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COMPARISON AMONG SIX DNA BARCODES FOR MOLECULAR AUTHENTICATION OF *OTOSTEGIA FRUTICOSA*

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Abstract

Otostegia fruticosa (Forssk.) Schweinf. ex Penz. plant is native to eastern Africa and the Middle East. This study is the first work for barcoding of *Otostegia fruticosa* subsp. *fruticosa* in Saudi Arabia with six specific primers (ITS, ITS2, matK, rbcL, TrnH-trnH2 and TrnH-GUG) and evaluating which one of these primers is more efficient than the other to characterize this plant. The results reveled from GenBank showed that there are only two rbcL reference sequences, one ITS sequence and no reference data for ITS2, matK, TrnH-trnH2 and TrnH-GUG sequences are available for *Otostegia fruticosa* plant. The results of DNA barcoding showed that ITS and matK loci recorded higher values of sequence length and variable sites than those of ITS2 and rbcL. The phylogenetic trees of ITS, TrnH-trnH2 and TrnH-GUG segregated *O. fruticosa* in a separate clade demonstrating that these loci performed better than ITS2, matK and rbcL for determining the genetic identity of this plant. This study contribute to supply molecular data about *O. fruticosa* based on DNA barcoding in Gene Banks, can be used for taxonomical studies of *O. fruticosa* and detect its relationship with other Lamiaceae species and determine the position of the *Otostegia fruticosa* collected from Saudi Arabia in the new generic classification.

Key words: *Otostegia fruticosa*; rbcL; TrnH; ITS; ITS2; matK.

Introduction

Otostegia fruticosa is widely distributed in Middle Eastern countries including Saudi Arabia (Ansari *et al*., 2020). The genus *Otostegia* (Lamiaceae) includes 31 species, of which *Otostegia fruticosa* subsp. *fruticosa* is the only subspecies existed in Saudi Arabia (Al-Musayeib *et al*., 2000). *O. fruticosa* is belonging to family Lamiacaea. The plant is undershrub, erect, perennial. The stem is circular, solid and its texture is tomentose. The leaves are simple, its arrangement is opposite decussate, petiolate, ovate, the leaf margin is crenate, apex is acute, leaf base is Cuneate-Truncate, leaf color is dark green, leaf texture is tomentose & wrinkled, number of leaves 3-7, Bract was present while bracteoles are absent. Calyx is tubular with 2 lippes without teeth. The corolla is White, bilobed densely villous. The basic number of stamens is four. Nutlet is trigonous, the color of nutlet is shiny green, nutlet texture is warty. The areole is absent. (Ya'ni *et al*., 2018)

Otostegia fruticosa is used in traditional medicine to treat arthritis, stomach ache and asthma (Bahta *et al*., 2020). It is antispasmodic, antiulcer, anxiolytic, antidepressant, antiparalytic, anti-inflammatory for eyes inflammation and sedative effects. Essential oil, diterpenoids and flavonoids of *O. fruticosa* showed antimicrobial characteristics against Gram (-) and Gram (+) bacteria and some species of fungal pathogens (Aboutabl *et al*., 1995; Khan & Syed, 2013; Ali *et al*., 2017; Rosselli *et al*., 2019; Ansari *et al*., 2020).

Loss of wild plants due to environmental degradation, deforestation, agricultural expansion, population pressure and overgrazing, have been reported in many countries including Saudi Arabia (Shinwari & Qaiser, 2011, Al-Rowaily *et al*., 2015, Abdel-Hamid, 2020). DNA barcodes has been described as easy way to not only identify living organisms but also can trace its evolutionary traits (Shinwari *et al*., 1994). Therefore, such detailed investigation on DNA barcodes in *O. fruticosa* will be very valuable to conserve its genetic resource and biodiversity. However, accurate and rapid identification of plant species is difficult, and this might be due to the immense diversity of plant species and the closely related species suffered contemporary radiation or repeated hybridization. DNA barcoding was instead tested for universality, discriminatory power and identification of organisms (Hebert & Gregory, 2005). DNA regions that were taken from the plastid genome (rbcL, matK and trnH–psbA) and the nuclear genome (e.g., ribosomal DNA ITS or ITS2) have been examined for these purposes.

The previous researches with regard to *O. fruticosa* were mainly concerned with the floristic diversity and vegetation analyses (Abdel Khalik *et al*., 2013; Aldhebiani & Howladar, 2015; Alsherif & Fadl, 2016), and the phytochemical and pharmacological studies (Al-Musayeib *et al*., 2000; Howladar, 2014; Bahta *et al*., 2020). So far, there is no molecular data about *O. fruticosa* collected from Saudi Arabia. As a powerful tool, DNA barcoding was used for species identification particularly for those used in traditional medicine (Palhares *et al*., 2015; Nithaniyal *et al*., 2017). ITS2 was suggested as the most variable site for identifying and comparing medicinal herbal species (Han *et al*., 2012; Michel *et al*., 2016). Another study reported that matK among three tested plastid loci (matK, rbcL and TrnH) was the potential barcode for species-level identification of Lamiaceae plants possessing medicinal products used in Pakistan (Zahra *et al*., 2016).

The purposes of the present work are (1) a considerable identification for *O. fruticosa* collected from Taif depending on six DNA loci; ITS, ITS2, matK, rbcL, TrnH-trnH2 and TrnH-GUG, and (2) comparing the performance of these loci to choose the more applicable barcodes for the molecular authentication of *O. fruticosa.*

Materials and Methods

Plant materials: *Otostegia fruticosa* is a wild plant (family Lamiaceae) was gathered from Taif highlands, KSA. The plant was morphologically identified according to Migahid (1996) and Collenette (1999).

The extraction of DNA and the amplification process: By CTAB method (Doyle & Doyle, 1987), young fresh leaves were used for extracting the genomic DNA. The primers for the six regions (ITS, ITS2, matK, rbcL, TrnH-trnH2 and TrnH-GUG) were listed in Table 1. For PCR protocol, DNA was denatured for 5 mints at 94°C. Thirty five cycles were adjusted including another denaturation for 1 min at 94°C, then followed by annealing step with suitable temperature (Table 1) and finished by first extension for 2 min at 72°C. A final extension step was done for 8 min at 72°C.

The PCR sequencing: After purification, the PCR products of *O. fruticosa* for the six DNA barcodes were finally sequenced at Macrogen corporation (Republic of South Korea). *O. fruticosa* Sequences that were generated in this research, were also registered in GenBank (recorded accessions numbers of *O. fruticosa* for the six DNA barcodes are in Table 2).

Statistics and analysis of phylogeny:ITS, ITS2, matK, rbcL, TrnH-trnH2 and TrnH-GUG sequences of *O. fruticosa* were undergone to BLAST on http://blast.ncbi.nlm.nih.gov/Blast.cgi to retrieve the most related genera belonging to family Lamiaceae occurred in GenBank database. MUSCLE method was done for all alignments (Edgar, 2004). Statistics; sequence length, (%) Variable sites, overall mean distance and transition/ transversion bias (R) and nucleotide exchange rates were calculated by Maximum Likelihood method (Tamura *et al*., 2013) using MEGA6 software. A total of 1000 bootstrap replicates were used through the building of the Maximum likelihood (MLB) phylogenetic trees.

Results and Discussion

The world has tried to use drugs that were made from natural sources such as plant-based drugs instead of synthetic chemical compounds (Walter *et al*., 2011, Sarwat *et al*., 2012). *O. fruticosa* is one of these natural resources so, it is important to protect its genetic germplasm by using DNA barcoding. Due to the paucity of genome or related sequence data, no study has been performed on *O. fruticosa* from Saudi Arabia. Understanding the genetic structure is fundamental to enhance the conservation efforts to decline this endemic or native species into the world (Shinwari, 2002). Given the above, we sequenced some regions; ITS, ITS2, matK, rbcL, TrnH-trnH2 and TrnH-GUG, of the genome of this important species and performed comparative analysis with related retrieved species from the GenBank. Khan *et al*., (2020) sequenced the complete chloroplast genome of *E. smithii* and *E. larica* and carried out comparative analysis with related species (*E. tirucalli, E. esula, J. curcas, M. esculanta, R. communis, H. brasiliensis, C. tiglium, D. tonkinensis* and *V. fordii*).

Table 1. list of the investigated DNA barcoding primers.

Site	Primer		The sequences (5'–3')	Ann. temp
ITS	AB101	F	ACGAATTCATGGTCCGGTGAAGTGTTCG	52˚C
	AB102	R	TAGAATTCCCCGGTTCGCTCGCCGTTAC	
ITS2	ITS-S2F	F	ATGCGATACTTGGTGTGAAT	52˚C
	ITS4	R	TCCTCCGCTTATTGATATGC	
rbcL	rbcLa	F	ATGTCACCACAAACAGAGACTAAAGC	52˚C
	rbcLa	R	GTAAAATCAAGTCCACCRCG	
matK	matK-KIM1	F	ACCCAGTCCATCTGGAAATCTTGGTTC	52˚C
	matK-KIM3	R	CGTACAGTACTTTTGTGTTTACGAG	
TrnH-trnH2	psbAF	F	CGCGCATGGTGGATTCACAATCC	52˚C
	trnH2	R	GTTATGCATGAACGTAATGCTC	
TrnH-GUG	psbA	F	CGAAGCTCCATCTACAAATGG	52˚C
	trnH(GUG)	R	ACTGCCTTGATCCACTTGGC	

Table 2. Accession numbers *O. fruticosa* sequences recorded in GenBank.

ITS	ITS2	matK	rbcL	TrnH-trnH2	TrnH-GUG
LC372527.1	LC372528.1	LC372529.1	LC372530.1	LC372531.1	LC372532.1

Table 3. Statistics for the comparison of the six DNA barcodes.

Locus	ITS	ITS2	matK	rbcL	TrnH-trnH2	TrnH-GUG
Sequence length	676	329	888	527	316	227
% Variable sites	60.8	25.5	45.5	3.60	31.6	36.1
Overall mean distance	0.26	0.11	0.27	0.01	0.08	0.11
Transition/Transversion bias (R)	0.97	1.53	1.06	1.67	0.99	0.63
No. of the retrieved *Otostegia* species from GenBank	1	0	0	2	0	0
No. of the other retrieved genera from GenBank	10	10	10	10	12	12

Table 4. Mean nucleotide substitution rates in the six loci for *O. fruticosa*.

Locus	Transition				Transversion							
	A→G	G→A	T→C	C→T	A→T	T→A	A→C	C→A	T→G	G→T	C→G	G→C
ITS	10.52	6.62	24.19	11.05	3.63	4.72	7.95	4.72	7.51	3.63	7.51	7.95
ITS2	19.22	8.34	5.94	10.15	2.39	2.57	6.49	2.57	5.94	2.39	5.94	6.49
matK	8.36	16.30	9.67	19.88	8.08	7.20	3.93	7.20	3.69	8.08	3.69	3.93
rbcL	17.00	20.54	10.95	14.35	5.26	5.09	4.02	5.09	4.21	5.26	4.21	4.02
TrnH- trnH2	7.36	16.61	8.47	22.04	8.31	7.79	3.20	7.79	3.45	8.31	3.45	3.20
TrnH-GUG	3.84	8.46	8.91	22.18	9.75	10.06	3.92	10.06	4.57	9.75	4.57	3.92

Identification of *O. fruticosa*: Although the six studied sequences were presented for the BLASTing process, no sequence could be detected in GenBank database, thus we think that this study presents new DNA barcodes for *O. fruticosa* from Saudi Arabia. This finding agreed with Hollingsworth *et al*., (2009) who reported that species that contain one sample can be distinguished if the sequence is unique. This also may occur due to loss of complete gene region of the reference sequence data in GenBank. Accordingly, the partial query sequence of a gene may not lead to an accurate identification. Several medicinal species of Lamiaceae have no reference sequence data available in the GenBank (Zahra *et al*., 2016, Shinwari and Shinwari, 2010). However, from the GenBank sequences of ITS and rbcL retrieved only one and two species belonging to genus *Otostegia* respectively (Table 3). Statistics in Table 3 showed that matK recorded the highest sequence length (888 bp) followed by ITS (676 bp), rbcL (527 bp), ITS2 (329 bp), TrnH-trnH2 (316 bp) and TrnH-GUG (227 bp), whereas, ITS and matK recoded higher percentages of the variable sites than the others. All loci scored transitions more than transversions (Table 4) and Transition/Transversion bias (R) ranged from 0.63 to 1.67 reflecting a moderate molecular evolution within the genome of *O. fruticosa* gathered from Taif. ITS and matK revealed higher genetic distances between *O. fruticosa* and the genera retrieved from GenBank (0.26 and 0.27 respectively) (Table 3).

Comparison of the six barcodes: ITS and ITS2 sequences were examined for *O. fruticosa* identification. After the BLASTing process, only a single reference sequence available of *O. fruticosa* subsp. *schimperi* for ITS marker was detected, but there is not any reference sequence available for ITS2. Therefore, ten reference sequences of *Ballota hispanica, Brandisia hancei, Colquhounia elegans, Eremostachys* sp., *Eriophyton wallichii, Gomphostemma chinense, Lamium album, Leonurus sibiricus, Paraphlomis formosana* and *Phlomoides rotata* were retrieved from the GenBank to compare the performance of ITS and ITS2 through two phylogenetic trees as shown in Figure 1. The loss of reference sequences from the GenBank was also reported by Zahra *et al*., (2016), so they depend on the genus-level identification rather than species-level discrimination. ITS tree represented *O. fruticosa* in separate clade demonstrating high variability between it and the other retrieved genera, whereas, ITS2 tree grouped *O. fruticosa* with *Gomphostemma chinense* in one clade. Moreover, ITS locus recorded higher values of sequence length, variable sites (%) and overall mean distance than those of ITS2 (Table 3).

In GenBank only two rbcL reference sequences of *Otostegia* sp. and *O. tomentosa*, were available while matK sequence is not found (Table 3). The two plastid sequences (matK and rbcL) retrieved the same ten genera; *Ballota africana, Colquhounia coccinea, Eurysolen gracilis, Gomphostemma* sp., *Lagopsis supina, Leonotis leonurus, Leonurus japonicus, Leucas cephalotes, Marrubium vulgare* and *Stachys sylvatica* from the GenBank for reconstructing two phylogenetic trees as described in Figure 2. matK tree separate *O. fruticosa* with *Stachys sylvatica* in one clade and the other genera in another clade however, rbcL data grouped the three species of *Otostegia* in one clade revealing the symmetry in rbcL sequence of *Otostegia* species. matK and rbcL loci were relatively long (almost 888 and 527 bp respectively) but the rate of evolution (R) was not the same. matK locus recorded higher variable sites (%) and overall mean distance values than those of rbcL (Table 3). Similar results were obtained by Elansary *et al*., (2017).

For further identification of *O. fruticosa*, TrnH-trnH2 and TrnH-GUG sequences were investigated. Statistics of the two loci were relatively similar (Table 3). Twelve genera; *Colquhounia elegans, Eriophyton rhomboideum, Gomphostemma* sp., *Isodon japonicus, Lagochilus ilicifolius, Lamium galeobdolon, Leonotis nepetifolia, Leucas sp., Magnoliophyta* sp., *Roylea cinerea, Rydingia limbata* and *Stachyopsis oblongata* reconstructed the two phylogenetic trees of TrnH-trnH2 and TrnH-GUG as represented in Figure 3. The two trees revealed that *O. fruticosa* was represented in separate clade demonstrating that TrnH locus performed better than the two plastid genes (matK and rbcL) through determining the taxonomic identity of *O. fruticosa.* Because of the two TrnH spacers were shorter (227-316 bp) than matK and rbcL, so we use it as a much better and accurate barcode than using any of these plastid loci (Schori & Showalter, 2011). This limitation of rbcL gene and the effective performance of TrnH in the identification and discrimination of *O. fruticosa* supported findings of De Mattia *et al*., (2011), Hollingsworth *et al*., (2011) and Theodoridis *et al*., (2012). The separate clading of *O. fruticosa* in the phylogenetic analyses of ITS, TrnH-trnH2 and TrnH-GUG demonstrated its endemicity to Saudi Arabia. The non-monophyly of *Otostegia* was demonstrated by Bendiksby *et al*., (2011) leading to a taxonomic update within family Lamiaceae depending upon four chloroplast regions (rps16, matK, trnL intron and trnL-F spacer). China Plant BOL Group (2011) reported that trnH–psbA and matK were better at recognizing and distinguishing gymnosperms than in angiosperms, however, nrITS was relatively well in angiosperms, with moderately high universality but showing lower success in gymnosperms. The other plastid site (rbcL) scored the highest level of universality in both angiosperms and gymnosperms.

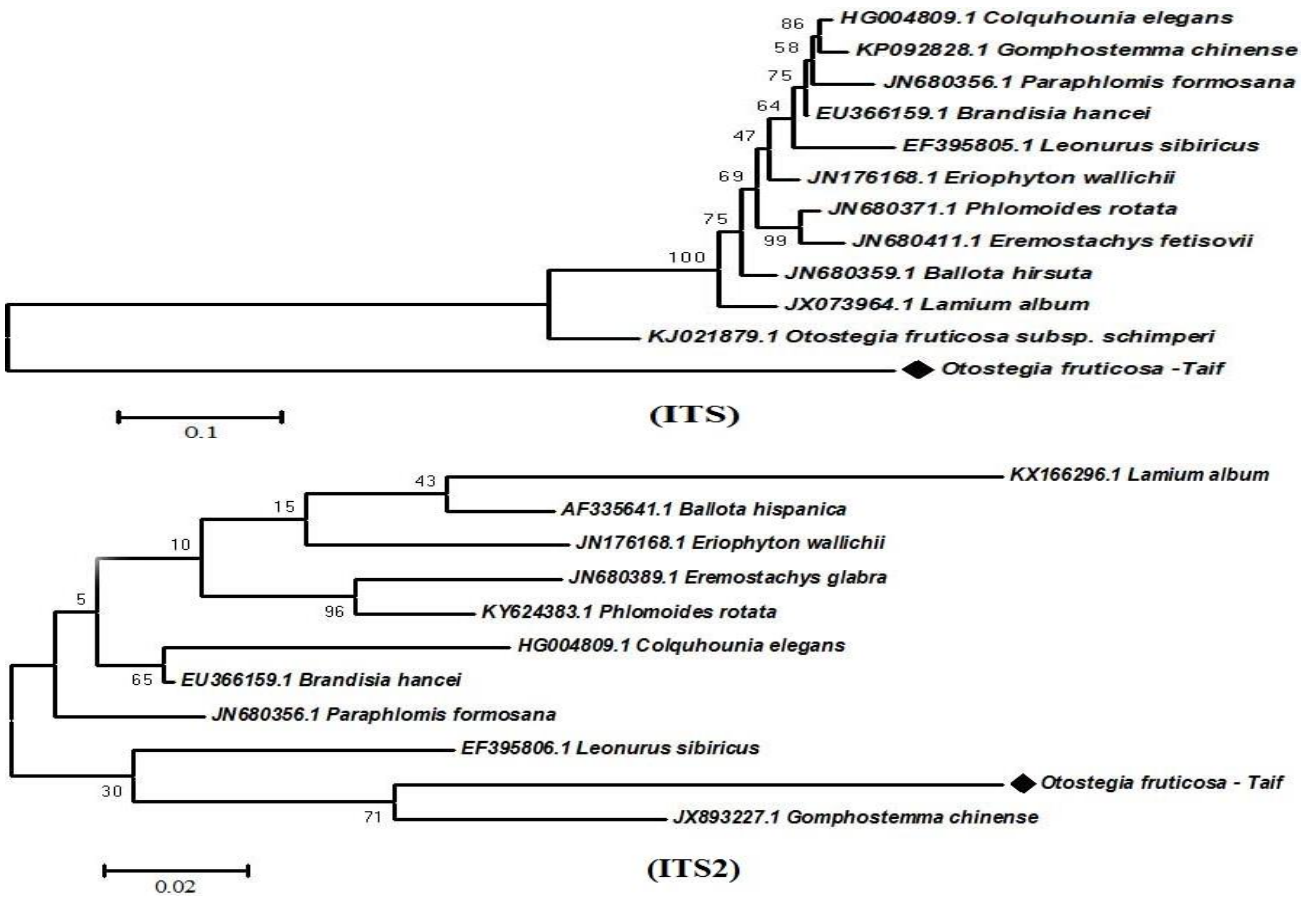

Fig. 1. Phylogeny analyses of *O. fruticosa* and the retrieved genera using ITS and ITS2 sequences.

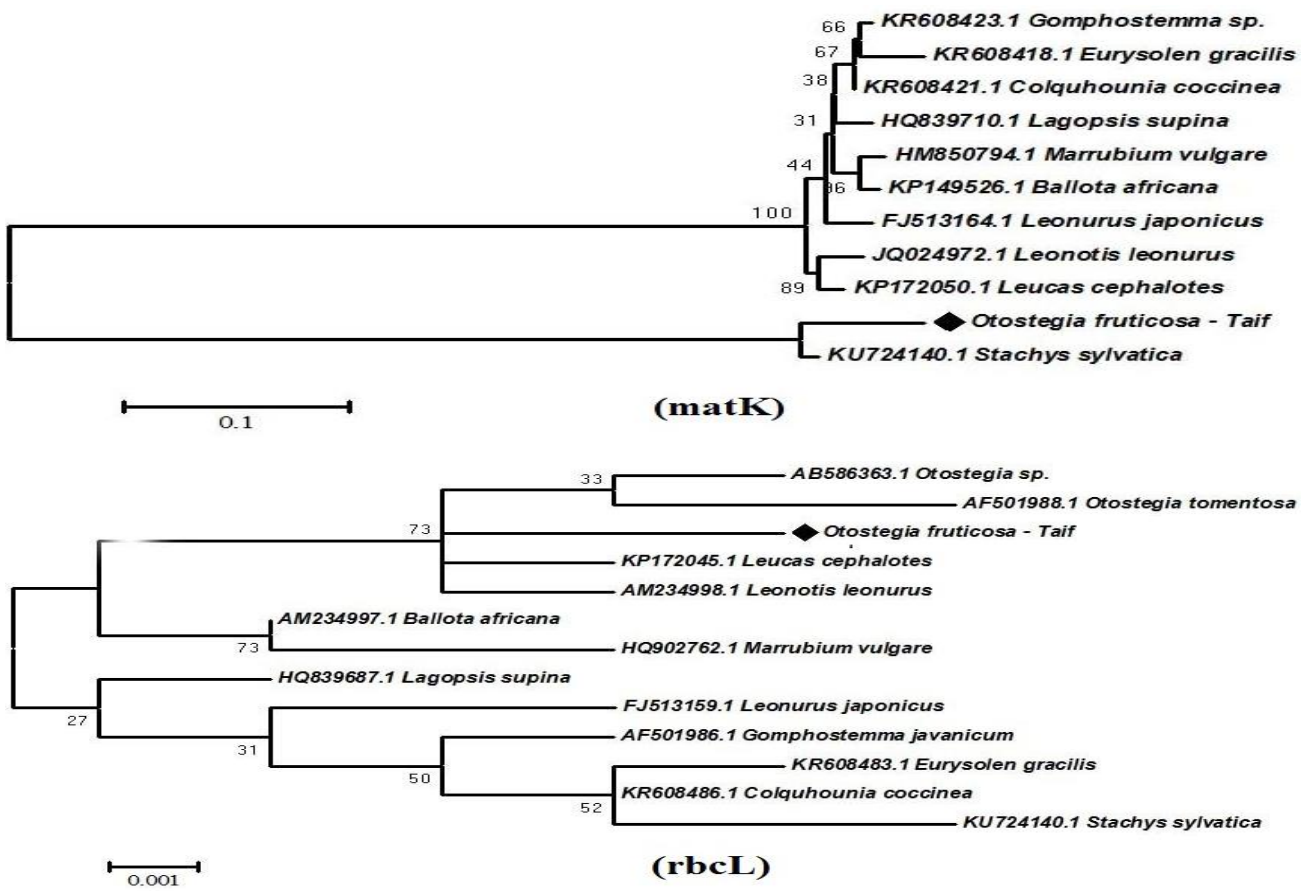

Fig. 2. Phylogeny analyses of *O. fruticosa* and the retrieved genera using matK and rbcL sequences.

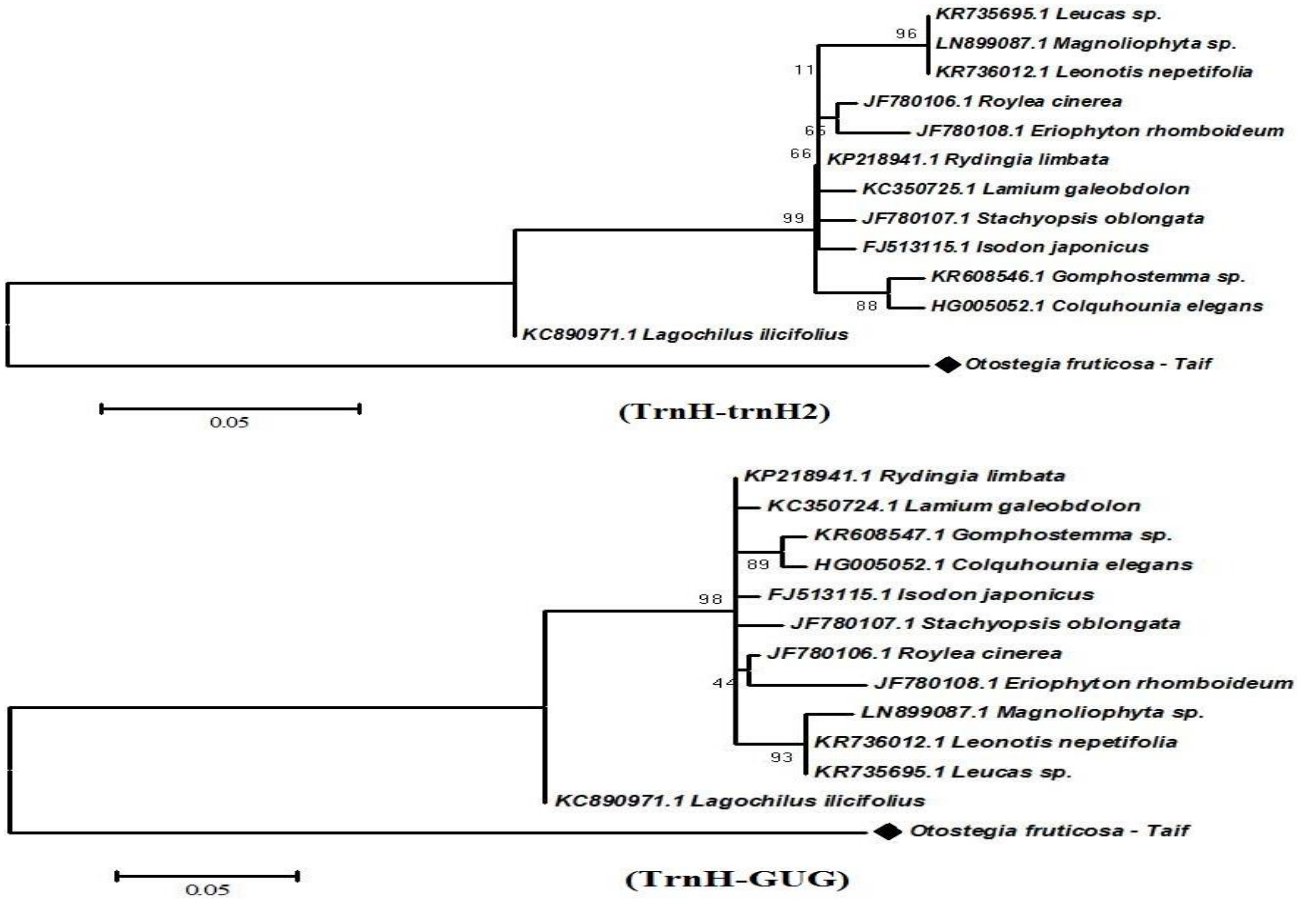

Fig. 3. Phylogeny analyses of *O. fruticosa* and the retrieved genera using TrnH- trnH2 and TrnH-GUG sequences.

Conclusions

DNA barcoding sequences by ITS2, matK, TrnH-trnH2 and TrnH-GUG of *O. fruticosa* gathered from Taif, KSA were registered for the first time in GenBank because there is no previous data available before on it. The phylogenetic trees of ITS, TrnH-trnH2 and TrnH-GUG segregated *O. fruticosa* in a separate clade demonstrating that loci have sufficient efficiency for characterization and species discrimination of *O. fruticosa* from other family member. This study contributed to supply Molecular data based on DNA barcoding in Gene Banks that can be used for taxonomical studies of *O. fruticosa* and its relationship with other Lamiaceae species.

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