, A., Thébaud, C., Chave, J., 2009. Identification of Amazonian Trees with DNA Barcodes. PLoS ONE 4. https://doi.org/10.1371/journal.pone.0007483.

Hashim, A.M., Alharbi, B.M., Abdulmajeed, A.M., Elkelish, A., Hozzein, W.N., Hassan, H.M., 2020. Oxidative Stress Responses of Some Endemic Plants to High Altitudes by Intensifying Antioxidants and Secondary Metabolites Content. Plants 9, 869. https://doi.org/10.3390/plants9070869.

Hollingsworth, P.M., 2008. DNA barcoding plants in biodiversity hot spots: Progress and outstanding questions. Heredity 101, 1–2. https://doi.org/10.1038/hdy.2008.16.

Hollingsworth, P.M., Li, D.-Z., van der Bank, M., Twyford, A.D., 2016. Telling plant species apart with DNA: from barcodes to genomes. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 371. https://doi.org/10.1098/rstb.2015.0338.

Hosein, F.N., Austin, N., Maharaj, S., Johnson, W., Rostant, L., Ramdass, A.C., Rampersad, S.N., 2017. Utility of DNA barcoding to identify rare endemic vascular plant species in Trinidad. Ecol. Evol. 7, 7311–7333. https://doi.org/10.1002/ece3.3220.

IUCN, 2015. Rosa arabica: Omar, K.: The IUCN Red List of Threatened Species 2017: e.T84120072A84120074. https://doi.org/10.2305/IUCN.UK.2017-3.RLTS.T84120072A84120074.en

IUCN, 2012, n.d. The IUCN Red List of Threatened Species [WWW Document]. IUCN Red List of Threatened Species. URL https://www.iucnredlist.org/en (accessed 3.21.20).

Jaccard, P., 1908. Nouvelles recherches sur la distribution florale. Rouge, Lausanne.

Joly, S., Davies, T.J., Archambault, A., Bruneau, A., Derry, A., Kembel, S.W., Peres-Neto, P., Vamosi, J., Wheeler, T.A., 2014. Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. Mol. Ecol. Resour. 14, 221–232. https://doi.org/10.1111/1755-0998.12173.

Kaky, E., Gilbert, F., 2020. Allowing for human socioeconomic impacts in the conservation of plants under climate change. Plant Biosyst. – Int. J. Dealing Asp. Plant Biol. 154, 295–305. https://doi.org/10.1080/11263504.2019.1610109.

Kang, Y., Deng, Z., Zang, R., Long, W., 2017. DNA barcoding analysis and phylogenetic relationships of tree species in tropical cloud forests. Sci Rep 7, 12564. https://doi.org/10.1038/s41598-017-13057-0.

Khafaga, O., Hatab, E.E., Omar, K., 2011. Predicting the potential geographical distribution of Nepeta septemcrenata in Saint Katherine Protectorate, South Sinai, Egypt using Maxent. Academia Arena 3, 45–50.

Khan, S., Al-Qurainy, F., Nadeem, M., 2012. Biotechnological approaches for conservation and improvement of rare and endangered plants of Saudi Arabia. Saudi J. Biolog. Sci. 19, 1–11. https://doi.org/10.1016/j.sjbs.2011.11.001.

Khedr, A.-H., Cadotte, M.W., El-Keblawy, A., Lovett-Doust, J., 2002. Phylogenetic diversity and ecological features in the Egyptian flora. Biodivers. Conserv. 11, 1809–1824. https://doi.org/10.1023/A:1020312108530.

Kikuchi, S., Bédard, J., Hirano, M., Hirabayashi, Y., Oishi, M., Imai, M., Takase, M., Ide, T., Nakai, M., 2013. Uncovering the Protein Translocon at the Chloroplast Inner Envelope Membrane. Science 339, 571–574. https://doi.org/10.1126/science.1229262.

Kress, W.J., Erickson, D.L., 2007. A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region. PLoS ONE 2. https://doi.org/10.1371/journal.pone.0000508.

Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A., Janzen, D.H., 2005. Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. U S A 102, 8369–8374. https://doi.org/10.1073/pnas.0503123102.

Lebonah, D.E., Dileep, A., Chandrasekhar, K., Sreevani, S., Sreedevi, B., Pramoda Kumari, J., 2014. DNA Barcoding on Bacteria: A Review [WWW Document]. Adv. Biol. https://doi.org/10.1155/2014/541787.

Letunic, I., Bork, P., 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. https://doi.org/10.1093/bioinformatics/btl529.

McGregor, C.E., Lambert, C.A., Greyling, M.M., Louw, J.H., Warnich, L., 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (Solanum tuberosum L.) germplasm. Euphytica 113, 135–144. https://doi.org/10.1023/A:1003925620546.

Mokni, R.E., Majdoub, S., Chaieb, I., Jlassi, I., Joshi, R.K., Hammami, S., 2019. Chromatographic analysis, antimicrobial and insecticidal activities of the

essential oil of Phlomis floccosa D Don. Biomed. Chromatogr. 33,. https://doi.org/10.1002/bmc.4603 e4603.
Moustafa, A.E.-R.A., El-Azeem, H., Omran, M.A., Nasr, S.A.M., Abdel-Nabi, I.M., Teleb, Z.A., 2015. Biochemical Effect of Nepeta Septemcrenata Growing in South Sinai. Egypt. American J. Ethnomed. 2.
Moustafa, A.E.-R.A., Zaghloul, M.S., 1996. Environment and vegetation in the montane Saint Catherine area, south Sinai Egypt. J. Arid Environ. 34, 331–349. https://doi.org/10.1006/jare.1996.0114.
Moustafa, A.G., 2017. Long Term Monitoring of Rosa arabica Populations as a Threatened Species in South Sinai, Egypt [WWW Document]. URL https://www.semanticscholar.org/paper/Long-Term-Monitoring-of-Rosa-arabica-Populations-as-Moustafa/a9209f600f5eef6928a0df021c7866cea10018f1 (accessed 4.2.20).
Moustafa, A.R.A., Klopatek, J.M., 1995. Vegetation and landforms of the Saint Catherine area, southern Sinai Egypt. J. Arid Environ. 30, 385–395. https://doi.org/10.1006/jare.1995.0033.
Mulpuri, S., Muddanuru, T., Francis, G., 2013. Start codon targeted (SCoT) polymorphism in toxic and non-toxic accessions of Jatropha curcas L. and development of a codominant SCAR marker. Plant Sci. 207, 117–127. https://doi.org/10.1016/j.plantsci.2013.02.013.
Newmaster, S.G., Fazekas, A.J., Ragupathy, S., 2006. DNA barcoding in land plants: evaluation of rbcL in a multigene tiered approach. Can. J. Bot. 84, 335–341. https://doi.org/10.1139/b06-047.
Ojeda, D.I., Santos-Guerra, A., Oliva-Tejera, F., Jaen-Molina, R., Caujape-Castells, J., Marrero-Rodriguez, A., Cronk, Q., 2014. DNA barcodes successfully identified Macaronesian Lotus (Leguminosae) species within early diverged lineages of Cape Verde and mainland Africa. AoB PLANTS 6, plu050–plu050. https://doi.org/10.1093/aobpla/plu050
Omar, K.A., 2014. Evaluating the effectiveness of in-situ conservation on some endemic plant species in South Sinai. Egypt. Am J Life Sci 2, 164–175.
Pennisi, E., 2007. Taxonomy. Wanted: a barcode for plants. Science 318, 190–191. https://doi.org/10.1126/science.318.5848.190.
Pieroni, A., Giusti, M.E., de Pasquale, C., Lenzarini, C., Censorii, E., Gonzáles-Tejero, M.R., Sánchez-Rojas, C.P., Ramiro-Gutiérrez, J.M., Skoula, M., Johnson, C., Sarpaki, A., Della, A., Paraskeva-Hadijchambi, D., Hadjichambis, A., Hmamouchi, M., El-Jorhi, S., El-Demerdash, M., El-Zayat, M., Al-Shahaby, O., Houmani, Z., Scherazed, M., 2006. Circum-Mediterranean cultural heritage and medicinal plant uses in traditional animal healthcare: a field survey in eight selected areas within the RUBIA project. J. Ethnobiol. Ethnomed. 2, 16. https://doi.org/10.1186/1746-4269-2-16.
Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breeding 2, 225–238. https://doi.org/10.1007/BF00564200.
Prevost, A., Wilkinson, M.J., 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor Appl Genet 98, 107–112. https://doi.org/10.1007/s001220051046.
Rohwer, J.G., Trofimov, D., Mayland-Quellhorst, E., Albach, D., 2019. Incongruence of morphological determinations and DNA barcode sequences: a case study in Cinnamomum (Lauraceae). Willdenowia 49 (3), 383–400.
Savolainen, O., Karhu, A., 2000. Assessment of Biodiversity with Molecular Tools in Forest Trees. In: Jain, S.M., Minocha, S.C. (Eds.), Molecular Biology of Woody Plants: Volume 1, Forestry Sciences. Springer, Netherlands, Dordrecht, pp. 395–406. https://doi.org/10.1007/978-94-017-2311-4_16.
Shahlaei, A., Torabi, S., Khosroshahli, M., 2014. Efficacy of SCoT and ISSR marekers in assessment of tomato (Lycopersicum esculentum Mill.) genetic diversity. https://doi.org/10.12692/ijb/5.2.14-22
Shaltout, K.H., Ahmed, D.A., Shabana, H.A., 2015. Population structure and dynamics of the endemic species Phlomis aurea Decne in different habitats in southern Sinai Peninsula Egypt. Global Ecol. Conservat. 4, 505–515. https://doi.org/10.1016/j.gecco.2015.10.002.
Tackholm, V., 1974. Students' flora of Egypt.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Uritu, C.M., Mihai, C.T., Stanciu, G.-D., Dodi, G., Alexa-Stratulat, T., Luca, A., Leon-Constantin, M.-M., Stefanescu, R., Bild, V., Melnic, S., Tamba, B.I., 2018. Medicinal Plants of the Family Lamiaceae in Pain Therapy: A Review [WWW Document]. Pain Res. Manage. https://doi.org/10.1155/2018/7801543.
Yesson, C., Bárcenas, R.T., Hernández, H.M., De La LUZ RUIZ-MAQUEDA, M., Prado, A., Rodríguez, V.M., Hawkins, J.A., 2011. DNA barcodes for Mexican Cactaceae, plants under pressure from wild collecting: DNA BARCODES FOR MEXICAN CACTACEAE. Molecular Ecology Resources 11, 775–783. https://doi.org/10.1111/j.1755-0998.2011.03009.x
Zahran, M.A., Willis, A.J., 2009. The Sinai Peninsula, in: Zahran, M.A., Willis, A.J. (Eds.), The Vegetation of Egypt, Plant and Vegetation. Springer Netherlands, Dordrecht, pp. 213–249. https://doi.org/10.1007/978-1-4020-8756-1_5
Zhang, J., Xie, W., Wang, Y., Zhao, X., 2015. Potential of Start Codon Targeted (SCoT) Markers to Estimate Genetic Diversity and Relationships among Chinese Elymus sibiricus Accessions. Molecules 20, 5987–6001. https://doi.org/10.3390/molecules20045987.