

Micropropagation and DNA-barcoding of the endangered endemic *Phlomis aurea* plant of Saint Katherine

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Abstract

Saint Katherine is considered a “biodiversity hotspot” because of the high level of endemism of reported plant species. In this study, conservation of the endangered endemic plant; *Phlomis aurea* of Saint Katherine, Southern Sinai, Egypt, was carried out through micropropagation and DNA barcoding. The first efficient micropropagation protocol for *Phlomis aurea* was established as a mean of *ex situ* conservation of the plant. Shoot tips and nodal segments of *in vitro* germinated seedlings were established on Murashige and Skoog medium supplemented with 0.54 μM β -naphthalene acetic acid (NAA) and 2.46 μM N6-(2-isopentenyl) adenine (2iP) in combination with 6-benzylaminopurine (BA) or kinetin (Kin). The medium supplemented with 3.48 μM Kin considered optimum for both explants. For multiplication, BA was the most efficient cytokinin. The percentage of rooted explants reached 100% at the concentration of 14.7 μM indolebutyric acid (IBA), whereas the highest number of roots was recorded for 4.90 μM , which considered the optimum concentration with a percentage of 80% of rooting. Rooted plantlets were transplanted in the greenhouse with 75% survival rate. The present study also aimed to carry out DNA barcoding of *Phlomis aurea* for accurate identification to provide a database for establishing an efficient conservation program for the plant. Three chloroplast DNA markers were used [ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcl*), maturase K (*matK*) and RNA polymerase C1 (*rpoC1*)] and all were successful in amplifying target regions, however the performance of both *rbcl* and *matK* markers seemed to be species-specific. The similarity percentage was maximum for *rbcl* (99.81%) and *matK* (100%) compared to the database of the same species.

Keywords: DNA barcoding; Lamiaceae; micropropagation; Sinai Jerusalem Sage; Southern Sinai.

Abbreviations: BA_ 6-benzylaminopurine; BLAST_ Basic Local Alignment Tool; CBOL_ Consortium for the Barcode of Life; IBA_ indolebutyric acid; 2iP_ N6_(2_isopentenyl) adenine; IUCN_ International Union for Conservation of Nature; Kin_ kinetin; *matK*_ maturase K; MS_ Murashige and Skoog; NAA_ β _naphthaleneacetic acid; NCBI_ National Centre of Biotechnology Information; *rbcl*_ ribulose_1,5_bisphosphate carboxylase/oxygenase large subunit; *rpoC1*_ RNA polymerase C1.

Introduction

The protection of endemic species is a worldwide need; endemic species are regularly the most defenseless plant species against the dangers of unmanaged human activities, due to their exceptional transformative history and low population size (Omar, 2017). *Phlomis aurea* Decne. is a wild golden-woolly perennial shrub, known as Sinai Jerusalem Sage and its Vernacular name is Awarwar, belonging to Lamiaceae family. According to the International Union for Conservation of Nature (IUCN, 2019), *Phlomis aurea* is an endangered species that needs an urgent conservation plan. It is classified as endangered, because it is endemic to the high mountain area of Saint Katherine Protectorate in Southern Sinai, Egypt and found just in two locations (Boulos, 2008). There is a continuing decline in the number of plant individuals because of the deterioration of habitat quality for this species, the destructive effect of climate change, drought, human activities such as over-collection for its economic importance and uprooting (Khafagi et al., 2012; Shabana, 2013). Because of these threads, the wild population of *Phlomis aurea* can be in extreme danger in the near future (Omar, 2017).

Genus *Phlomis* comprises about 100 species in the world, restricted to Mediterranean region to central Asia and China. Only two *Phlomis* species occur in Egypt; *Phlomis aurea* and *Phlomis floccosa* D. Don. (Täckholm, 1974; Boulos, 2002). *Phlomis* has been utilized by the Egyptian people in folk medicine as a sedative, stimulant, immune-suppressive, hemorrhoids, free radical scavenger, astringent, hemorrhoid, anti-diarrheal, anti-inflammatory, antimicrobial, anti-malarial, anti-diabetic, anti-mutagenic, anti-cough, anti-nociceptive, for mending of wound and to treat ulcers, gastric, intestinal and abdominal pains (Amor et al., 2009). Some *Phlomis* species are consumed as a tea in Mediterranean region (Serag et al., 2018). Genus *Phlomis* contains essential oil, phenolics and flavonoids, therefore the plant possesses antioxidant activity (Sobeh et al., 2016; Merouane et al., 2018). Also, because of the active constituents in the plant species, it has antibacterial (Turker and Yldrm, 2013; Bajalan et al., 2017), antifungal (Asgarpanah et al., 2017) and cytotoxic (Sobeh et al, 2016; Sarkhail et al., 2017) activities. Phlomisoside F is isolated from *P. younghusbandii* and is a promising potential anti-

inflammatory drug (Li et al., 2015). Moreover, diverse classes of glycosides including diterpenoids, phenylpropanoids, phenylethanoids and flavonoids had been described from genus *Phlomis*. Many of the phenyl propanoids showed biological significances, e.g. immunosuppressant, cytotoxic anti-inflammatory and anti-microbial activities (Serag et al., 2018).

Phlomis aurea is a chamaephyte and is used in the traditional medicine. It is aromatic and its essence can be promising in different industries as it is still an unknown species to trade. The plant species has a significant medicinal and ecological importance. Medicinally, the plant has anti-microbial, anti-diabetic, anti-inflammatory, immune-suppressant and cytostatic properties. Flavonoids had been recognized from that plant. Ecologically, *Phlomis aurea* is characteristic to arid lands and has a role in pollination due to its lipped flower that attracted honey bees (Serag et al., 2018). The plant is also used as fuel (Shabana, 2013) and as fodder for animals (Omar, 2017). Iridoid and megastigmane glycosides were extracted from the plant (Kamel et al., 2000).

Both *in situ* and *ex situ* conservation strategies are urgently needed to be applied in parallel to rare and endangered plant species, especially that are endemic to a specific area. One of the most efficient *ex situ* conservation methods is micropropagation of the target plant. Although, propagation by seeds and seed banking from different populations is extremely important for conserving genetic variations and reducing the chance of extinction of a plant population at a site (Bakker and Berendse, 1999), micropropagation is essential and has many advantages. It is particularly essential for conserving the plant species that do not produce seeds or that have recalcitrant seeds (Oseni, et al., 2018). Furthermore, it produces hundreds of plants in a limited space and comparatively short time, regardless of the season or weather (Akin-Idowu et al., 2009).

There could be a severe decline in the population of *Phlomis aurea* with time, unless precautions have been taken. In order to establish an efficient conservation program for a target plant species, providing a database on its biology and ecology is a must (Shaltout et al., 2016). For biodiversity conservation, accurate taxonomic assignment is important. DNA-based identification (barcoding) is simple and free from subjective errors, which is not the case in morphological identification. The phylogeny of the genus *Phlomis* was studied by Mathiesen et al. (2011) using trnL-F and rps16 intron and retrieved low interspecific resolution. El-Banhawy and Al-Juhani (2019) obtained equivalent results using DNA sequences of two chloroplast markers; *psb-A/trn-H* and *rbcl* to reconstruct the phylogeny of genus *Phlomis* alongside the endemic *Phlomis aurea* and they strongly recommended using other markers.

According to the critical situation of *Phlomis aurea* as an endangered species and also as an endemic plant, restricted to Saint Katherine, the aim of the study was to establish an effective conservation program for the plant through micropropagation and DNA barcoding. To our knowledge, this is the first report on the micropropagation of *Phlomis aurea*.

Results and Discussion

Micropropagation of *Phlomis aurea*

In vitro germination percentage of *Phlomis aurea* seeds was 90%. Germination began seven days after the seeds were

planted on Murashige and Skoog (MS; Murashige and Skoog, 1962) medium (Fig 1A). The presence of contamination was limited (20%), which did not represent a problem for germination. About 82% survival from contamination has been achieved. Shoot tips and stem nodal segments were excised from one-month old seedlings and transferred to the different establishment media for the micropropagation. Seedlings are the most preferred material for use to establish *in vitro* cultures when the objective is plant conservation for maximizing genetic diversity of the produced plants, which is desirable (Fay, 1994).

Culture establishment

Shoot tips and stem nodal segments established *in vitro* on all the tested treatments (Table 1). For shoot tips, survival percentage ranged between 63 and 100%. Growth percentage of the survived explants reached 100% on MS medium supplemented with 0.54 μM NAA and 2.46 μM 2iP only and with the addition of 1.11 or 3.33 μM BA or 3.48 μM Kin. Mean number of axillary shoots explant⁻¹ was significantly the maximum of 6 axillary shoots when the medium was supplemented with 2.22 μM BA, but the mean length of axillary was the least (0.25 cm) on the same medium, comparing to the tested treatments. The concentration of 2.32 μM Kin ranked next and gave 4 axillary shoots explant⁻¹. The significantly longest axillary shoot of 3.5 cm was obtained on the nutrient medium supplemented with 3.48 μM Kin and gradually became shorted by decreasing Kin concentration. The same observation was recorded with *Teucrium capitatum* (Papafotiou and Martini, 2016).

It was noticed that the media containing BA gave axillary shoots suffering from vitrification, which is not favourable for further stages. Therefore, MS medium supplemented with 0.54 μM NAA and 2.46 μM 2iP in addition to 3.48 μM Kin considered the best establishment medium of shoot tips.

Concerning stem nodal segments, the survival percentage and growth percentage of the survived explants ranged between 50 and 100%. MS medium supplemented with 0.54 μM NAA and 2.46 μM 2iP in addition to 3.33 μM BA produced the highest number of axillary shoots of 4.5 explant⁻¹. This number decreased by decreasing the concentration of BA.

Concerning the mean length of axillary shoots, it attained its significantly maximum value of 2.75 cm on MS medium supplemented with 3.48 μM Kin, with a mean of 3.5 axillary shoots explant⁻¹.

In general, plant growth regulators are crucial in regulating differentiation and organogenesis. Tsafouros and Roussos (2019) reported that in many plants, the supplementation of the nutrient medium with only cytokinins is not sufficient to induce the formation of axillary shoots. Synthetic auxins, such as NAA are utilized in combination with cytokinin in the micropropagation of many plant species with better results than cytokinin individually (Akhtar et al., 2016). It is well established that the nutrient media supplemented with cytokinin promotes shoot formation and a higher ratio of cytokinin to auxin is required for shoot regeneration (Rossopoff et al., 2017; Pernisova et al., 2018). Combining NAA with BA enhances shoots proliferation in many other species such as *Clitoria ternatea* (Rout, 2004), *Prunella vulgaris* (Rasool et al., 2009) and *Cymbopogon schoenanthus* (Abdelsalam et al., 2017).

A preliminary study on the establishment of *Phlomis aurea* shoot tips and stem nodal segments was carried out using

only BA or Kn with NAA and resulted in dwarf axillary shoots. The addition of 2iP to the medium enhanced the elongation. It is well known that a synergistic effect of different cytokinins can result in effective regeneration rates (Pernisova et al., 2009, 2018).

The length of axillary shoots is more important in this stage than their number. The aim of this stage is obtaining axillary shoots that could be handled and divided into nodal sections to be transferred to the multiplication medium for proliferation. Therefore, the medium supplemented with 3.48 μM Kin considered optimum for the *in vitro* establishment of both shoot tip and stem nodal segment explants of *Phlomis aurea* (Fig 1B and C). This confirms the significant role for Kin in shoot formation and regeneration (Wang et al., 2018). The results are in harmony with Tsafouros and Roussos (2019), who clarified the adenine efficiency and found that BA induced high number of nodes and Kin characterized by the high shoot length induction.

Multiplication

Table (2) shows the results of culturing the axillary shoots developed from the establishment stage on different concentrations of BA, Kin or 2iP supplemented medium. These cytokinins were chosen in this experiment because there are the most widely used cytokinin (Yancheva and Kondakova, 2016). In this experiment, the concentrations of cytokinins were reduced in comparison to the establishment stage to eliminate the vitrification of axillary shoots that was observed at the higher concentrations of BA. For the multiplication stage, it is important to select a proper concentration of cytokinin, because higher concentrations cause a reduction in shoot size, somaclonal variations and vitrification (Lebedev et al., 2019).

Multiplication of axillary shoots was affected both by cytokinin type and its concentration. All tested treatments produced axillary shoots. The highest recorded mean number of axillary shoots explant⁻¹ was obtained on MS medium supplemented with 1.78 μM BA. Decreasing the concentration of BA in the nutrient medium gradually decreased the number of axillary shoots. Concerning the other tested cytokinins; 2iP ranked next as it produced higher number of axillary shoots than Kin. For both, the number of axillary shoots increased by increasing their concentration. The present study indicated that the number of axillary shoots was directly correlated to the cytokinin concentration. The same observation was recorded for *Dendrocalamus giganteus* (Devi et al., 2012) and *Ziziphora tenuior* (Dakah et al., 2014). It is obvious that the correct choice of cytokinin is one of the most important factors in the multiplication of axillary shoots and it varied from species to another. It is well known that cytokinins play multiple roles in the development of plant; such as induction of cell division and enlargement and stimulation of protein synthesis and enzymes activity (Arab et al., 2014).

The maximum mean length of axillary shoots was 2.6 cm on MS medium supplemented with 0.93 μM Kin, but it is insignificantly different compared to the best medium for multiplication. Therefore, MS medium supplemented with 1.78 μM BA considered the optimum for axillary shoots multiplication (Fig 1D).

The variation in the effectiveness of different cytokinins could be partly assigned to their different metabolism within the plant. In this study, BA was more appropriate for shoot multiplication than Kin or 2iP. Higher shoot promoting ability of BA compared with Kin and 2iP has also been reported for

other Lamiaceae species; *Teucrium capitatum* (Papafotiou and Martini, 2016), or compared with Kin as for *Salvia fruticosa* (Arikat et al., 2004), *Ocimum kilimandscharicum* (Saha et al., 2010) and *Thymus satureioides* (Aicha et al., 2013). Also, BA induced higher shoot production compared with 2iP in *Sideritis athena* (Papafotiou and Kalantzis, 2009). Moreover, BA induced multiplication in many *Lavandula* species (Zuzarte et al., 2010; Gonçalves and Romano, 2013). The high efficiency of BA could be attributed to that it is not a suitable substrate of cytokinin oxidase, the enzyme responsible for the endogenous cytokinin balance (Kieber and Schaller, 2004; George et al., 2008; Tsafouros and Roussos, 2019). Some of the conjugated forms of BA produced during BA metabolism, extending the action of BA. Furthermore, BA considered as the most stable among the tested cytokinins because of that the stability of the aromatic side chain substituted at N6 is higher than the isoprenoid chain of 2iP, the furan chain of Kin (Zhang et al., 2010; Tsafouros and Roussos, 2019).

Rooting and acclimatization

Rooting percentage was positively affected by increasing the concentration of IBA (Table 3). It reached 100% using 14.7 μM IBA in half strength MS medium, while the medium without IBA produced the lowest rooting percentage (only 40%). However, the mean number and length of roots explant⁻¹ were maximum at the concentration of 4.90 μM IBA (Fig 1E). The number of roots is an important factor for increasing the percentage of plant survival during acclimatization stage and considered a sign of qualitative rooting response (Dolcet-Sanjuan et al., 2004). Therefore, the concentration of 4.90 μM IBA considered the optimum concentration for the rooting of *Phlomis aurea*, although it produced lower percentage of rooting (80%). It is well known that rooting response depends on many factors, among which the type of auxin used, the applied concentration, the species or even the clone of a specific species (Dolcet-Sanjuan et al., 2004). Regarding to other species from Lamiaceae family, many plant species rooted on medium supplemented with only IBA; such as *Teucrium capitatum* rooted at higher percentages when cultured on ½ strength MS medium supplemented with IBA, regardless of its concentration (Papafotiou and Martini, 2016), with 4.90 μM IBA for *Teucrium stocksianum* (Bouhouche and Ksiksi, 2007) and 2.46 μM IBA for *Teucrium fruticans* (Frabetti et al., 2009). *Sideritis athena* was difficult to root and the best rooting (46 to 53%) was achieved with 9.80 μM IBA (Papafotiou and Kalantzis, 2009).

Rooted plantlets were transplanted into pots and gradually transferred from the growth chamber to the greenhouse with 75% survival rate (Fig 1F).

DNA barcoding of *Phlomis aurea*

It is highly important to distinguish and recognize the endemic species to help in the conservation of natural resources, especially the medicinally important species such as *Phlomis aurea* (de Groot et al., 2011), from Wadi El Arbaen, Saint Katherine, Southern Sinai. DNA barcoding is a reliable approach to verify a plant identity; a short genetic sequence from a standard part of the genome can be sufficient for plant species identification. The outputs of BLAST matching and tree analysis of *Phlomis aurea* are represented in Tables (4, 5 and 6) and Fig (2, 3, 4 and 5) for

Table 1. Effect of MS medium supplemented with 0.54 μM NAA and 2.46 μM 2iP in addition to BA or Kin at different concentrations on the *in vitro* establishment of *Phlomis aurea* shoot tip and nodal segment explants.

| Treatments | Cytokinin conc. (μM) | | Shoot tip | | | | Stem nodal segment | | | |
|------------|-----------------------------------|------|--------------|------------|---|-------------------------------------|--------------------|------------|---|-------------------------------------|
| | BA | Kin | Survival (%) | Growth (%) | Mean no. of axillary shoots explant ⁻¹ | Mean length of axillary shoots (cm) | Survival (%) | Growth (%) | Mean no. of axillary shoots explant ⁻¹ | Mean length of axillary shoots (cm) |
| T1 | 0.00 | 0.00 | 100 | 100 | 3.25 ^b | 1.52 ^b | 75 | 100 | 2.88 ^b | 0.67 ^c |
| T2 | 1.11 | 0.00 | 100 | 100 | 3.25 ^b | 1.75 ^b | 100 | 100 | 3.13 ^b | 0.65 ^c |
| T3 | 2.22 | 0.00 | 100 | 75 | 6.01 ^a | 0.25 ^c | 75 | 100 | 3.31 ^{ab} | 0.94 ^c |
| T4 | 3.33 | 0.00 | 63 | 100 | 2.00 ^c | 0.52 ^c | 100 | 75 | 4.50 ^a | 0.30 ^c |
| T5 | 0.00 | 1.16 | 100 | 75 | 1.63 ^c | 1.25 ^b | 50 | 100 | 3.13 ^b | 0.40 ^c |
| T6 | 0.00 | 2.32 | 100 | 75 | 4.00 ^b | 1.70 ^b | 100 | 75 | 3.88 ^{ab} | 1.74 ^b |
| T7 | 0.00 | 3.48 | 75 | 100 | 1.50 ^c | 3.50 ^a | 75 | 50 | 3.50 ^{ab} | 2.75 ^a |



Fig 1. *Phlomis aurea* *in vitro* propagation stages. (A) Germinated seeds, (B) established shoot tip, (C) Established stem nodal segments. (D) Multiple shoots, (E) Development of roots (F) Acclimatized plantlets grown in greenhouse.

Table 2. Effect of MS medium supplemented with BA, 2iP or Kin at different concentrations on the multiplication of *Phlomis aurea* axillary shoots.

| Treatments | Cytokinin conc. (μM) | | | Mean no. of axillary shoots explant ⁻¹ | Mean length of axillary shoots (cm) |
|------------|----------------------|------|------|---|-------------------------------------|
| | BA | Kin | 2iP | | |
| T1 | 0.00 | 0.00 | 0.00 | 2.4 ^e | 1.90 ^{ab} |
| T2 | 0.44 | 0.00 | 0.00 | 2.2 ^e | 2.20 ^{ab} |
| T3 | 0.89 | 0.00 | 0.00 | 3.6 ^d | 2.20 ^{ab} |
| T4 | 1.78 | 0.00 | 0.00 | 7.4 ^a | 2.15 ^{ab} |
| T5 | 0.00 | 0.46 | 0.00 | 2.2 ^e | 2.15 ^{ab} |
| T6 | 0.00 | 0.93 | 0.00 | 2.6 ^e | 2.60 ^a |
| T7 | 0.00 | 1.86 | 0.00 | 3.4 ^d | 1.71 ^{ab} |
| T8 | 0.00 | 0.00 | 0.49 | 3.6 ^d | 1.31 ^b |
| T9 | 0.00 | 0.00 | 0.98 | 5.2 ^c | 1.45 ^b |
| T10 | 0.00 | 0.00 | 1.97 | 6.0 ^b | 1.10 ^b |

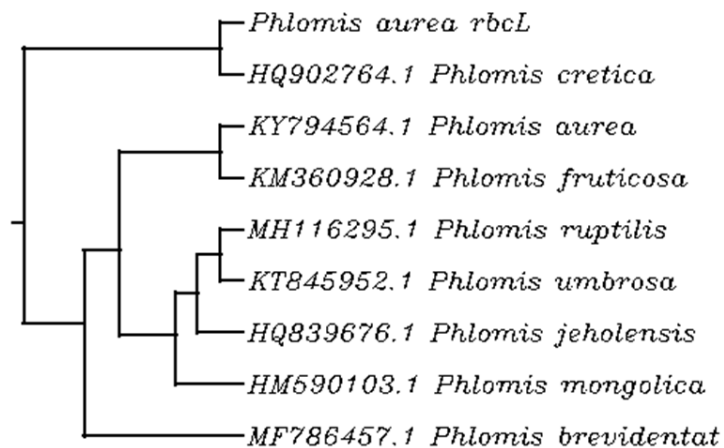


Fig 2. Phylogenetic tree of *Phlomis aurea* using the cpDNA marker; *rbcL* showing names of plant species and accession numbers.

Table 3. Effect of ½ MS medium supplemented with IBA at different concentrations on the rooting of *Phlomis aurea* axillary shoots.

| Treatments | IBA conc. (µM) | Rooting (%) | Mean no. of roots explant ⁻¹ | Mean length of roots (cm) |
|------------|----------------|-------------|--|---------------------------|
| T1 | 0.00 | 40 | 1.0 ^b | 4.7 ^a |
| T2 | 2.46 | 60 | 1.6 ^b | 5.1 ^a |
| T3 | 4.90 | 80 | 3.7 ^a | 5.7 ^a |
| T4 | 9.80 | 80 | 1.0 ^b | 5.1 ^a |
| T5 | 14.70 | 100 | 1.4 ^b | 4.8 ^a |

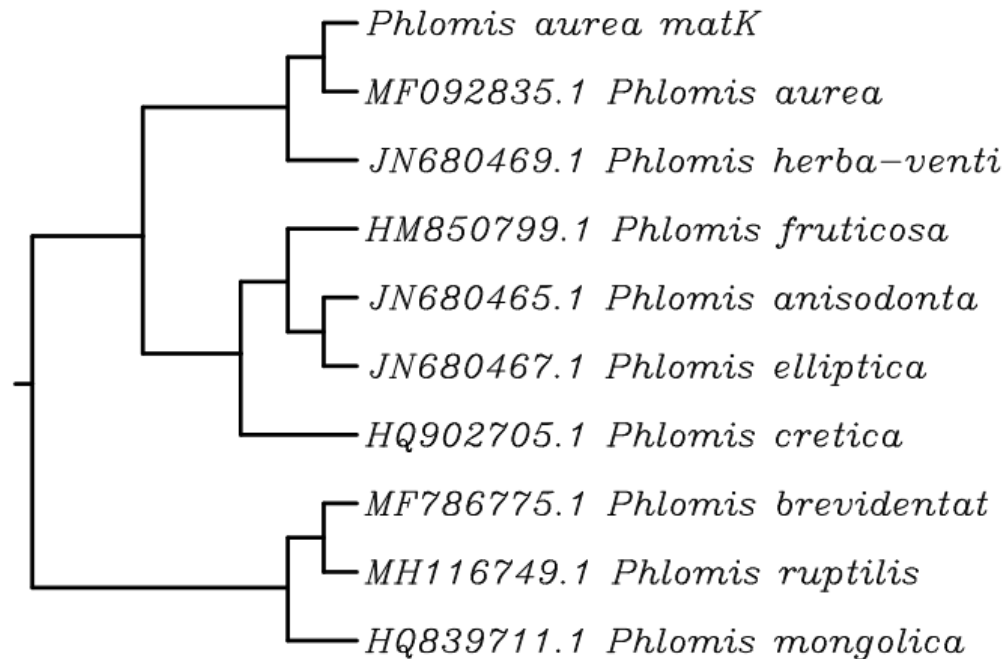


Fig 3. Phylogenetic tree of *Phlomis aurea* using the cpDNA marker; *matK*, showing names of plant species and accession numbers.

Table 4. *rbcL* DNA barcode of related plant species with similarity percentage of more than 98.3%, downloaded from GenBank database.

| Plant species | Accession no. | E-value | Query coverage (%) | Similarity (%) |
|-----------------------------|---------------|---------|--------------------|----------------|
| <i>Phlomis aurea</i> | KY794564.1 | 0.0 | 76 | 99.81 |
| <i>Phlomis fruticosa</i> | KM360928.1 | 0.0 | 78 | 98.90 |
| <i>Phlomis rutilis</i> | MH116295.1 | 0.0 | 78 | 98.53 |
| <i>Phlomis umbrosa</i> | KT845952.1 | 0.0 | 76 | 99.43 |
| <i>Phlomis jeholensis</i> | HQ839676.1 | 0.0 | 78 | 98.53 |
| <i>Phlomis mongolica</i> | HM590103.1 | 0.0 | 78 | 98.53 |
| <i>Phlomis cretica</i> | HQ902764.1 | 0.0 | 75 | 99.43 |
| <i>Phlomis brevidentata</i> | MF786457.1 | 0.0 | 76 | 98.31 |

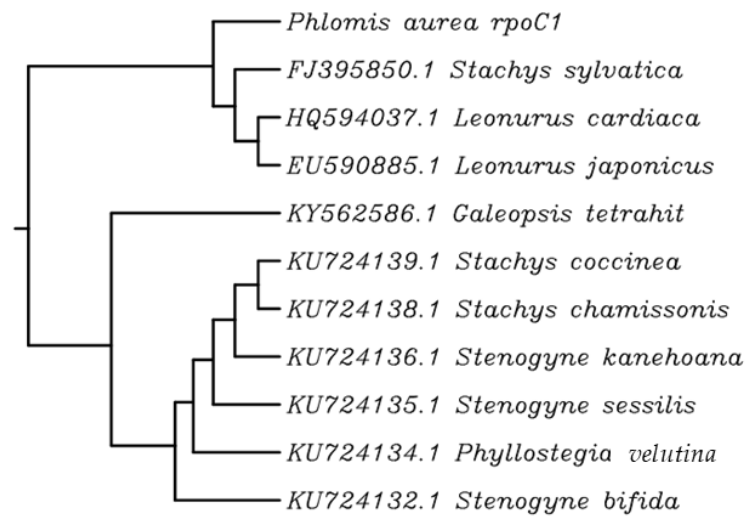


Fig 4. Phylogenetic tree of *Phlomis aurea* using the cpDNA marker; *rpoC1*, showing names of plant species and accession numbers.

Table 5. *matK* DNA barcode of related plant species with similarity percentage of more than 97.5%, downloaded from GenBank database.

| Plant species | Accession no. | E-value | Query coverage (%) | Similarity (%) |
|-----------------------------|----------------------------|---------|--------------------|----------------|
| <i>Phlomis fruticosa</i> | HM850799.1 | 0.0 | 85 | 99.51 |
| <i>Phlomis cretica</i> | HQ902705.1 | 0.0 | 84 | 99.26 |
| <i>Phlomis brevidentata</i> | MF786775.1 | 0.0 | 81 | 97.95 |
| <i>Phlomis mongolica</i> | HQ839711.1 | 0.0 | 80 | 97.54 |
| <i>Phlomis ruptilis</i> | MH116749.1 | 0.0 | 68 | 98.16 |
| <i>Phlomis anisodonta</i> | JN680465.1 | 0.0 | 63 | 99.84 |
| <i>Phlomis elliptica</i> | JN680467.1 | 0.0 | 64 | 99.67 |
| <i>Phlomis herba-venti</i> | JN680469.1 | 0.0 | 63 | 99.51 |
| <i>Phlomis aurea</i> | MF092835.1 | 2e-131 | 26 | 100 |

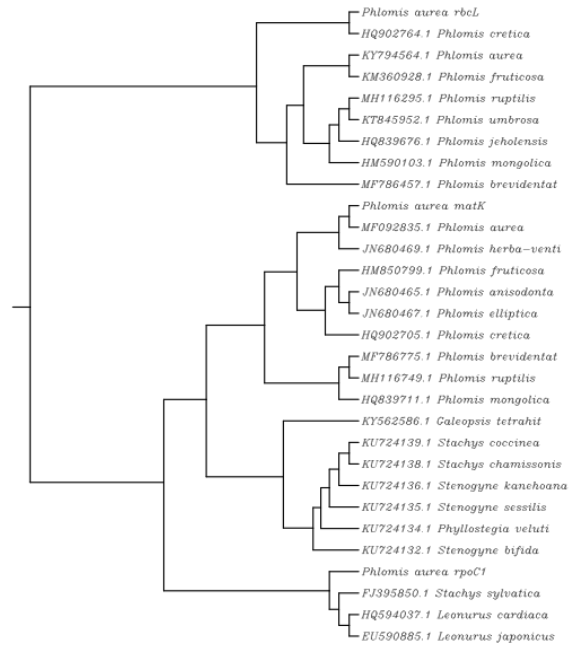


Fig 5. Phylogenetic tree of *Phlomis aurea* using the three cpDNA markers; *rbcL*, *matK* and *rpoC1* (combined data) showing names of plant species and accession numbers.

Table 6. *rpoC1* DNA barcode of related plant species with similarity percentage of more than 97%, downloaded from GenBank database.

| Plant species | Accession no. | E-value | Query coverage (%) | Similarity (%) |
|------------------------------|---------------|---------|--------------------|----------------|
| <i>Galeopsis tetrahit</i> | KY562586.1 | 0.0 | 94 | 97.57 |
| <i>Stachys sylvatica</i> | FJ395850.1 | 0.0 | 94 | 97.36 |
| <i>Stachys coccinea</i> | KU724139.1 | 0.0 | 94 | 97.36 |
| <i>Stachys chamissonis</i> | KU724138.1 | 0.0 | 94 | 97.36 |
| <i>Stenogyne kanehoana</i> | KU724136.1 | 0.0 | 94 | 97.36 |
| <i>Stenogyne sessilis</i> | KU724135.1 | 0.0 | 94 | 97.36 |
| <i>Phyllostegia velutina</i> | KU724134.1 | 0.0 | 94 | 97.36 |
| <i>Stenogyne bifida</i> | KU724132.1 | 0.0 | 94 | 97.36 |
| <i>Leonurus cardiaca</i> | HQ594037.1 | 0.0 | 89 | 98.51 |
| <i>Leonurus japonicus</i> | EU590885.1 | 0.0 | 89 | 98.50 |



Fig 6. *Phlomis aurea* grown naturally in Wadi El Arbaien, Saint Katherine, Southern Sinai showing flowers with a close up view of a fruit.

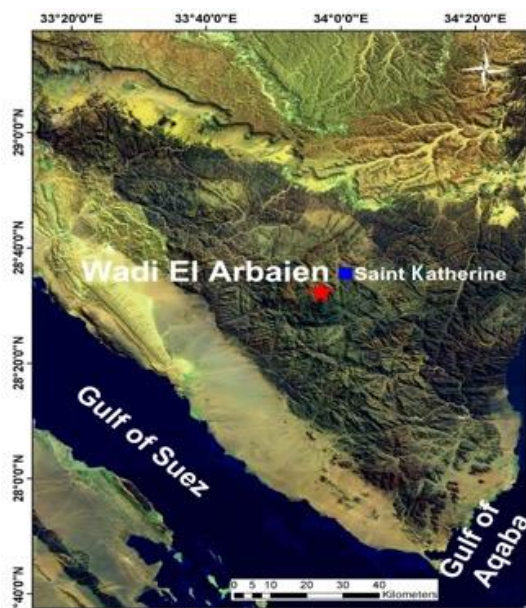


Fig 7. A map showing the location of *Phlomis aurea* at Wadi El Arbaien, Saint Katherine, South Sinai, Egypt.

the plant species that have the highest percentages of similarity. The newly generated sequences of the three cpDNA markers; *rbcl*, *matK* and *rpoC1* were used as barcodes. The maximum query coverage percentages were 78, 85 and 94% for *rbcl*, *matK* and *rpoC1*, respectively. de Groot et al. (2011) recommended that species identification is considered successful when the percentage of similarity scores more than 95% and involves a single species. Also, on the generic level, DNA barcoding is considered successful when all BLAST searches score more than 95% similarity and include a single genus (de Groot et al., 2011). In the present study, the maximum similarity percentages were 99.81% for *rbcl*, 100% for *matK* and 98.51% for *rpoC1*. The identification of *Phlomis aurea* on species and genus levels was successful for both *rbcl* and *matK* markers. Although for *rpoC1*, it was successful only on the family level.

The phylogenetic trees including the plant species that have the highest percentages of similarity have a fan shape (Fig 2, 3, 4 and 5), showing closely related species clustering together and relatively distantly related species scattering. DNA barcoding should replace morphological classification of plant species for species identification and genetic relationships (Hosein et al., 2017). The choice of the three genes in this study based on the Consortium for the Barcode of Life (CBOL) plant working group (2009), who has recommended *rbcl* and *matK* genes as 'core barcodes' for the identification of all land plants. Recently, plant diversity was determined using *rbcl* gene sequences as a core DNA barcoding marker (Kesanakurti et al., 2011). Moreover, *matK* is one of the most useful genes for identification at family, genus and species levels (Dong et al., 2012). Nowadays, methods for studying the molecular phylogeny of plants depend mainly on chloroplast genome sequencing, because of the simple and stable genetic structure of the chloroplast genome and universal primers are used for the amplification of these target sequences, such as *rbcl*, *matK* and *rpoC1* have been heavily relied upon for development of markers for plant DNA barcoding (Dong et al., 2012).

The universality of barcode markers is restricted due to geographical and morphological variations and plant species evolution (Roy et al., 2010). Therefore, the universal DNA barcoding markers and the choice of the correct loci are challenging (de Groot et al., 2011).

The results of the present study show a successful identification of *Phlomis aurea* on the species and genus levels and it is recommended to examine other markers, such as nuclear gene markers to entirely identify the plant more accurately.

Materials and methods

Samples collection

Phlomis aurea leaf specimens and seeds were collected from shrubs grown in Wadi El Arbaien, Saint Katherine, Southern Sinai (N: 28.54123, E: 33.95736, Alt: 1704) as shown in Fig (6 and 7). Plant was identified by Dr. Omran Ghaly, Head of Plant Taxonomy Unit, Desert Research Center, Egypt and given the voucher number CAIH-1002-R. Voucher Herbarium specimens were deposited in the Herbarium of Desert Research Center (CAIH).

In vitro experiments

Plant material collection and sterilization

Phlomis aurea seeds were collected and used as plant material. Seeds were washed with detergent solution for 10 min to remove foreign debris. Seeds were surface sterilized in a solution of 40% commercial bleach containing 5.25% sodium hypochlorite solution for 20 min, followed by three washes with sterile distilled water. Surface sterilization of the seeds was performed under complete aseptic conditions in a laminar air flow hood (Holten LaminAir HVR 2448, USA).

In vitro germination

Seeds were planted aseptically into jars containing MS medium (Duchefa, Haarlem, the Netherlands) supplemented with 3% (w v⁻¹) sucrose and gelled with 0.3% (w v⁻¹) phytagel (Duchefa, Haarlem, the Netherlands) and adjusted to pH of 5.7±0.1 before autoclaving at a pressure of 1.06 kg cm⁻² and 121°C for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). Cultures were incubated at 25±1°C at a photoperiod of 16/8 h light/darkness under cool white fluorescent tubes of 2500-3000 lux (F140t9d/38, Toshiba) for germination and seedling development. After one month of seed germination, the shoot tips and nodal segments of the emerged seedlings were excised and used as explants for micropropagation.

Micropropagation

Culture establishment

Shoot tips and stem nodal segment explants were cultured aseptically on MS medium supplemented with 3% (w v⁻¹) sucrose and 0.3% (w v⁻¹) phytagel and 0.54 μM β-naphthalene acetic acid (NAA), 2.46 μM N6-(2-isopentenyl) adenine (2iP) in combination with 6-benzylaminopurine (BA) at concentrations 1.11, 2.22 and 3.33 μM or kinetin (Kin) at

concentrations of 1.16, 2.32, 3.48 μM (Sigma Cell Culture, min. 90%, St. Louis, USA), in addition to MS medium without growth regulators as a control, to induce new growth development. The pH of the medium was adjusted to 5.7 ± 0.1 and media were autoclaved at a pressure of 1.06 kg cm^{-2} and 121°C for 15 min. Cultures were incubated at $25 \pm 1^\circ\text{C}$ at a photoperiod of 16/8 h light/darkness under cool white fluorescent tubes of 2500-3000 lux. The percentage of survived explants (survival %), percentage of explants forming growth (growth %), the mean number and length of axillary shoots explant⁻¹ were scored after eight weeks of culture on the establishment medium.

Axillary shoot multiplication

The newly developed axillary shoots were excised and cultured into jars containing MS medium supplemented with 3% (w v⁻¹) sucrose and 0.3% (w v⁻¹) phytagel and BA (0.44, 0.89 and 1.78 μM), Kin (0.46, 0.93 and 1.86 μM) or 2iP (0.49, 0.98 and 1.97 μM), each individually. The pH of the medium was adjusted, autoclaved and cultures were incubated as mentioned in the establishment stage. The mean number and length of axillary shoots explant⁻¹ were scored after eight weeks of culture. This process was performed onto fresh medium for four successive subcultures of eight weeks each.

Rooting

Axillary shoots were transferred to rooting medium of $\frac{1}{2}$ strength MS medium supplemented with 2.46, 4.90, 9.80 and 14.7 μM of indolebutyric acid (IBA; Sigma Cell Culture, min. 90%, St. Louis, USA) and $\frac{1}{2}$ strength MS medium without growth regulators (as a control) for rooting induction. The pH of the medium was adjusted, autoclaved and cultures were incubated as mentioned the previous stages. The percentage of rooted shoots (rooting %), the number and length of roots explant⁻¹ were scored after four weeks of culture on the rooting medium.

Acclimatization

Rooted plantlets were removed from the nutrient medium and washed with distilled water to remove the debris on the roots. Hardening and acclimatization of the rooted plantlets were taken place gradually from the *in vitro* conditions to the greenhouse ($28 \pm 2^\circ\text{C}$, 70-80% relative humidity) and was done in plastic pots containing a mixture of peat moss and soil (1:1 v:v) (Peat moos, PROMIX®). Plants were irrigated, then covered with transparent polythene bags. The bags were perforated during the first seven days, then removed gradually to complete acclimatization.

Experimental design and statistical analysis of data

In vitro experiments were subjected to completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1990), were performed to analyze the obtained data. Each experiment contained at least 15 replicates and was repeated twice. Means followed by the same letter are not significantly different at $P \leq 0.05$.

DNA barcoding

DNA extraction and purification

Phlomis aurea leaf specimens were collected and ground under liquid nitrogen to a fine powder using a sterile mortar and pestle. DNeasy Plant Kit (Qiagen, Germany) was used for DNA extraction and purification. The extracted DNA concentration and quality were estimated by running on 1% agarose gel electrophoresis, using a DNA size marker (Lambda DNA *Hind III* digest Phi X 174/*HaeIII* digest).

PCR and gene sequencing

The PCR reaction was performed as mentioned by Ibrahim et al. (2016) in a total volume of 50 μL PCR master mixture consisted of the following: 1x buffer (Promega, USA), 15 mM MgCl_2 , 0.2 mM dNTPs (Promega, USA), 20 pcoml of each primer (Invitrogen, USA), 1 u of Taq DNA polymerase (GoTaq, Promega, USA), 40 ng DNA and ultra-pure water to the final volume.

DNA barcoding analysis was performed with the chloroplast DNA (cpDNA) genes; ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcl*), maturase K (*matK*) and RNA polymerase C1 (*rpoC1*) regions. For PCR amplification and sequencing of *rbcl*, *matK* and *rpoC1*, the primer pairs *rbcl*-F (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3'), *rbcl*-R (5'-TCG CAT GTA CCT GCA GTA GC-3'), *matK*-F (5'-CGA TCT ATT CAT TCA ATA TTT C-3'), *matK*-R (5'-TCTAGCACACGAAAGTCGAAGT-3') and *rpoC1*-F (5'-GGC AAA GAG GGA AGA TTT CG-3'), *rpoC1*-R (5'-CCA TAA GCA TAT CTT GAG TTG G-3'), were used for the PCR. Average amplicon size/bp were 692, 957 and 522 for *rbcl*, *matK* and *rpoC1*, respectively.

The PCR was performed with a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, USA) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C . Each cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 50°C for 30 s and an elongation step at 72°C for 30 s. The primer extension segment was extended to 7 min at 72°C in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug ml^{-1}) in 1X TBE buffer at 95 volts. A 100 bp DNA ladder (Promega, USA), was used as a molecular size standard for PCR product sizes determination. Gel images were visualized using UV transilluminator and photographed using a Gel Documentation System (BIO-RAD 2000, USA).

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, USA). The sequencing of the PCR product was carried out using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3730XL, Applied Biosystems) (Microgen, Korea) and a BigDye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems, USA) following the protocol supplied by the manufacturer.

Assignment of species

DNA barcoding of *Phlomis aurea* was carried out using the Basic Local Alignment Tool (BLAST) available on the National Centre of Biotechnology Information (NCBI) website. All sequences were submitted to GenBank, USA. GenBank

provided accession numbers for the nucleotide sequences of MK628683, MK628684 and MK628686.

BLAST searches were applied to all produced sequences using the available online databases (DDBJ/EMBL/GenBank) and were analyzed using BLASTN 2.8.1 program (<http://www.ncbi.nlm.nih.gov/BLAST>) and aligned using Align Sequences Nucleotide BLAST. Species identification was considered successful only when the highest maximal percent identity included a single species and scored >97% (de Groot et al., 2011). Phylogenetic analysis was conducted using MAFFT v6.864, <http://www.genome.jp/tools-bin/mafft>, then the phylogenetic trees were generated.

Conclusion

Phlomis aurea is an important plant ecologically, medicinally and economically and thus, it is an endangered plant that needs a strict conservation plan. In this study, identification of the plant using DNA barcoding was carried out and nuclear markers should be tested for the complete identification of the plant. Also, the first protocol for efficient micropropagation of *Phlomis aurea* was established in the present study. This protocol should be urgently applied to conserve the plant even in a botanical garden as a first step.

Acknowledgement

The authors are thankful to Dr. Ibrahim Abdelrafee El Gamal, Nature Conservation Sector, Egyptian Environmental Affairs Agency, Southern Sinai, Egypt for his help in collection of plant specimens.

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