MOLECULAR IDENTIFICATION AND PHYLOGENETIC RELATIONSHIPS OF *Origanum syriacum* L. AND *Origanum vulgare* L. USING CHLOROPLAST *rbcL* AND *matK* BARCODES GENES

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Genus *Origanum* is considered as an important multipurpose aromatic perennial herb used in folk medicine and as food additive since ancient times which belongs to the family *Lamiaceae* or *Labiatae*, tribe *Mentheae* subfamily *Nepetoideae*. It is comprises 43 species and 18 hybrids widely distributed in Eurasia and North Africa (Ietswaart, 1980; Duman *et al*., 1998). Ietswaart (1980) identified six subspecies within *O. vulgare* based on differences in indumentums, number of sessile glands on leaves, bracts and calyces, and in size and color of bracts and flowers. *Lamiaceae* family contains about 236 genera having 6900 to 7200 species (Harley *et al*., 2004). *Origanum vulgare* L. is a perennial aromatic herb, widely naturally distributed all over Euroasia and North Africa and is one of the most traded and consumed spice (Kokkini, 1997). *Origanum vulgare* L. is used as medicinal plant because of the essential oils produced in the aerial parts (Skoula and Harborne, 2002). Thymol and carvacrol as a major constituent of its essential oil were isolated by Baser *et al*., (2003). It is especially antimicrobial, economic importance and specific biological characters (Asdal *et al*., 2006) and antioxidant properties (Mastelic *et al*., 2008). In Egypt, *Origanum syriacum* L. subsp. *Sinaicum* (Boiss.), commonly known as 'Syrian marjoram' is an aromatic, herbaceous and perennial plant growing wild in the Sinai desert of Egypt (Tackholm, 1974). It is endemic species grow in mountainous areas of Saint Katherine, south of Sinai in dry rocky habitats, also have a vernacular local name as "Za'atar Katherine" or Bardaqwish and from the conservation point of view, it is an endangered plant (Tackholm, 1974; Boulos, 2002). *Origanum syriacum* L. species, as one of wild plant species, is endangered and has limited distribution. There are many challenges affecting both conservation and the distribution of wild plants in SKP (Saint Katherine Protectorate) such as; feral donkeys, plant over collection, tourist intrusions (e.g., tres-

Egypt. J. Genet. Cytol., 49:191-211, July, 2020
Web Site (*www.esg.net.eg*)

passing beyond trail borders and collection of firewood during camping), overgrazing, collection for scientific research, urbanization and settlement expansion and quarries as recorded by Guenther *et al*., (2005), Assi (2007), Hatab (2009) and Khafagi *et al*., (2013). The taxonomy of *Origanum* was found to be rather complex and nearly all of the sections are afflicted with some kind of taxonomic uncertainties (Lukas, 2010).

For molecular characterization and identification of the biological species based on using DNA sequence data were widely used for DNA barcoding in the last decades. Several international organizations, including iBOL (the International Barcode of Life Project) http:// www.ibol.org/, CBOL (the Consortium for the Barcode of Life) and ECBOL (the European Consortium for the Barcode of Life), have applied in large-scale DNA barcoding projects aiming to identify and classify all life on earth. Recently, the Consortium for the Barcode of Life **(**CBOL**)** Plant Working Group (CBOL 2009 and 2010) proposed two other chloroplast regions, the protein coding *rbcL* and *matK*, as a 2-locus combination barcode. DNA barcoding techniques play an important role in the identification of polymorphic plant species having a problematic taxonomic identity for the biodiversity investigation (Ajmal *et al*., 2014). DNA barcoding technique in plants with high effective and robust conserved regions is a characterizing using a partial DNA sequence from a standard and agreed-upon position in the genome of all species (http://barcoding.si.edu/DNABarCoding.htm). For instance, barcoding strategies have been deployed for the verification of plant products from several medicinal plants (Asahina *et al*., 2010; Xue and Li, 2011). Large data information gathered from two chloroplast chDNA barcodes (*rbcL* and *matK*) at molecular level used a universal barcode system far beyond taxonomic studies of land plants. Some investigators have chosen a combination of two regions (*matK* and *rbcL*) as a satisfactory compromise that best meets the DNA barcoding criteria (Hebert, 2003; Chase *et al*., 2005; De Vere *et al*., 2012 and; Fazekas *et al*., 2008& 2012). Using four candidate barcode regions (*rpo*B, *rbcL*, *matK* and *trnH-psbA* to evaluate the utility of using markers plant DNA barcodes in 64 species specimens, encompassing six different genera (i.e. *Mentha*, *Ocimum*, O*riganum*, *Salvia*, *Thymus* and *Rosmarinus*) to reduce cost and time for species identification (De Mattia *et al*., 2011 & 2012). Using one region barcodes from *rbcL*, *matK*, *psbA-trnH* loci of 14 *Labiatae* species were identified and analyzed with species identification from Pakistan (Schori and Showalter, 2011). Universal primers were used for amplification of *matK* and *rbcL* Loci in 2 different plant species (covering 14 families) from Saudi Arabia (Bafeel *et al*., 2011). To classify between 36 samples of *Thymus* spp. the core barcode regions (*matK* and *rbcL*) and the plastid intergenic spacer *trnH-psbA* were compared (Federici *et al*., 2013). Three cpDNA loci (*matK*, *rbcL* and *trnH-psbA*) as single region or as multi-region barcodes based on CBOL were used and analyzed for medicinal

plants of the *Labiatae* (*Lamiaceae*) family (Theodoridis *et al*., 2012). DNA barcoding as part from authentication of traded medicinal plants, also finds application in biodiversity monitoring, conservation impact assessment, monitoring of illegal trading, forensic botany, etc. (Nithaniyal *et al*., 2014; Ferri *et al*., 2015 and Mishra *et al*., 2016). For more than a decade, other several applications has been widely tested in the DNA barcoding, molecular systematics, identification at molecular level and community phylogenetic of some medicinal plants (Vohra and Khera, 2013; Techen *et al*., 2014; Zhou *et al*., 2014; Parveen *et al*., 2016; Bezeng *et al*., 2017 and Chen *et al.,* 2019).

The importance of *Origanum* species is due to active ingredients and essential oils which confer their medicinal, culinary and pharmaceutical properties. Moreover, threatens such as over-collecting of plants and human constructions in plants natural habitats which put them in their way to extinction. Therefore, the current investigation was carried out to identify and authenticate the endemic wild *Origanum* species, *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) in Egypt and compare them with cultivated species; *Origanum vulgare* L. using two chloroplast genes (*rbcL* and *matK* genes) as the most important DNA barcode at the molecular level. In addition, to distinguish between the two *Origanum* species under study and with the other available species on NCBI database to fulfill a strict conservation plan and maintenance of the studied species. Phylogenetic relationship analysis and homology modeling of both sequences (*matK* + *rbcL* genes) using Basic Local Alignment Search Tool (BLASTn) and MEGA 7 software program to apply comparative sequences between of them.

MATERIALS AND METHODS

Plant materials collection

The two *Origanum* species available in Egypt; *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) (Ecotype wild species) and *Origanum vulgare* L. (cultivated species) were collected for the present investigation. The fresh young leaves were collected as bulk for each species in spring of 2018, transferred into liquid nitrogen, and kept frozen at - 80 °C till use. The *Origanum* species chosen for the present study were wild type *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) which was collected from the mountains of Saint Catherine Protectorate (SKP), South Sinai Governorate, Egypt and the other is cultivated species; *Origanum vulgare* L. which was collected from private farms at Kirdasa region, Giza Governorate, Egypt.

DNA extraction, primers design and PCR amplification

Genomic DNAs were extracted from 100 mg frozen tissue and loaded in 2.0 ml eppendorf tubes with one 5 mm stainless steel bead. Samples were ground using Tissue Lyser II (Qiagen, Ltd.) until the material became powder (frequency 20

Hz x 2 x ~ 30 sec) following the procedure described by DNeasy Plant Mini Kit (Qiagen Inc., Cat.no.69104, USA), this was performed following the manufacturer's instruction. The concentrations and quality of the genomic DNA samples were estimated on spectrophotometer ND-2000 (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 40 ng/ µl with TE buffer (10 mM Tris-HC1, pH 8.0 and 1 m M EDTA), then stored at -20°C for further use. DNA fragments were amplified *via* standard polymerase chain reaction (PCR). The entire coding plastid *rbcLa* (first part of *rbcL* gene ~ 700bp or less) was amplified using the primer pairs *rbcLa*_1Fwd (5′-ATG TCA CCA CAA ACA GAG ACT AAA GC-3′) and *rbcLa*_599Rev (5′-GTA AAA TCA AGT CCA CCR CG-3′) and PCR fragment a 599 bp of the *rbcL* gene as previously described by Levin *et al*., (2003) and Kress and Erickson, (2007). For *matK* primer design, 13 ORF full length sequence *matK* genes from different species belong to *Lamiaceae* family were retrievable from the National Center for Biotechnology Information (NCBI) database (GenBank). Accession numbers GU381790.1 (*Thymus pulegioides*), AY840173.1 (*Thymus serpyllum*), GU381789.1 (*Thymus caespititius*), GU381791.1 (*Thymus broussonetii* subsp. *hannonis*), GU381792.1 (*Thymus vulgaris*), GU381802.1 (*Origanum vulgare*), GU381799.1 (*Origanum elongatum*), GU381798.1 (*Origanum dictamnus*), GU381797.1 (*Origanum rotundifolium*), GU381801.1 (*Origanum microphyllum*), GU381800.1 (*Origanum dayi*), AY840165.1 (*Origanum vulgare*), and MG256495.1 (*Mentha spicata*) were used for multiple sequences alignment of nucleotide (BLSTn) to design specific primers pair of *matK* gene. The entire coding plastid maturase fragment (*matK*) was amplified ~ 884 bp or less using the primer pairs *matK*_466Fwd (5`- GTC CAT GTG GAA ATC TTG ATT C -3`) and *matK*_1349Rev (5`- CGT ACA GTA CTT TTG TGT TTA CG -3`) and PCR fragment ~ 884 bp or less 850 bp according to CBOL Plant Working Group (2009). Phusion® *Taq*, the High-Fidelity DNA polymerase (Thermo Scientific, Product codes: F-530L, 500 Unit) was used. For *rbcl* and *matK* master mix: The amplification reaction was carried out in 25 µl reaction volume contains; 2 µl DNA, 5 µl 5X Phusion HF buffer, 0.5 µl 10mM dNTP mix, 1.25 µl Fwd. primer (10 µM), 1.25 µl Rev. primer (10 µM), 0.25 µl Phusion DNA polymerase (Thermo Scientific, Product codes: F-530L, 500 Unit), 14.75 µl DEPC H_2O and was spin for 15 Sec. The reaction mixture was incubated in a Perkin-Elmer thermo cycler 9700. PCR program for *rbcL* and *matK* regions, the temperature profile in different cycles was as follows: an initial strand separation cycle at 98°C for 3 min followed by 35 cycles comprised of a denaturation step at 98°C for 30 seconds, an annealing step at 55°C for 30 seconds and an extension step at 72°C for 45 seconds. The final cycle

was a polymerization cycle for 7 min at 72°C. PCR amplifications samples were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TAE buffer at 95 volts. A 1Kbp DNA ladder was used as DNA standard size marker. PCR products were visualized on UV light using a Gel Documentation System (BIO-RAD 2000). PCR product were purification or fast cleanup from the agarose gel for sequence up to 10 µg can bind to each QIA quick column by using QIA quick gel extraction kit (Qiagen, Cat. No. 28704-28706).

Sequence editing, alignment and phylogenetic inference

Sequencing chromatograms of obtained two *rbcL* and two *matK* regions were analyzed by Macrogen, Seoul, south of Korea and translated into amino acid sequences by the *ExPASy* online program (https://web.expasy.org/translate) for each studied two *Origanum* species. All nucleotide sequences of *rbcL* and *matK* gene, Open Reading Frame (ORF) were searched in NCBI database, The National Center for Biotechnology Information GenBank Database, (http://www.ncbi.nlm.nih.gov). The homology searches were performed with Basic Local Alignment Search Tool of several sequences (BLASTn online program) on the basis of their homologies with sequences published in DDBJ/EMBL/GenBank database which are available using NCBI database (Altschul *et al*., 1990).

RESULTS AND DISCUSSION

Molecular description

Successful application for isolation with high quality and pure molecular size of DNA is quite a challenge to reduce degraded DNA of plant material especially in medicinal plants. DNA concentration were obtained using nanodrop spectrophotometer observes and ratio of 260 and 280 nm were (i.e., 27–35 ng mL-1). PCR based amplification of conserved regions (*matK and rbcL*) is primarily required to establish DNA barcodes for species identification. With both universal primers *rbcL* and *matK*, good results of PCR amplification were observed as about 700 or less 600 bp for the first part of partial fragment length of *rbcL* gene and about 900-850 bp for partial *matK* gene in two *Origanum* species (*Origanum syriacum* L. subsp. *Sinaicum* (Boiss.), (Ecotype of wild species) and *Origanum vulgare* L., respectively. The single fragment was purified from agarose gel and sequenced. Specific single fragment was obtained for each sample, size and reading sequence were determined by Macrogen analysis, and then aligned by BLASTn web sites to identify sequences similarities. The nucleotide sequences isolated in this paper have been deposited in the GenBank database

NCBI (National Center for Biotechnology Information) by BankIt, online from website (https://www.ncbi.nlm.nih.gov/ WebSub/) after it was processed by email (gb-admin@ncbi.nlm.nih.gov). Two *rbcL* gene sequences were available to DDBJ/EMBL/GenBank database with accession no. MT679150.1/QNJ99666 from *Origanum vulgare* L. (528bp) which predicted to encode a protein of 176 amino acids and with GenBank accession no. MT679151.1/QNJ99667 from *Origanum syriacum* L. subsp. *Sinaicum* (532 bp), which predicted to encode a protein of 177 amino acids as shown in Fig. (1.A). In the same context, the two sequenced *matK* genes sequences were available with GenBank accession no. MT679152.1/QNJ99668 from *Origanum vulgare* L. (745 bp) which predicted to encode a protein of 248 amino acids and accession no. MT679153.1/QNJ99669 from *Origanum syriacum* L. subsp. *Sinaicum* (775bp), which predicted to encode a protein of 258 amino acids as shown in Fig. (1. B).

Molecular phylogenetic analysis based on sequences of *rbcL* gene

Sequence homology of each nucleotide of the chloroplast 60 *rbcL* gene searches (Code 1 to 2) for *Origanum* species (current studies), (Code 3 to 7) for *Origanum* genus, (Code 8 to 10) for *Conradina* genus, (Code 11 to13) for *Dicerandra* genus, (Code 14 to 16) for *Monarda* genus, (Code 17 to 19) for *Pycnanthemum* genus, (Code 20) for *Bystropogon* genus, (Code 21 to 23) for *Clinopodium* genus, (Code 24 to 26) for *Satureja* genus, (Code 27 to 29) for *Mentha* genus, (Code 30 to 35) for *Thymus* genus, (Code 36 to 38) for *Agastache* genus, (Code 39 to 41) for *Dracocephalum* genus, (Code 42 to 44) for *Nepeta* genus, (Code 45 to 46) for *Prunella* genus, (Code 47 to 49) for *Lepechinia* genus, (Code 50 to 52) for *Rosmarinus* genus, (Code 53 to 55) for *Salvia* genus and (Code 56 to 60) for 5 different species; *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1), *Chenopodium album* (JX848451.1) and *Triticum aestivum* (AY328025.1) as out group of the family were tested by top-scoring hits through NCBI database using Basic Local Alignment Search Tool (BLASTn) as shown in Table (1). Multiple Sequence Alignments (MSA) of 60 *rbcL* gene was carried out between sequences of selected nucleotide sequences of 17 different genera belonging to the family *Lamiaceae* or *Labiatae* available in GenBank databases, will be discussed briefly. The results revealed that *Origanum vulgare* L. in this investigation was closely related with high identity and similarity of 99.62% with 5 accessions of *Origanum* genus and less than 99.62 - 96.97% with 49 accessions from 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species (89.22%,

89.77%, 90.55% and 89.20%) with *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1) and *Chenopodium album* (JX848451.1), respectively with E-value = Zero and 87.31% *Triticum aestivum* (AY328025.1) with E-value = 2e-176 as out group of the family *Lamiaceae* or *Labiatae*. The Multiple Sequence Alignments (MSA) of nucleotide partial *rbcL* gene from *Origanum syriacum* L. subsp. *sinaicum* (current study) exhibited high identity and similarity of 100.00% to 5 accessions with E-value = Zero. Moreover, *Origanum syriacum* subsp. *sinaicum* showed similarity less than 100.00-97.37% with 49 accessions from 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species (89.29%, 90.21% 90.99% and 89.66%) with *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1) and *Chenopodium album* (JX848451.1), respectively with E-value = Zero and 87.38% *Triticum aestivum* (AY328025.1) with E-value = 5e-178 as out group of the family *Lamiaceae* or *Labiatae*. Based on these results, phylogenetic relationships analyses enable us to check the closest of species from 17 different genera belonging to the family *Lamiaceae* were conducted in MEGA 7.0 software program by Maximum Likelihood (ML) tree with the highest log likelihood (-2836.15) is shown (Kumar *et al*., 2016). The phylogenetic tree was constructed based on the 60 amino acid sequences encoded from the *rbcL* gene that have the highest percentages of similarity with species from 17 different genera, showing closely related species clustering together from the family *Lamiaceae* and they showed low percentages of similarities towards relatively distantly related species scattering with 5 different species from families as out group of the family as shown in Fig. (2).

Molecular phylogenetic analysis based on sequences of maturase gene (*matK*)

Sequence homology of each nucleotide of the chloroplast 60 maturase gene (*matK*) searches (Code 1 to 2) for *Origanum* species (current studies), (Code 3 to 6) for *Origanum* genus, (Code 7 to 10) for *Conradina* genus, (Code 11 to13) for *Dicerandra* genus, (Code 14 to 17) for *Monarda* genus, (Code 18 to 20) for *Pycnanthemum* genus, (Code 21-23) for *Bystropogon* genus, (Code 24 to 26) for *Clinopodium* genus, (Code 27 to 29) for *Satureja* genus, (Code 30 to 32) for *Mentha* genus, (Code 33 to 36) for *Thymus* genus, (Code 37 to 39) for *Agastache* genus, (Code 40 to 42) for *Dracocephalum* genus, (Code 43 to 46) for *Nepeta* genus, (no. record) for genus *Lepechinia*, (Code 47 to 49) for *Prunella* genus, (Code 50 to 53) for *Rosmarinus* genus, (Code 54 to 55) for *Salvia* genus and (Code 56 to 60) for 5 different species with *Linum usitatissimum* (HM544115.1), *Arabidopsis thaliana* (KM892769.1), *Glycine max* (EF550007.1), *Chenopodium album* (KX133100.1) and *Triticum aestivum* (AF164405.1) as out group of the family

were tested by top-scoring hits through NCBI database using Basic Local Alignment Search Tool (BLASTn) as shown in Table (2). The Multiple Sequence Alignments (MSA) of 60 maturase gene (*matK*) were carried out between sequences of selected nucleotide sequence with 17 different genera belonging to the family *Lamiaceae* or *Labiatae* available in GenBank databases, which will be discussed briefly. The results revealed that *Origanum vulgare* L. in this investigation was closely related with high identity and similarity 100.00% with four accessions of *Origanum* genus and less than 100.00% - 92.73% with 40 accessions with 17 different genus belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species with 71.02% *Linum usitatissimum* (HM544115.1) with E-value = 1e-76, 75.68% *Arabidopsis thaliana* (KM892769.1) with E-value = 2e-134, 74.46% *Glycine max* (EF550007.1) with E-value = 3e-136 and 76.85% *Chenopodium album* (KX133100.1) with E-value = 6e-85, 70.99% *Triticum aestivum* (AF164405.1) with E-value = 1e-102 as out group of the family *Lamiaceae* or *Labiatae*. The Multiple Sequence Alignments (MSA) of nucleotide partial maturase gene (*matK*) from *Origanum syriacum* L. subsp. *sinaicum* (current study) exhibited high identity and similarity of 100.00% to 4 accessions with E-value = Zero. Moreover, *Origanum syriacum* L. subsp. *sinaicum* showed similarity less than 100.00-92.73% with 50 accessions of 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other five different species with 71.02% *Linum usitatissimum* (HM544115.1) with E-value =1e-76, 75.68 % *Arabidopsis thaliana* (KM892769.1) with E-value =2e-134, 74.64% *Glycine max* (EF550007.1) with E-value = 4e 142 and 76.81% *Chenopodium album* (KX133100.1) with E-value = 2e-168 and 71.76% *Triticum aestivum* (AF164405.1) with E-value = 2e-114 as out group of the family *Lamiaceae* or *Labiatae*. Based on these results, phylogenetic relationships analyses enable us to check the closest of species from 17 different genera belonging to the family *Lamiaceae* were conducted in MEGA 7.0 software program by Maximum Likelihood (ML) tree with the highest log likelihood (-5692.60) is shown (Kumar *et al*., 2016). The phylogenetic tree was constructed based on the 60 amino acid sequences encoded from the *matK* gene have the highest percentages of similarity with species from 17 different genera, showing closely related species clustering together from the family *Lamiaceae* and they showed low percentages of similarities towards relatively distantly related species scattering with 5 different species from families as out group of the family as shown in Fig. (3).

Primer universality and species identification are two crucial criterions for an ideal DNA barcode. The two DNA barcodes showed high rates of amplification and sequencing successes, among which *rbc*L and *matK* genes had the best performance of universality. Phylogenetic

analysis using tree-based method is an important approach to determine the DNA region and evaluate its ability to verify whether it can identify and detect species-specific clusters of species from the same genus. In this study, ML analysis produced phylogenetic tree with better resolution for all tested barcodes. Maximum likelihood tree analysis was performed to evaluate the discriminatory power of the *rbcL* and *matK* genes.

Our data analyses were in agreement for used two plastid markers as the most rapidly evolving plastid coding regions and it consistently showed high levels of discrimination among angiosperm species (CBOL Plant Working Group, 2009). and Theodoridis *et al*., (2012) tested three cpDNA regions (*matK*, *rbcL*, *trnH-psbA*) that were proposed by previous studies in *Labiatae* species. The efficacy of a DNA barcoding approach as clear evidences to the recognition of commercial spices within the family *Lamiaceae*. Fazekas *et al*., (2008) and (2012) examined the suitability of different leading candidate markers and proposed the two-locus combination of *matK* and *rbcL* as the core plant barcode could be important used supplementary marker in appropriate cases. Other authors, Bafeel *et al*., (2011) used universal *matK* primer for *matK* as a barcode. The efficacy of a DNA barcoding approach as clear evidences to the recognition of commercial spices within the family *Lamiaceae* (De Mattia *et al*., 2011). Schori and Showalter (2011) analyzed 14 species from *Labiatae* in Pakistan and found that the *rbcL*, *matK*, *psbA-trnH* loci, could serve as single-region barcodes depending on plant to be identified, one region was preferred over the other to aid in species identification. Meanwhile, Bafeel *et al*., (2012) tested the potential of the *rbcL* marker for the identification of wild plants belonging to diverse families of arid regions. Recently, Federici *et al*., (2013) showed clear amplification and sequencing 36 samples of *Thymus* spp. using the molecular analysis of the core barcode regions (*matK* and *rbcL*) and the plastid intergenic spacer *trnH-psbA*. For herbal plant identification, *matK*, *rbcL*, *trnH-psbA*, ITS, trnL-F, 5S-rRNA and 18S-rRNA have been used as successful DNA barcodes by Mishra *et al*., (2016). While, Parveen *et al*., (2016) proposed that DNA barcoding as a means to identify herbal ingredients and to detect adulteration. However, general barcoding techniques using universal primers have been shown to provide mixed results with regard to data accuracy. More recently, Skuza *et al*., (2019) observed that nucleotide sequences had a high variability within *matK* and *rbcL* regions and the *matK* region is suitable for differentiation and discrimination between the studied species in the genus *Secale*. In this study, our results indicated that two plastid regions (*rbcL* and *matK*) could be a better choice for barcoding with excellent primer universality. They could also help to understand the relationships of co-occurring species and the species assembly within community when combining more information including species abundance and the functional traits of all the species in the future.

SUMMARY

Genus *Origanum* is one of the most species-rich as medicinal plants and pharmaceutically in the family *Lamiaceae* for several multipurpose used, and thus it is an endangered plant that needs a strict conservation plan. This genus contains many plants with medical uses, and thus an objective identification method is urgently needed. DNA barcoding is a sample fast technique at molecular level in the field of identification, authentication, classification and differentiation between two *Origanum* species and with other species. The current investigation was conducted to identify, discriminate and authenticate *Origanum vulgare* L. and *Origanum syriacum* L. subsp. *sinaicum* using two chloroplast genes (coding sequences) as the most common DNA barcodes, ribulose 1, 5-biphosphate carboxylase large subunit (*rbcL*) and maturase K (*matK*) genes. The partial sequence length of *rbcL* gene of two *Origanum* species were 528bp and 532bp with *Origanum vulgare* L. (MT679150.1/QNJ99666), and *Origanum syriacum* L. subsp. *sinaicum* (MT679151.1/QNJ99667), and similarly with *matK* gene were 745bp and 775 bp with *Origanum vulgare* L. (MT679152.1/QNJ99668), and *Origanum syriacum* L., subsp. *Sinaicum* (MT679153.1/QNJ99669), respectively. The alignments of the sequence chloroplast genes (*rbcL* and *matK*) were able to distinguish two *Origanum* species under study with high similarities and to the closely related species of *Origanum* genus, other 17 genera belonging to family *Lamiaceae* and take them away from five plant species from different families as out group of the family *Lamiaceae*. The obtained results revealed that *rbcL* and *matK* genes nucleotide sequence isolated from the two *Origanum* species in this investigation showed high similarities and closely related to NCBI recoded 17 genera belonging to the family *Lamiaceae*. Furthermore, a phylogenetic tree analyses were constructed based on amino acid sequence of 60 *rbcL* and 60 *matK* genes using MEGA 7 program by Maximum Likelihood (ML) method with the highest log likelihood (-2836.15) for *rbcL* gene and (-5692.60) for *matK* gene.

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Table (1): Homology of nucleotide sequences for 60 selected accession lists and its related Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* for chloroplast, *rbcL* gene sequenced in this study, BLASTn top hits against GenBank database, similarity score and GenBank accession no.

Family *Lamiaceae* Tribe: *Mentheae*	Code	Scientific name of chloroplast *rbcL* gene (length bp)	GenBank Accession No.	Similarity% (bp)	
				**Origanum vulgare*	**O. syriacum* subsp. *sinaicum*
1. Genus *Origanum*	1	**Origanum vulgare* (528bp)	MT679150.1	----	99.62
	2	**O. syriacum* subsp. *sinaicum* (532bp)	MT679151.1	99.62	----
	3	*Origanum sipyleum*	HQ902792.1	99.61	100.00
	4	*Origanum vulgare*	MG224341.1	99.62	100.00
	5	*Origanum majorana*	KX783936.1	99.62	100.00
	6	*Origanum laevigatum*	MF349689.1	99.62	100.00
	7	*Origanum vulgare*	MF694999.1	99.62	100.00
2. Genus *Conradina*	8	*Conradina glabra*	KY765542.1	99.37	99.58
	9	*Conradina grandiflora*	MG582632.1	99.23	99.23
	10	*Conradina verticillata*	MH749055.1	99.24	99.62
3. Genus *Dicerandra*	11	*Dicerandra thinicola*	MG582633.1	98.84	99.23
	12	*Dicerandra immaculate*	KY765541.1	98.93	99.15
	13	*Dicerandra frutescens*	MG592699.1	98.84	99.23
4. Genus *Monarda*	14	*Monarda punctate*	MK526204.1	99.22	99.61
	15	*Monarda punctate*	KY627431.1	99.23	99.62
	16	*Monarda fistulosa*	MF349566.1	99.24	99.62
5. Genus *Pycnanthemum*	17	*Pycnanthemum incanum*	MG221861.1	99.23	99.62
	18	*Pycnanthemum tenuifolium*	MG223821.1	99.24	99.62
	19	*Pycnanthemum virginianum*	MG224042.1	99.24	99.62
6. Genus *Bystropogon*	20	*Bystropogon origanifolius*	KJ595607.1	99.43	99.81
7. Genus *Clinopodium*	21	*Clinopodium vulgare*	HQ590041.1	99.43	99.81
	22	*Clinopodium chinense*	FJ513146.1	99.43	99.81
	23	*Clinopodium repens*	MH116120.1	99.43	99.81

Table (1): Cont''.

8. Genus *Satureja*	24	*Satureja pilosa subsp. origanita*	KR063652.1	99.05	99.44
	25	*Satureja Montana*	MF349309.1	99.05	99.44
	26	*Satureja hortensis*	MG224422.1	99.05	99.44
9. Genus *Mentha*	27	*Mentha spicata*	KY400629.1	99.43	99.81
	28	*Mentha Canadensis*	KC473279.1	99.43	99.81
	29	*Mentha pulegium*	KY656718.1	99.43	99.81
10. Genus *Thymus*	30	*Thymus serpyllum*	KF997486.1	99.05	99.43
	31	*Thymus pulegioides*	JN892334.1	99.03	99.42
	32	*Thymus praecox*	MG221401.1	99.01	99.41
	33	*Thymus drucei*	MG221346.1	99.05	99.44
	34	*Thymus vulgaris*	Z37471.1	98.86	99.25
	35	*Thymus vulgaris* (II)	Z37472.1	99.24	99.62
11. Genus *Agastache*	36	*Agastache rugosa*	FJ513154.1	97.92	98.31
	37	*Agastache foeniculum*	MG222708.1	97.92	98.31
	38	*Agastache nepetoides*	MG222557.1	97.92	98.31
12. Genus *Dracocephalum*	39	*Dracocephalum moldavica*	HM590077.1	96.97	97.37
	40	*Dracocephalum rupestre*	HQ839685.1	97.16	97.56
	41	*Dracocephalum ruyschiana*	KF307354.1	97.54	97.93
13. Genus *Nepeta*	42	*Nepeta cataria*	MG946943.1	97.35	97.74
	43	*Nepeta bracteata*	MH998002.1	96.97	97.37
	44	*Nepeta cataria*	MN601459.1	97.35	97.74
14. Genus *Prunella*	45	*Prunella vulgaris*	MH116337.1	98.11	98.12
	46	*Prunella grandiflora*	FR865137.1	97.28	97.28
15. Genus *Lepechinia*	47	*Lepechinia chamaedryoides*	AY570387.1	98.11	98.50
	48	*Lepechinia fragrans*	AY570388.1	98.11	98.50
	49	*Lepechinia calycin*	AY570386.1	98.11	98.50
16. Genus *Rosmarinus*	50	*Rosmarinus officinalis*	HE963635.1	98.62	99.02
	51	*Rosmarinus officinalis*	HQ619754.1	98.67	99.06
	52	*Rosmarinus officinalis*	KM360960.1	98.86	99.25
17. Genus *Salvia*	53	*Salvia deserta*	JQ933991.1	99.05	99.44
	54	*Salvia fruticosa*	HM590078.1	99.05	99.44
	55	*Salvia officinalis*	JQ934010.1	99.05	99.44
Family *linaceae*	56	*Linum usitatissimum* (Out group)	MG946893.1	89.22	89.29
Family *Brassicaceae*	57	*Arabidopsis thaliana* (Out group)	AB917053.1	89.77	90.20
Family *Fabaceae*	58	*Glycine max*(Out group)	Z95552.1	90.59	90.99
Family *Amaranthaceae*	59	*Chenopodium album* (Out group)	JX848451.1	89.20	89.66
Family *Poaceae*	60	*Triticum aestivum* (Out group)	AY328025.1	87.31	87.38

Note:* *Origanum vulgare* L. and * *Origanum syriacum* L. subsp. *sinaicum* were used as current study.

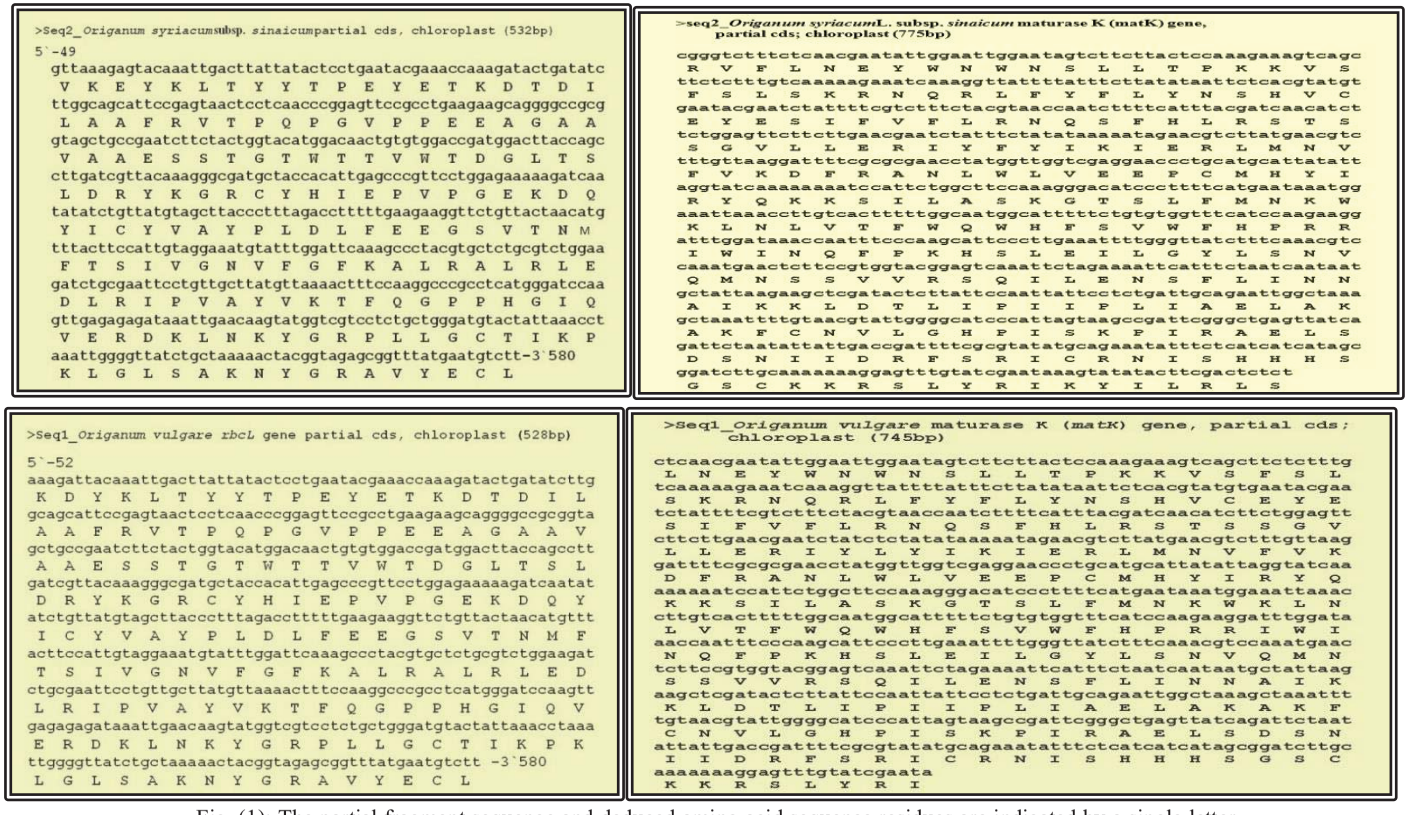
Table (2): Homology of nucleotide sequences for 60 selected accession lists and its related Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* for chloroplast maturase gene (*matK*) sequenced in this study, BLAST top hits against GenBank database, similarity score and accession no.

Family: *Lamiaceae* Tribe: *Mentheae*	Code	Scientific name of chloroplast *matK* gene (length bp)	GenBank Accession No.	Similarity% (bp)	
				**Origanum vulgare*	**O. syriacum* subsp. *sinaicum*
1- Genus *Origanum*	1	**Origanum vulgare* (745bp)	MT679152.1	---	100.00
	2	**O. syriacum* subsp. *sinaicum* (775bp)	MT679153.1	100.00	---
	3	*Origanum laevigatum*	MF350147.1	100.00	100.00
	4	*Origanum majorana*	MN167195.1	99.87	99.87
	5	*Origanum vulgare*	MN167194.1	100.00	100.00
	6	*Origanum vulgare*	MK520369.1	100.00	100.00
2. Genus *Conradina*	7	*Conradina grandiflora*	KY607200.1	97.31	97.31
	8	*Conradina verticillata*	MH748917.1	97.58	97.61
	9	*Conradina glabra*	KY607199.1	97.14	97.14
	10	*Conradina canescens*	KJ772673.1	97.24	97.24
3. Genus *Dicerandra*	11	*Dicerandra christmanii*	KY607212.1	97.16	97.16
	12	*Dicerandra cornutissima*	KY607213.1	96.84	96.84
	13	*Dicerandra immaculata*	KY607214.1	97.14	97.14
4. Genus *Monarda*	14	*Monarda fistulosa var. mollis*	KT176605.1	97.58	97.68
	15	*Monarda didyma*	MG224897.1	97.49	97.52
	16	*Monarda clinopodia*	KP642819.1	97.36	97.40
	17	*Monarda fistulosa*	MF350057.1	97.58	97.64
5. Genus *Pycnan.*	18	*Pycnanthemum albescens*	MF350277.1	97.62	97.65
	19	*Pycnanthemum virginianum*	MG225271.1	97.64	97.66
	20	*Pycnanthemum albescens*	MH748968.1	97.71	97.74
6. Genus *Bystropogon*	21	*Bystropogon canariensis*	GU381726.1	98.07	97.98
	22	*Bystropogon origanifolius*	GU381727.1	98.02	98.09
	23	*Bystropogon origanifolius*	GU381728.1	98.07	98.14
7-Genus *Clinopodium*	24	*Clinopodium vulgare*	KJ592905.1	97.79	97.81
	25	*Clinopodium macrostemum*	MK601827.1	97.45	97.55
	26	*Clinopodium wardii*	KX526681.1	97.79	97.82

Table (2): Cont''.

8. Genus *Satureja*	27	*Satureja horvatii*	KX954592.1	98.39	98.44
	28	*Satureja subspicata*	KX954591.1	98.39	98.44
	29	*Satureja Montana*	MF350242.1	98.31	98.34
9. Genus *Mentha*	30	*Mentha Canadensis*	JN407140.1	98.39	98.45
	31	*Mentha spicata*	MN167204.1	98.52	98.58
	32	*Mentha suaveolens*	LC126645.1	98.52	98.45
10. Genus *Thymus*	33	*Thymus decussatus*	MN972469.1	99.31	98.53
	34	*Thymus longicaulis*	HE819415.1	99.13	98.31
	35	*Thymus vulgaris*	HE819430.1	99.27	98.45
	36	*Thymus serpyllum*	MF350183.1	99.33	99.35
11. Genus *Agastache*	37	*Agastache nepetoides*	MK509382.1	95.38	95.44
	38	*Agastache scrophulariifolia*	MK509383.1	95.37	95.43
	39	*Agastache foeniculum*	AY840146.1	94.54	94.56
12. Genus *Dracocephalum*	40	*Dracocephalum parviflorum*	MK520021.1	93.29	93.37
	41	*Dracocephalum tanguticum*	MF786820.1	92.73	92.73
	42	*Dracocephalum forrestii*	MF786791.1	93.36	93.36
13. Genus *Nepeta*	43	*Nepeta x faassenii*	MF349917.1	93.96	94.04
	44	*Nepeta italic*	HQ902725.1	94.63	94.55
	45	*Nepeta cataria*	MG224812.1	93.69	93.67
	46	*Nepeta bracteata*	MG946967.1	93.56	93.47
14. Genus *Prunella*	47	*Prunella vulgaris*	KX676737.1	95.55	94.85
	48	*Prunella vulgaris*	KP402374.1	95.44	95.44
	49	*Prunella vulgaris*	MF158707.1	95.20	94.56
15. Genus *Lepechinia*	----	No record	----	----	----
16. Genus *Rosmarinus*	50	*Salvia rosmarinus*	MF694874.1	95.34	94.61
	51	*Salvia rosmarinus*	KX783771.1	95.32	94.50
	52	*Rosmarinus officinalis*	KP172065.1	95.41	94.55
	53	*Rosmarinus officinalis*	FR719112.1	95.34	94.51
17. Genus *Salvia*	54	*Salvia rosmarinus*	MF349943.1	95.34	94.70
	55	*Salvia officinalis*	KC473367.1	95.57	95.58
Family *linaceae*	56	*Linum usitatissimum*	HM544115.1	71.02	71.02
Family *Brassicaceae*	57	*Arabidopsis thaliana*	KM892769.1	75.68	75.68
Family *Fabaceae*	58	*Glycine max*	EF550007.1	74.46	74.64
Family *Amaranthaceae*	59	*Chenopodium album*	KX133100.1	76.85	76.81
Family *Poaceae*	60	*Triticum aestivum*	AF164405.1	70.99	71.76

Note:* *Origanum vulgare* and * *Origanum syriacum* subsp. *sinaicum* were used as current study.



(A) **(B)**

Fig. (1): The partial fragment sequence and deduced amino acid sequence residues are indicated by a single letter code used by (https://web.expasy.org/translate/).

(A): *rbcL* gene from *Origanum vulgare* and *Origanum syriacum* subsp. *sinaicum* (528 and 532 bp) with G enBank accession no. MT679150.1/QNJ99666 and MT679151.1/QNJ99667, respectively.

(B): *matK* gene from *Origanum vulgare* L. and *Origanum syriacum* L. subsp. *sinaicum* (745 and 775bp) with GenBank accession no. MT679152.1/QNJ99668 and MT679153.1/QNJ99669, respectively.

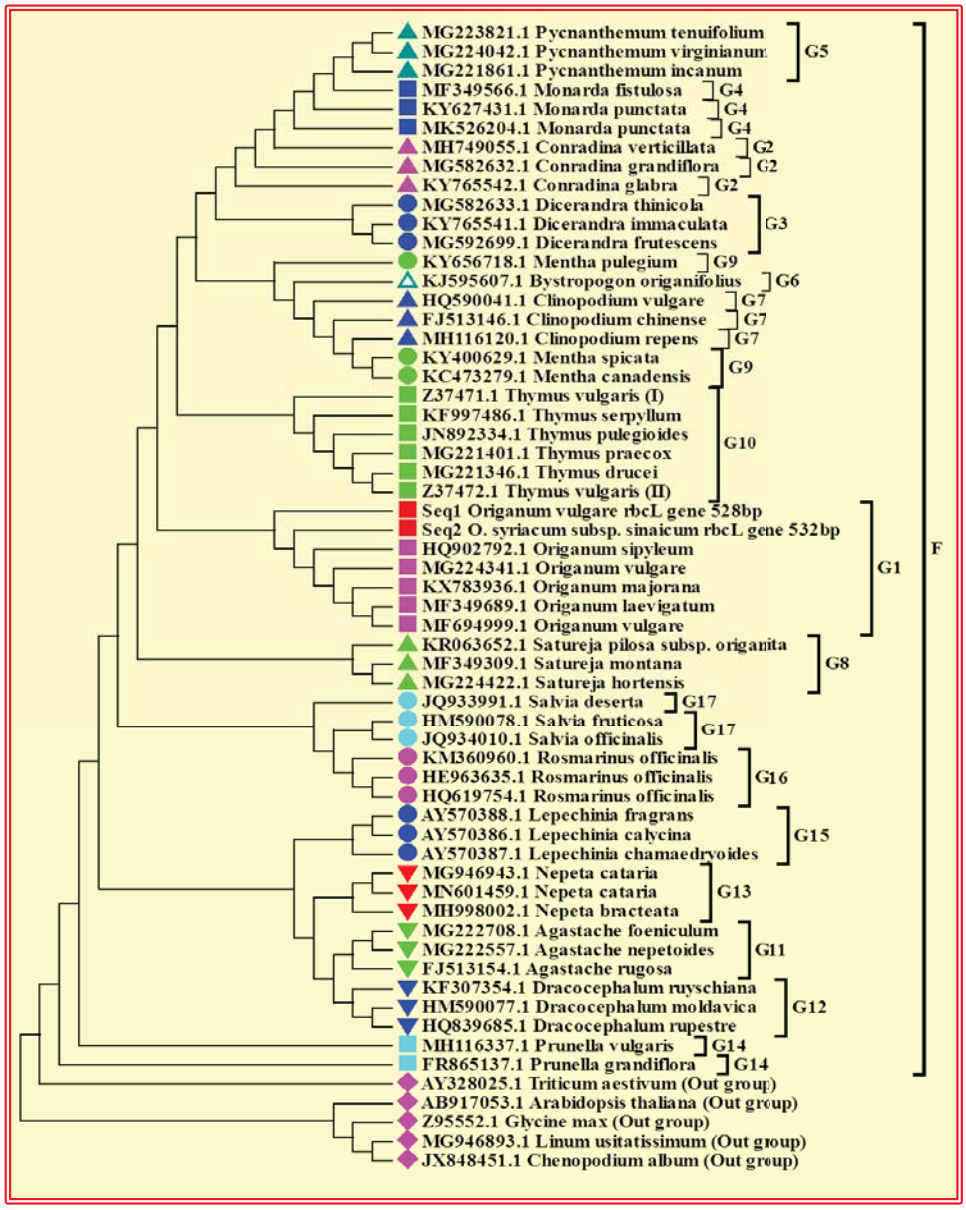


Fig. (2): Molecular phylogenetic analyses involved 60 amino acid sequences of *rbcL* gene were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-2836.15) is shown. Note: G1: *Origanum* species, G2: *Conradina* species, G3: *Dicerandra* species, G4: *Monarda* species, G5: *Pycnant.* Species, G6: *Bystropogon* species, G7: *Clinopodium* species, G8: *Satureja* species, G9: *Mentha* species, G10: *Thymus* species, G11: *Agastache* species,G12: *Dracocephalum* species, G13: *Nepeta* species, G14: *Prunella* species, G15: *Lepechinia* species, G16: *Rosmarinus* species, G17: *Salvia* species and 4 different species from different family as out group. F: family *Lamiaceae.* Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* were used as current study.

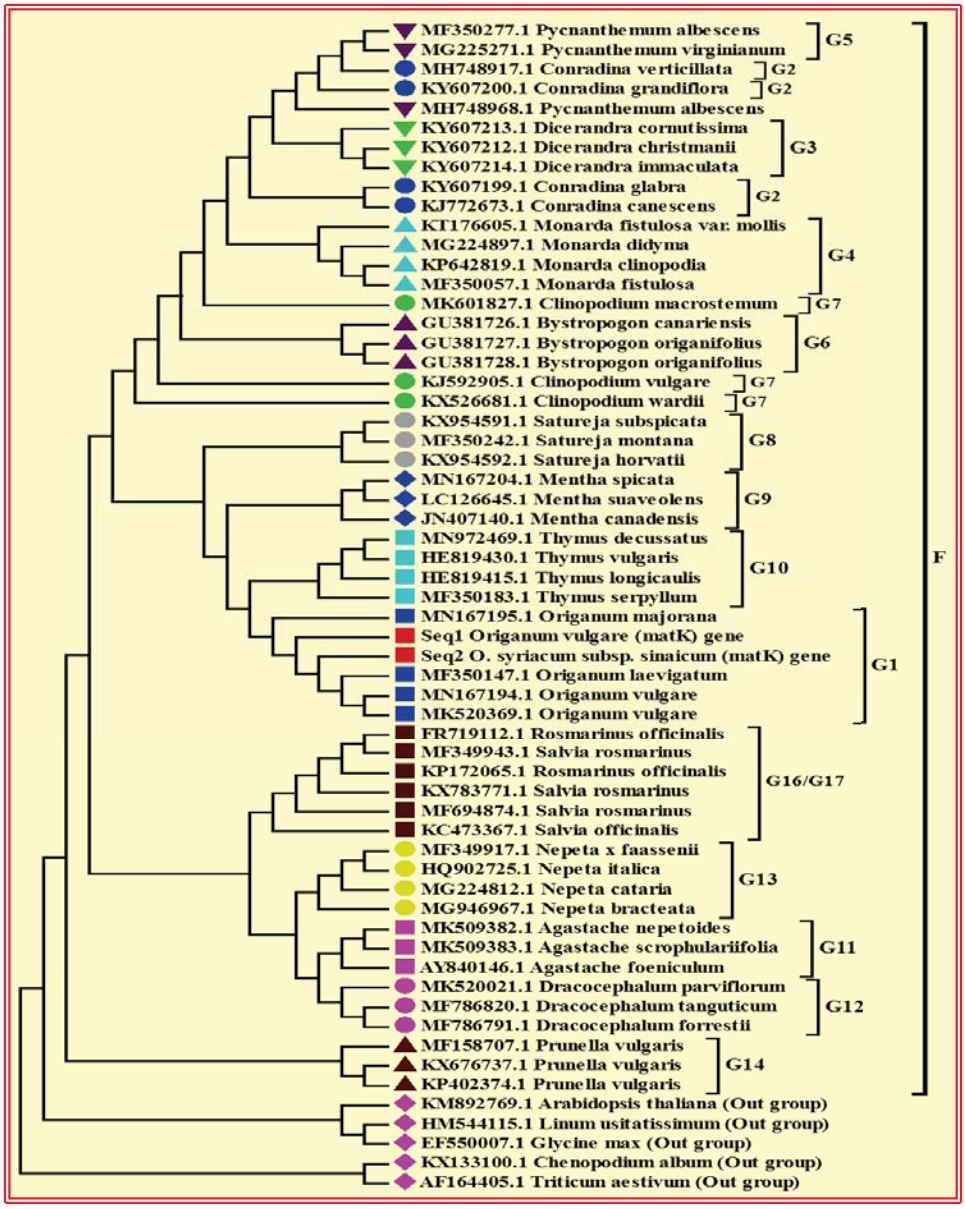


Fig.(3): Molecular phylogenetic analysis involved 60 amino acid sequences of maturase gene (*matK*) were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-5692.60) is shown. Note: G1: *Origanum* species, G2: *Conradina* species, G3: *Dicerandra* species, G4: *Monarda* species, G5: *Pycnant.* Species, G6: *Bystropogon* species, G7: *Clinopodium* species, G8: *Satureja* species, G9: *Mentha* species, G10: *Thymus* species, G11: *Agastache* species,G12: *Dracocephalum* species, G13: *Nepeta* species, G14: *Prunella* species, G15: *Lepechinia* species (no. record), G16: *Rosmarinus* species, G17: *Salvia* species and 4 different species from different family as out group. F: family *Lamiaceae.* Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* were used as current study.