PHYLOGENETIC ANALYSES OF SOME EGYPTIAN GENERA OF Lamiaceae FAMILY USING rbcL SEQUENCES

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ABSTRACT

Six local Egyptian commercial cultivars of family Lamiaceae, two cultivars of genus Ocimum L. (Basil), two cultivars of genus Menthe L. (Mint), and two cultivars of genus Thymus L. (Thyme) were analyzed for ribulose 1,5-bisphosphate carboxylase Large (rbcL) gene at the level of DNA sequences. All samples successfully amplified the ± 630 bp fragment. Additionally, the results of alignment analysis using BLASTN tools divulged that the sequence of DNA rbcL for the two local basil cultivars (Basil1 & Basil2) has similarities with (Ocimum basilicum, Ocimum tenuiflorum, Ocimum kilimandscharicum and Ocimum gratissimum) 100, 99.69, 99.37 and 99.06 respectively. In addition, two local mint cultivars (Mint 1 and Mint 2) has similarities with Mentha spicata, Mentha pulegium, Mentha canadensis and Mentha menthaefolia, 99.85, 99.84, 99.69 and 99.53 respectively. For thyme local cultivars (Thyme1 and Thyme2), Thym1 cultivar sample genotype is genetic closely with species, (Thymus alsinoides and Thymus sibthorpii) with 99.69 and 99.84 respectively and they located nearest from the cluster (Thymus genus) members in phylogenetic trees while, Thym2 is located after the cluster with Artemisia genus belonging to family Asteraceae. The reason of this result may be occurring due to that a Thym2 genotype comes from local marketing, which some of them are selling it as a thymus genus however, it is belongs to Artemisia genus and has similarities with with seven species of Artemisia genus (Artemisia absinthium, Artemisia gmelinii, Artemisia selengensis, Artemisia scoparia, Artemisia maritima, Artemisia capillaris and Artemisia tukudo). Using of rbcL DNA barcode proves to be effective in identifying the plants from the family level up to the genus level. This study demonstrates the efficiency of using rbcL barcoding primer to classify family Lamiaceae phylogenetically. It is also concluded that the rbcL gene showed genuine potentials to distinguish the plant Egypt species under investigation into the proper family and genus.

Keywords: DNA barcode; rbcL; Family lamiaceae; BLASTN; Phylogenetic

INTRODUCTION

From ancient times, medicinal plants have been used to treat diseases and even today, in many tribal and primitive societies, these plants are used on a large scale. Metabolic by-products of plants such as alkali, glycosides, steroids or other active compounds are also used to treat various diseases such as cancer, malaria, diabetes and dysentery. (Biradar, 2015). Therefore, the precise knowledge and molecular identification of medicinal plants are essential for developing new healthcare products from plants.

Lamiaceae family, which includes many cultivars of plant species is characterized by its economic importance, which is used for the most part as cooking herbs and the other part is used to extract a lot of drug-active compounds (Venkateshappa and Sreenath, 2013), which have been used for producing pesticides, cosmetic and food (Khaled-Khodja et al 2014). Today, the essential oils of a few Lamiaceae plants have become an inexorably significant crude material for the food, pharmaceutical and cosmetic industry (Edris, 2007).
Using DNA barcoding for molecular identification of medicinal plants could be very tricky and challenging at the same time in term of generating barcodes data and in analyzing these data to stands on the discrimination power (Cowan and Fay, 2012). Practically, DNA barcoding technique depends on a short and unique DNA sequence for one locus or a few loci utilized together as an altogether unit. The generating data from a unique species used for fingerprinting and copyright protection for this species and marketplace regulation in general. (Kress and Erickson, 2007. Elansary et al. 2017)

Mainly, to distinguish a difficult taxa, DNA barcoding markers will be the best option by generating a phylogenetic tree (ElAtroush et al 2015). moreover, the plant barcode, such as rbcL, should be multi-locus, preferably comprising a conserved coding region or vice versa, more rapidly evolving region that is probably non-coding (Kress et al 2009).

The rbcL (Ribulose-1,5 - bisphosphate carboxylase/oxygenase large subunit) gene that is coding for large subunit of the enzyme RuBisCo, consider one of the most barcoding genes used in the phylogeny of plants.

By the consent of consortium for the barcode of life (CBOL) in 2009 they consider matK (the chloroplast gene) and rbcL as the main barcodes of plant species, in addition to intergenic sequence trnH-psbA and nuclear gene ITS as the addition barcodes (CBOL, 2009). Meanwhile, rbcL is well-known by its comparability, universality and easy amplification (Hollingsworth et al 2016). rbcL genes used successfully to classification of angiosperm, and even among the different groups of the seed plants (Chase et al 2007). take into consideration that the variation in rbcL sequence commonly exists at only the above-species level, and rarely found at the species level (Newmaster et al 2006), resulting in poor discrimination power at species level (Gonzalez et al 2009). At a study of Newmaster et al (2006) they aligned approximately 10,300 rbcL sequences collected from GenBank, and found that rbcL gene cannot be able to distinguish between all plant species but can clearly distinguished plants belong to the same genus Newmaster et al (2006).

Another study of Sundari et al (2019), they used the rbcL for identification of red jaben and gofasa plants collected from Indonesia stated that the effectiveness of using rbcL in identify plants have been limited into the family level up to the genus Sundari et al (2019).

So, our study will try to found some genetic variations able to build a clear phylogenetic relationship between some plants belong to Lamiaceae family utilizing rbcL genes barcoding.

MATERIALS AND METHODS

MATERIALS

Fresh leaves of six local Egyptian commercial cultivars belong to Lamiaceae family were collected from private and public nurseries in Egypt (Table 1).

Table 1. The six medicinal plants information’s used in this study collected from a private and public nursery in Egypt

| No | Sample name | Genus | Collection site (city, Governorate) |
|----|-------------|-------|-----------------------------------|
| 1  | Basil1      | Ocimum L. (basil) | Kaf Shukr, Al-Qalyubiyya |
| 2  | Basil2      | Menthe L. (mint) | Ossim, Giza |
| 3  | Mint1       | Thymus L. (thyme) | Shibin El Qanater, Al-Qalyubiyya |
| 4  | Mint2       |                | Sinnuris, Fayyum |
| 5  | Thym1       |                | Qalyub, Al-Qalyubiyya |
| 6  | Thym2       |                | El Hawamdeya, Giza |

METHODS

DNA barcoding

Genomic DNA purification

Six fresh plant leaf samples from local cultivars belonging to three genera of Lamiaceae family were collected from different governorates of Egypt. DNA extraction was carried out using Plant High Molecular DNA extraction KIT (SIGMA, USA). The quality of DNA was assist using agarose gel electrophoresis, visualized by pre-added RedSafe® (5ul /100ml) under UV light. The quantities and purities of DNA were assisting using UV spectrophotometer on 260 nm and 280 nm (BIO RAD- SmartSpec Plus spectrophotometer).

PCR and sequencing

The primer sequences used to amplify rbcL fragment was:

rbcL- Forward (5’-ATGTCACCACAACAGGAAC-3’)
rbcL- Reverse (5’TGCATGTACCTGCAGTAGC-3’)

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The PCR amplification was carried out in a Peltier Thermal Cycler (Techne Laboratories, USA). The thermal cycling conditions consisted of 1 cycle at 95°C for 5 min, followed by 30 cycles of denaturation at 92°C for 15 sec, annealing temperatures for 30 sec, and extension at 72°C for 30 sec and last extension step at 72°C for 5 min.

The PCR reaction mixture consisted of 1 μL (~50 ng) DNA, 12.5 μL master mix (Transgen Biotech Company), and 1.0 μL (2.5 μmol/L) each primers in a final volume of 25 μL. Standard PCR profile with 50°C annealing temperature was used to rbcL.

**Taxa assignment**

Basic local alignment tools (BLAST) were applied to all produced sequences using online NCBI databases (http://www.ncbi.nlm.nih.gov/ Blast). The hits with maximal percent identity scores > 95% was considered successful when all involved a single genus.

**Molecular identification and phylogenetic analysis**

The maximum likelihood (ML) analysis method was applied to align rbcL sequences using MEGA10 (Tamura et al. 2018) with the following parameters:

1. Tree inference options were set to Nearest Neighbor Interchange.
2. Gaps/missing data were treated as partial deletions with site coverage cut off = 95%.
3. A bootstrap analysis with 100 replicates was carried out in order to study the clade support values.

**RESULTS AND DISCUSSION**

**DNA purification**

The concentration of genomic DNA isolated from the leaves of six plants ranged from 11.6 to 27.1 ng/ml with purities from 1.12 to 1.86 (Table 2).

**Table 2. Results of quantitative measurements of DNA isolation**

| No | Sample name | Genus       | Concentration (ng/ml) | A260/280 |
|----|-------------|-------------|-----------------------|----------|
| 1  | Basil1      | Ocimum L.   | 11.6                  | 1.20     |
| 2  | Basil2      | (basil)     | 13.4                  | 1.32     |
| 3  | Mint1       | Menthe L.   | 17.7                  | 1.12     |
| 4  | Mint2       | (mint)      | 19.3                  | 1.73     |
| 5  | Thym1       | Thymus L.   | 27.1                  | 1.29     |
| 6  | Thym2       | (thyme)     | 21.6                  | 1.86     |

**The amplification of rbcL gene**

The obtained results of rbcL gene amplification from Lamiaceae plant samples examined by agarose gel electrophoresis are shown in Fig. (1).

**Fig. 1.** Agarose gel electrophoresis (1.5%) showing the PCR amplification of the amplified rbcL gene from the six samples (Lane 1: Basil1; Lane 2: Basil2; Lane 3: Mint1; Lane 4: Mint2; Lane 5: Thym1; Lane 6: Thym2). M= OneMARK 100, GeneDireX.

The amplification of the rbcL gene showed DNA bands with a size of ± 630 bp for all the amplified samples.

**Phylogenetic Analysis**

In order to identify phylogenetic analysis, six samples of this study were compared with data from the Gene Bank of BLAST search results at NCBI. From this phylogenetic analysis the genetic relation within and between two cultivars of genus Menthe L. (Mint), two cultivars of genus Ocimum L. (Basil) and two cultivars of genus Thymus L. (Thyme) were detected. Data from kinship analysis (phylogenetic) are shown in Figs. (2, 3 and 4).

Identification of genus ability of each DNA barcodes was assessed using the BLAST method. In this study was directed as follows: firstly, every DNA sequences of the six collected samples of family Lamiaceae was downloaded from NCBI database was established utilizing the downloaded sequences (Burgess et al. 2011). Secondly, each sequence measured was BLAST against the sequence in Gene bank database, and the percentage of identical sites was calculated and was taken as the genus discrimination rate of the measured sequence. If the
percentage of identical sites of a sequence calculated between intraspecific individuals were higher than interspecific individuals were, then the sequence was taken as the purpose one of the studied species. Finally, the identification success rate of DNA barcoding was calculated as the result of sequencing success rate and genus discrimination rate (Kress et al 2009).

The chloroplast gene (rbcL) was used for phylogenetic comparative analysis of six local Egyptian commercial cultivars belong to Lamiaceae family. The results showed in Tables (3, 4, 5, and 6) Figs. (2, 3, and 4).

The Results of phylogenetic tree for the two local Egyptian basil cultivars (Basil1 and Basil2) showed two main clusters, the first one included with five different basil species (Ocimum basilicum, Ocimum americanum, Ocimum kilimandscharicum, Ocimum tenuiflorum and Ocimum gratissimum), while the second cluster included two species (Salvia bulleyana and Salvia przewalskii) (Table 3 and Fig. 2).

The two basil Egyptian cultivars (Basil1 and Basil2) were found to be linked together and have closer relationship (100%) with Ocimum basilicum species.

The rbcL gene of two local Egyptian basil cultivars (Basil1 and Basil2) were highly similar to species Ocimum basilicum, Ocimum tenuiflorum, Ocimum kilimandscharicum and Salvia bulleyana with 100, 99.69, 99.37 and 99.06, respectively.

The Blast results for rbcL gene of tested samples (Mint1 and Mint2) are shown in Table (4) and Fig. (3). The Results of phylogenetic tree for the two mint cultivars (Mint1 and Mint2), indicated two main clusters, the first one included two local Egyptian mint (Mint1 and Mint2) with six different mint species (Mentha rotundifolia, Salvia officinalis, Mentha spicata, Mentha canadensis, Mentha longifolia and Mentha suaveolens), while the second cluster included two species (Mentha menthaefolia and Mentha pulegium).

The rbcL gene of the two mint cultivars (Menth1 and Menth2) were closely genetic related to genus (Mentha spicata and Mentha longifolia) and (Mentha pulegium and Mentha suaveolens) with 99.85 and 99.84, respectively.

The Blast results for rbcL gene of tested samples (Thym1 and Thym2) are shown in Tables (5 and 6) and Fig. (4).

The results for rbcL gene of tested samples (Thym1 and Thym2) are shown in Tables (5 and 6) and Fig. (4). The phylogram could be separated into two distinct clusters. Cluster one contained Thym2 with seven species of Artemisia genus (Artemisia absinthium, Artemisia gmelini, Artemisia selengensis, Artemisia scoparia, Artemisia maritima, Artemisia capillaris and Artemisia fukudo). Whereas Thym1 with three species of Thymus genus (Thymus alsinoides, Thymus vulgaris and Thymus sibthorpii) and three species of Salvia genus (Salvia japonica, Salvia nemorosa and Salvia rosmarinus) were grouped in second cluster. The phylogenetic tree of tested samples from the rbcL sequences showed that Thym1 genotype was genetic closely related with species, Thymus alsinoides and Thymus sibthorpii in cluster one forming clearly distinctive clades (monophyletic groups) whereas in second cluster, only Artemisia genus form clearly distinctive clades while Thym2 genotype not positioned within which was this clade (Figure 4). Thym1 plant samples are located nearest from the cluster (Thymus genus) members in phylogenetic trees while Thym2 was located next to the cluster with Artemisia genus. The reason of this result may be occurs due to that a Thym2 genotype comes from local marketing, which some of them are selling it as an thymus genus however, it is belongs to Artemisia genus.

The results of phylogenetic analysis using Neighbor Join (NJ) method note that the rbcL gene can used to clarify taxon positions in the identification of a species. A specimen from a different area may be together on the same cluster (Che et al 2012). Our results showed that the identification of Basil, Mint and Thyme plants were effective. Basil1 and Basil2 genotypes were identical (100%) to Ocimum basilicum species. For Mint1 and Mint2 genotypes, it has a 99.85% similarity with Mentha spicata and Mentha longifolia NCBI database. Actually, the rbcL gene can amplified with a high achievement rate with one or two universal primers. It was additionally stated that when compared to other barcode gene candidates, the rbcL gene has a high achievement rate of bidirectional sequencing (Consortium for the Barcode of Life sequencing with forward and reverse primers) (CBOL, 2009).

The study of rbcL gene sequencing was efficient to differentiate between some genera and species of family Lamiaceae due to sufficient data in GenBank (Molins et al 2011).

A many researchers have recommended that rbcL gene should be incorporated as a standard for comparison to different markers due the advantage that this gene is handily amplified and sequenced in many plants and it is viewed as a benchmark locus in phylogenetic examinations by providing a reliable
placement of a species into plant genus and/or family (Hassel et al. 2013).

In conclusion, this study gives a fundamental appraisal information that will be valuable for more extensive utilization of DNA barcoding in medicinal plants. It was discovered that rbcL was valuable for the barcoding of some medicinal plant species in family Lamiaceae, where it has a good resolution toward species identification. However, further protocol development to improve clean DNA extraction, PCR amplification programs, including the development of new primers, and local confirmed databases could assume significant important roles in efficient utilization of plant barcoding.

Table 3. The BLAST results for Basil1 and Basil2 of tested samples. Database search match for similarities and phylogenetic relationship using rbcL gene sequences

| Species from NCBI          | Accession | Query cover | % identity | E value |
|---------------------------|-----------|-------------|------------|---------|
| Ocimum basilicum          | KY623639  | 99%         | 100%       | 0.0     |
| Ocimum tenuiflorum        | NC043873  | 99%         | 99.69%     | 0.0     |
| Ocimum americanum         | MF468188  | 99%         | 99.69%     | 0.0     |
| Ocimum kilimandscharicum  | MF468191  | 99%         | 99.37%     | 0.0     |
| Salvia bulleyana          | NC_041092 | 99%         | 99.06%     | 0.0     |
| Salvia przewalskii        | NC_041091 | 99%         | 99.06%     | 0.0     |
| Ocimum gratissimum        | MF468194  | 99%         | 99.06%     | 0.0     |

Fig. 2. Phylogenetic relationship of gene rbcL sequence for basil (Basil1 and Basil2) samples based maximum likelihood tree
Table 4. The Blast results for Mentha1 and Mentha2 of tested samples. Database search match for similarities and phylogenetic relationship using *rbcL* gene sequences

| Species from NCBI               | Accession     | Query cover | % identity | E value |
|---------------------------------|---------------|-------------|------------|---------|
| Mentha_spicata                  | NC_037247     | 100%        | 99.85%     | 0.0     |
| Mentha_longifolia               | KU956042      | 100%        | 99.85%     | 0.0     |
| Mentha_canadensis               | NC_044082     | 100%        | 99.69%     | 0.0     |
| Mentha_rotundifolia             | Z37417        | 99%         | 99.69%     | 0.0     |
| Mentha_menthaefolia             | Z37420        | 99%         | 99.53%     | 0.0     |
| Salvia_officinalis              | NC_038165     | 100%        | 99.38%     | 0.0     |
| Mentha_pulegium                 | KY656718      | 98%         | 99.84%     | 0.0     |
| Mentha_suaveolens               | KP172040.1    | 97%         | 99.84%     | 0.0     |

Fig. 3. Phylogenetic relationship of gene rbcL sequence for mint (Mint1 and Mint2) samples based maximum likelihood tree.

Table 5. The Blast results for Thym1 of tested samples. Database search match for similarities and phylogenetic relationship using *rbcL* gene sequences

| Species from NCBI               | Accession     | Query cover | % identity | E value |
|---------------------------------|---------------|-------------|------------|---------|
| Thymus_alsinoides               | Z37470        | 99%         | 99.69%     | 0.0     |
| Thymus_vulgaris                 | Z37472        | 99%         | 99.53%     | 0.0     |
| Thymus_sibthorpii               | KR063653      | 97%         | 99.84%     | 0.0     |
| Salvia_japonica                 | KY646163      | 99%         | 98.76%     | 0.0     |
| Salvia_nemorosa                 |               | 99%         | 98.76%     | 0.0     |
| Salvia_rosmarinus               |               | 99%         | 98.76%     | 0.0     |
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Table 6. The Blast results for Thym2 of tested samples. Database search match for similarities and phylogenetic relationship using rbcL gene sequences

| Species from NCBI         | Accession    | Query cover | % identity | E value |
|---------------------------|--------------|-------------|------------|---------|
| Artemisia scoparia        | NC_045286    | 100%        | 98.91%     | 0.0     |
| Artemisia maritima        | NC_045093    | 100%        | 98.91%     | 0.0     |
| Artemisia fukudo          | NC_044156    | 100%        | 98.91%     | 0.0     |
| Artemisia capillaris      | KY073391     | 100%        | 98.91%     | 0.0     |
| Artemisia absinthium      | MK188885     | 100%        | 99.76%     | 0.0     |
| Artemisia selengensis     | NC_039647    | 100%        | 99.76%     | 0.0     |
| Artemisia gmelinii        | KY073390     | 100%        | 99.76%     | 0.0     |

Fig. 4. Phylogenetic relationship of gene rbcL sequence for Thyme (Thym1 and Thym2) samples based maximum likelihood tree

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دراسة القرابة الوراثية لبعض الأجناس المصرية التابعة لعائلة الشفوية (Lamiaceae) باستخدام تتابعات جين الـ rbcL.

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الموجز

تم دراسة القرابة الوراثية لستة أصناف تجارية مصرية من العائلة الشفوية (Lamiaceae)، صنفين من جنس الريحان (Ocimum L.)، وصنفين من جنس النعناع (Menthe L.) باستخدام التتابعات النيوكليريدية لجين ريبولايز (rbcL). نجحت جميع العينات في تضخيم حزمة بلغ حجمها ±0.36 زوج من القواعد. كما أظهرت تتابعات التماثل باستخدام أدوات BLASTN أن تتابعات الحمض النووي للجين (rbcL) الخاصة بصنفي الريحان المحلي تحت الدراسة لها قرابة وراثية مع أجناس الريحان التالية (Ocimum basilicum, Ocimum tenuiflorum, Ocimum kilimandscharicum and Ocimum gratissimum) بنسب تماثل (99.69 to 99.96) على التوالي. كما أشارت النتائج إلى أن صنفي النعناع المحلي تحت الدراسة لها قرابة وراثية مع أجناس النعناع النباتية (Mentha spicata, Mentha pulegium, Mentha canadensis and Mentha menthaefolia) بنسب تماثل (99.85, 99.84, 99.69 and 99.78) على التوالي.

الكلمات المفتاحية: باركورود الحمض الريبوزي، rbcL، القرابة الوراثية، العائلة الشفوية، BLASTN.