# MOLECULAR IDENTIFICATION OF SOME SURROUNDING SPECIES OF FAMILY BORAGINACEAE WITHIN ELBA MOUNTAIN

**Esraa A. Elsherbeny**
Genetic and Cytology Unit, Genetic Department of Genetic Resources, Ecology and Dry Land Agriculture Division, Desert Research Center, Cairo, Egypt
E-mail: eamalesraa@yahoo.com

Floral identification has expanded to include additional aspects including the DNA barcoding technique to aid in the identification and delimitation of wild and uncertain plant species. In the current study an area with little focus, Elba Mountain in the far southern east corner of Egypt that have been explored and surveyed for the dominant plant species. The samples were collected, and were subjected to DNA extraction, PCR and sequencing using two molecular regions (chloroplast rbcL and nuclear ITS). A total of nine species belong to three plant families are reported in this work (i.e., the Capparaceae, Lamiaceace and Moraceace). Based on both BLAST search and phylogenetic analysis, the species identification procedure and comparative analysis were explained and shown while the identification decision was taken. In conclusion, six out of the nine species were identified, five were identified based on one DNA barcode sequence, and one based on the two regions. The identified and identified cases are shown and discussed in detail. This is a part of serial studies targeting the identification and delimitation of floral species dominating Elba Mountain of Egypt.

**Keywords:** DNA barcodes, NCBI, ITS, rbcL, Lamiaceace, Capparaceae, Moraceace, Egypt

## INTRODUCTION

In taxonomy, the need for species identification at the genetic level has been increasingly recognized. The term “DNA barcode” was first coined by Hebert et al. (2003) and has gained worldwide attention in the scientific community (Blaxter, 2003; Gregory, 2005 and Schindel and Miller, 2005). DNA barcoding is a molecular marker-based technique in which short fragments of DNA from either the nuclear genome or organellar genomes are utilized, that exhibit a sufficient level of variation to discriminate among

species. This technique identifies plant species more definite than the traditional taxonomic tools, with unrecognizable plant parts and without requiring taxonomic experience (Saddhe and Kumar, 2018 and Fouad et al., 2019). The essential characteristics of an ideal DNA barcoding are universality, short sequence length, flanked by the conserved regions, specificity on variation, easiness of employment, suitability for a wide range of taxa, and adequate variability in sequences to discriminate between the species (Kress and Erickson, 2008; Ford et al., 2009 and Hollingsworth et al., 2009). The potential of DNA barcoding applications is expansive, that is used in biodiversity monitoring, conservation impact assessment, monitoring of illegal trading, and powerful tool for non-professional users such as customs officers, traditional drug producers, forensic botany, etc. (Nithaniyal et al., 2014; Verma and Goswami, 2014 and Ferri et al., 2015). DNA barcoding helped in systematics and the identification of species bounder, as well as the cryptic species and the adulteration of the medical plants with non-medicinal substitutes of a closely related species with lower efficiency (Ragupathy et al., 2009). Additionally, this technique is useful to be able to identify species from materials such as roots, seeds, and pollen; or in mixtures of plants sampled from the air, soil or water (de Vere et al., 2012).

Recently, DNA barcode data have been widely and regularly used to provide additional evidence at the molecular level for plant taxonomic studies. The trend of combining morphological characteristics, chemical, and genetic markers into a dataset for species identification becomes very important for systematic studies, in which DNA barcoding has become one of the most efficient tools for species identification of medicinal plants (Pham et al., 2021). In 2009, a large consortium of researchers, the Consortium for the Barcode of Life (CBOL) Plant Working Group, proposed portions of two coding regions from the plastid (chloroplast) genome, rbcL and matK, as a "core barcode" for plant identification; these regions are supplemented with additional regions as required (CBOL Plant Working Group, 2009). The China Plant BOL Group (CPBG) confirmed that the ITS/ITS2 regions should be incorporated into the core barcode for seed plants (China Plant BOL Group, 2011 and Li et al., 2015). These loci have also proved valuable in the assessment of relationships at lower taxonomic levels and have been used to distinguish closely related species and intraspecific taxa (e.g., Pang and Chen, 2006; Chen et al., 2010; Kesanakurti et al., 2011; Kang et al., 2017 and Cahyaningsih et al., 2022). However, DNA barcoding markers are universal, and choosing the correct loci is challenging (de Groot et al., 2011).

The objective of the present study was to characterize nine plant species using rbcL and ITS regions to confirm their identification as economical and/or medicinal plants, compare the discriminatory power of the standard barcode regions, and explore the taxonomic implications in the studied taxa. Results could help generate DNA barcode libraries of the Elba

Nature Reserve plant flora, which could be a step toward completing Egypt and global DNA barcoding libraries.

## MATERIALS AND METHODS

### 1. Study Area

The study was conducted in Elba Nature Reserve (Elba Mountain), which is in the eastern south triangle of the Eastern Desert in Halai'b city, 250 km south of Marsa Alam city (Fig. 1). This mountain range is considered a continuation of the granitic formation of the Red Sea highland complex between Egypt and Sudan, situated between 36º and 37º of the eastern longitudes and about 22º of the northern latitude (El-Ghani and Abdel-Khalik, 2006). It is considered one of the most important protected areas in Egypt; it has a complete environmental tropical system and includes 2000 unique wild plant types and extinct animals (Seif El-Nasr, 2017).

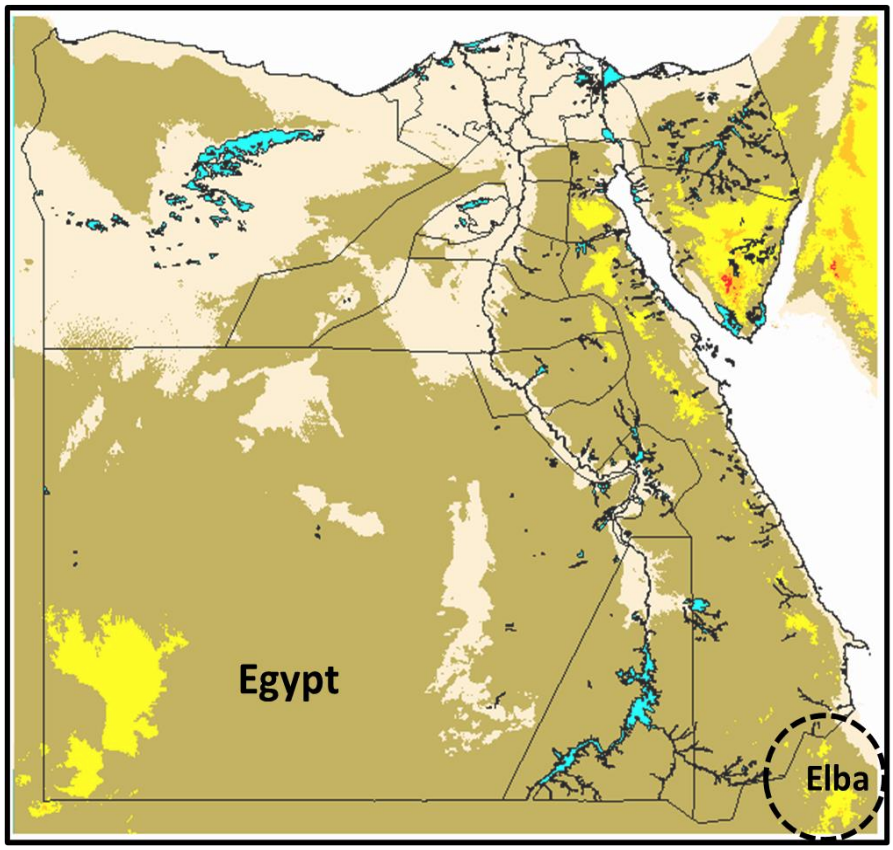


**Fig. (1).** Location of Elba Mountain in south-east Egypt.

**2. Sample Collection and Identification**

Green leaves were collected from nine individuals from their natural populations in Elba Nature Reserve during the Summer of 2019. Leaf tissue (approximately 2 $cm^2$) was collected from each fresh specimen and dried in silica gel for DNA barcoding analysis. The collected materials were developed based on morphological identification (Täckholm, 1974 and Boulos, 2009).

**3. DNA Extraction, PCR Amplification, and Sequencing**

The DNA extractions were performed on healthy dried leaf tissues using the WizPrep™ gDNA Mini Kit (Cell/Tissue; Korea), according to the manufacturer's instructions, with a final elution volume of 50 ml. The concentration and quality of the extracted DNA were checked by 1% gel electrophoresis and visualized under UV light using the Ingenius3 Gel documentation system (Syngene, UK).

PCR amplification was performed for the core barcoding markers rbcL (plastid barcode region) and ITS (nuclear ribosomal barcode region), using Taq DNA polymerase with universal primers. The forward and reverse primers for rbcL and ITS used were rbcLa-F 5′-TGTCACCACAAACAGACTAAAGC-3′, rbcLa-R 5′-GTAAAATCAAGTCCACCRCG-3′ (Levin et al., 2003), and ITS-U1 5′-GGAAGKARAAGTCGTAACAAGG-3′, ITS-U4 5′-RGTTTCTTTTCCTCCGCTTA-3′ (Cheng et al., 2016), respectively. The two candidate DNA barcodes were amplified by PCR amplification using One PCR™ Plus (Genedirex®, Taiwan) master mix in a total volume of 25 μL, which contained 12.5 μL of OnePCR™, 1 μL of each primer (forward and reverse, each of 10 μM), and 1 μL of extracted DNA (~100 ng/μL). The optimized PCR profile for both rbcL and ITS comprised of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing for rbcL and ITS at 50°C for 30 sec, extension at 72°C for 90 s, and a final extension segment at 72°C for 10 min. PCR products were examined electrophorectically using 1.5% agarose gels and purified using EasyPure® PCR Purification Kit (TransGen Biotech, Beijing, China), following the manufacturer's instructions). Samples were sequenced commercially using the amplification primers. The bidirectional sequencing was completed using the Sanger method (Macrogen Inc., Seoul, South Korea).

**4. Sequence Alignment and Data Analysis**

The quality estimation and assembly for the newly generated sequences were performed using Geneious R10 (Kearse et al., 2012). All the newly acquired sequences were confirmed via the BLAST search tool in the NCBI database against the online nucleotide database and further deposited in GenBank. The sequence alignment for each locus was initially performed using the MAFFT aligner (Katoh and Standley, 2013), implemented in Geneious R10. The genetic distances were calculated using the maximum likelihood methods computed by MEGA X (Tamura et al., 2018), according to the Kimura 2-Parameter (K2P) model.

## RESULTS

### 1. BLAST Based Identification

#### 1.1. Chloroplast rbcL gene

The rbcL average sequence length was 270±3 bp, with a minimum and a maximum length of 267 to 273 bp, respectively. The overall sequence quality was Q20 = 96.2%. Based on BLAST search by similarity, the top hits for each plant sample rbcL sequence were retrieved independently. Based on the morphological inspection and limited by the BLAST nucleotide database filtered by > 95% pairwise identity (PI), the species (01) matched *Capparis decidua* (NC_053386; PI = 99.30%), species (02) matched *Maerua oblongifolia* (NC_058240; PI = 98.70%), species (03) matched *Lavandula coronopifolia* (KY656732; PI = 99.60%), species (04) matched *Otostegia* sp. (AB586362; PI = 98.60%), species (05) matched *Ocimum gratissimum* (NC_057196; PI = 100%), species (06) matched *Lavandula coronopifolia* (KY656732; PI = 97.30%), species (07 and 08) matched *Ficus platypoda* (MT325958; PI = 98.80 and 99.70%, respectively) and species (09) matched *Ficus carica* (MK286386; PI = 99.80%). In the case of species (05) additional species matched at the same PI value, namely, *Hanceola exserta* (NC_056915) (Table 1).

**Table (1).** List of the top-hits of the rbcL based BLAST results for each of the sampled species from Elba Mountain in south-east Egypt.

| Species | Sample_ID | Organism | rbcL | PI |
|---|---|---|---|---|
| **S01** | Capparaceae_S01 | *Capparis decidua* | NC_053386 | 99.3% |
| **S02** | Capparaceae_S02 | *Maerua oblongifolia* | NC_058240 | 98.7% |
| **S03** | Lamiaceae_S01 | *Lavandula coronopifolia* | KY656732 | 99.6% |
| **S04** | Lamiaceae_S02 | *Otostegia* sp. | AB586362 | 98.6% |
| **S05** | Lamiaceae_S03 | *Ocimum gratissimum*<br>*Hanceola exserta* | NC_057196<br>NC_056915 | 100.0% |
| **S06** | Lamiaceae_S04 | *Lavandula coronopifolia* | KY656732 | 97.3% |
| **S07** | Moraceae_S01 | *Ficus platypoda* | MT325958 | 98.8% |
| **S08** | Moraceae_S02 | *Ficus platypoda* | MT325958 | 99.7% |
| **S09** | Moraceae_S03 | *Ficus carica* | MK286386 | 99.8% |

#### 1.2. Nuclear ITS region

The ITS sequences recorded average length of 624±29 bp, with a minimum and a maximum length of 553 to 646 bp, respectively. The overall sequence quality was Q20 = 99.90%. Based on BLAST similarity search, the top hits for each plant sample sequence were retrieved independently. Based on the morphological inspection and limited by the BLAST nucleotide database filtered by > 91% pairwise identity (PI), the species (01) matched *Pergularia tomentosa* (KF850578; PI = 99.80%), species (04) matched

*Leucas chinensis* (MH768220; PI = 91.60%), species (06) matched *Lavandula coronopifolia* (MN907383; PI = 99.80%), species (07) matched *Ficus cordata* (KF850595; PI = 99.80%), species (08) matched *Ficus virens* (KJ845958; PI = 99.90%), species (09) matched *Ficus palmata* (AY730125; PI = 100%). In the case of species (07 and 09) additional species were matched at the same PI value, namely, *Ficus virens* (MT955652) and *Ficus johannis* (AY730123), respectively. In the case of species (02, 03 and 05) multiple-peak were detected, which reflect a multicopy of the ITS sequences and subsequently a sign of hybridization and mostly interspecific hybridization that cause a severe shift in the ITS region (Table 2).

**Table (2).** List of the top-hits of the ITS based BLAST results for each of the sampled species from Elba Mountain in south-east Egypt.

| Species | Sample_ID | Organism | ITS | PI |
|---|---|---|---|---|
| **S01** | Capparaceae_S01 | *Pergularia tomentosa* | KF850578 | 99.80% |
| **S02** | Capparaceae_S02 | - | - | - |
| **S03** | Lamiaceae_S01 | - | - | - |
| **S04** | Lamiaceae_S02 | *Leucas chinensis* | MH768220 | 91.60% |
| **S05** | Lamiaceae_S03 | - | - | - |
| **S06** | Lamiaceae_S04 | *Lavandula coronopifolia* | MN907383 | 99.80% |
| **S07** | Moraceae_S01 | *Ficus cordata* | KF850595 | 99.80% |
| | | *Ficus virens* | KJ845958 | 99.80% |
| **S08** | Moraceae_S02 | *Ficus virens* | KJ845958 | 99.90% |
| **S09** | Moraceae_S03 | *Ficus palmata* | AY730125 | 100.00% |
| | | *Ficus johannis* | AY730123 | 100.00% |

## 2. Phylogeny Based Identification

### 2.1. Chloroplast rbcL gene

The rbcL sequences along with the BLAST top 5 hits were aligned together and trimmed of equal length. The retained total nucleotide alignment was 270 bp, with total identical sites of 231 (85.6%), PI = 94%, and Q20 of at least 98% of the retained nucleotides. The nucleotide frequencies of non-gaped sites were 25.6%, 22.2%, 24% and 28.3% for A, C, G and T, respectively, with GC% = 46.1%. Based on the aligned sequences, the maximum likelihood tree was constructed and visualized as a rooted cladogram (Fig. 2).

The phylogenetic status was not similar to the published taxonomical information but the species from the same family are clustered together. In detail, two major clades corresponding to the Lamiaceace and Capparaceae/Moraceace were distinguished. Species (01 and 02) were both

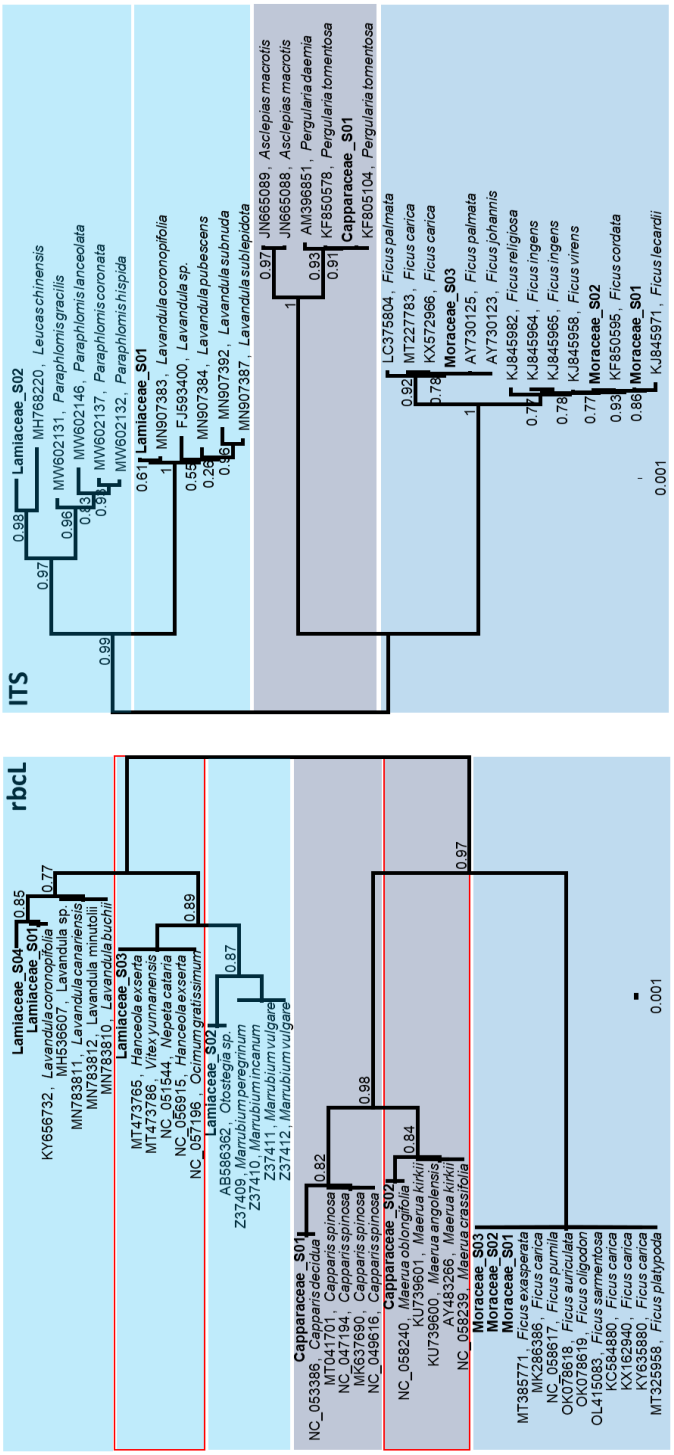


**Fig. (2)**. Comparative phlogenetic analysis based on two DNA barcoding markers (rbcL and ITS). Each of the identified families was shaded in a different color, whil missing individual in ITS were marked with red box on the rbcL tree.

clustered within the Capparaceae subcluster, each belong to different genera, *Capparis* and *Maerua*, respectively. The final assigned species names were, *Capparis decidua* and *Maerua oblongifolia*, respectively. Species (03, 04, 05 and 06) were all clustered within the Lamiaceace cluster, in which the species (03 and 06) were both identified as *Lavandula coronopifolia* with high bootstrap value (0.85). In case of species (04), it was identified to unidentified species belong to genus *Otostegia* and the proximate species found in the database were belong to genus *Marrubium*. The species (05) was clustered with several species from different genera (i.e., *Hanceola*, *Vitex*, *Nepta* and *Ocimum*) all from Lamiaceae family, uncertain identification was labeled for this species. Similarly, species (07, 08 and 09) all from the Moraceae family were clustered to all other *Ficus* species without any structure, which cause a confusion for the species identification (Fig. 2).

**2.2. Nuclear ITS region**

The ITS sequences along with the BLAST top 5 hits were aligned together and trimmed of equal length. The retained total nucleotide alignment was 705 bp, with total identical sites of 242 (34.3%), PI = 67.6%, and Q20 of at least 99.90% of the retained nucleotides. The nucleotide frequencies of non-gaped sites were 16.9%, 30.8%, 32.5% and 19.9% for A, C, G and T, respectively, with GC% = 63.2%, while the gaps were 10.5% of the total alignment.

Based on the aligned sequences, the maximum likelihood tree was constructed and visualized as a rooted cladogram (Fig. 2). The phylogenetic status was not similar to the published taxonomical information but the species from the same family are clustered together. In detail, two major clades corresponding to the Lamiaceace and Capparaceae/Moraceace were distinguished. Species (04) and (06) represented the Lamiaceae family but of two different genera, *Lavandula* and *Leucas*, respectively. The first, was highly clustered to *Leucas chinensis* with a bootstrap of 0.98. The other species was highly clustered to *Lavandula coronopifolia* with a bootstrap of 0.61. The Capparaceae family was represented by species (01) and was clustered with *Pergularia tomentosa* with high bootstrap value of 0.91. In the case of the members of the family Moraceae, the species (07) was highly clustered with *Ficus lecardii* (bootstrap value = 0.86), species (08) was highly clustered with *Ficus cordata* (bootstrap value = 0.93), and species (09) was equally clustered to *Ficus palmata* and *Ficus johannis* with 0.8 bootstrap value (Fig. 2).

**3. Comparative Phylogenetic Analysis**

The comparison between two DNA barcoding regions and the identification methods (i.e., BLAST vs. Phylogeny) showed agreements as well as discrepancies. Based on the BLAST results, the molecular identification using both molecular loci agreed for species (06) as *Lavandula coronopifolia*, but not the same for its similar species (03), as the ITS showed

multiple copies with no valid sequence to be used for identification. For species (02, 03 and 05), the same ITS issue was found and relied only on the rbcL to identify, and finally identified as *Maerua oblongifolia*, *Lavandula coronopifolia* and *Ocimum gratissimum*. The ITS phylogenetic analysis showed enough genetic variation to delimit species by paraphyletic clustering for species (07 and 08) in contrast to the rbcL monophyletic clustering for those species and were recorded as *Ficus lecardii* and *Ficus cordata*. None of the two DNA barcoding regions were able to delimit species (04 and 09) and still identified as Lamiaceae sp. and *Ficus* sp., with no certain match, respectively. Likewise, species (01), was identified as two different species for each of the two markers, as *Pergularia tomentosa* for ITS and *Capparis decidua* for rbcL. The same case was found for species (09), where the ITS was unable to distinguish between two *Ficus* species, namely *Ficus palmata* and *Ficus johannis*. All the cases of conflicts detected between the identification based on the two DNA barcodes would require an explanation (Table 3).

**Table (3).** Comparative identification results obtained for rbcL and ITS DNA sequencing from nine samples collected from Elba Mountain in south-east Egypt.

| N | BLAST-ITS | Phylo-ITS | BLAST-rbcL | Phylo-rbcL | Decision |
|---|---|---|---|---|---|
| **S01** | *Pergularia tomentosa* | *Pergularia tomentosa* | *Capparis decidua* | *Capparis decidua* | *Hybrid* |
| **S02** | - | - | *Maerua oblongifolia* | *Maerua oblongifolia* | *Maerua oblongifolia* |
| **S03** | - | - | *Lavandula coronopifolia* | *Lavandula coronopifolia* | *Lavandula coronopifolia* |
| **S04** | *Leucas chinensis* | *Leucas chinensis* | *Otostegia* sp. | *Otostegia* sp. | *Leucas* sp. |
| **S05** | - | - | *Ocimum gratissimum* | Unidentified | *Ocimum gratissimum* |
| **S06** | *Lavandula coronopifolia* | *Lavandula coronopifolia* | *Lavandula coronopifolia* | *Lavandula coronopifolia* | *Lavandula coronopifolia* |
| **S07** | *Ficus cordata* | *Ficus lecardii* | *Ficus platypoda* | *Ficus* sp. | *Ficus lecardii* |
| **S08** | *Ficus virens* | *Ficus cordata* | *Ficus platypoda* | *Ficus* sp. | *Ficus cordata* |
| **S09** | *Ficus palmata /johannis* | *Ficus palmata /johannis* | *Ficus carica* | *Ficus* sp. | *Ficus* sp. |

## DISCUSSION

The phylogenetic based identification using the ITS tree was better in identifying the samples at the familiar or lower levels in contrast to the rbcL. The ITS was efficient in identifying some of the studied species but it cannot be used alone due to paralogous issues that cause intraspecific variation (Kress et al., 2005; El-Atroush et al., 2015 and El-Sakaty et al., 2022). In the current

analysis, six out of the nine species were identified, five were daintified based on one DNA barcode sequence, and one based on the two regions.

In the case of the uncertain or unidentified, the lack of enough database barcodes would explain the in-between case found for species no 4 (*Leucas* vs. *Otostegia*). None of the two barcodes were successful to find a highly similar match (i.e., > 99%), and were phylogenetically clustered apart from other species of another genus. Both species are known to be part of a problematic sister genera, that cause conflict in identification and taxonomical positioning particularly among members of the subfamily Lamioideae (Scheen and Albert, 2007). However, in DNA barcoding technique, the main factors that may affect how barcode works is the database and sequence search strategies (Kress and Erickson, 2008). Other observed case is species (01) that was identified from two different species of two different families, the intercrossing between the same genera is more likely, however, crossing between plant species of different taxonomical ranks is possible (e.g., Xiu-Cheng et al., 2008).

The floral distribution of the detected species is peculiar, some are not originated from Egypt or approximate areas, e.g., *Ficus cordata*, a species dominates the southern parts of the African continent (Botanic Gardens Conservation International, 2018). Others were rarely occurring in Egypt, and has a known medical importance, e.g., *Maerua oblongifolia* (GBIF Secretariat, 2021a). As well as a frequent species from the area and proximate species that are known for its essential oils and uses for aromatic and cosmetic purposes, e.g., *Lavandula coronopifolia* (GBIF Secretariat, 2021b). Based on the analysis of this study, an *in-situ* conservation plan for the studied plant species is recommended; the species are of a valuable botanical role as part of the mountain peculiar and exotic genetic resources, that will allow a continued adaptation and evolution of migrated plant genotypes. Additionally, a local database of authenticated DNA barcodes should be created so that plant DNA barcoding techniques can be used and applied effectively.

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# التعريف الجزيئي لبعض أنواع النباتات المحيطة بالعائلة الحمحامية في جبل علبة

**إسراء عطية الشربيني**
وحده الوراثة والسيتولوجى، قسم الأصول الوراثية، شعبة البيئة وزراعات المناطق الجافة، مركز بحوث الصحراء، القاهرة، مصر

تم استخدام تقنية باركود الحمض النووي DNA في تحديد أنواع النباتات البرية والغير مؤكدة. في هذه الدراسة تم عمل استكشاف ومسح للنباتات البرية السائدة في منطقة جبل علبة الواقع في الركن الجنوبي الشرقي من مصر. تم جمع العينات واستخلاص الحمض النووي، وعمل PCR والتسلسل باستخدام منطقتين جزيئيتين (كلوروبلاست rbcL وITS النووية). حيث تم دراسة تسعة أنواع تنتمي إلى ثلاث عائلات نباتية (Capparaceae وLamiaceace وMoraceace) استنادًا إلى كل من بحث بلاست وتحليل النشوء والتطور، تم تحديد الأنواع والتحليل المقارن وعرضها أثناء اتخاذ قرار تحديد الهوية. وأخيرًا تم التمكن من تحديد ستة أنواع من أصل تسعة، وتم تحديد خمسة بناءً على تسلسل رمز شريطي واحد للحمض النووي وآخر بناءً على منطقتي الجينين rbcL وITS حيث أن هذه الدراسة جزء من دراسات متسلسلة تستهدف تحديد وتعريف وتوصيف أنواع النباتات البرية بجبل علبة في مصر.