Turkish Journal of Agriculture - Food Science and Technology, 7(6): 908-912, 2019
DOI: https://doi.org/10.24925/turjaf.v7i6.908-912.2504

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology

Characterization of *rbcL* and *trnL* Plastid DNA Sequences of *Vuralia turcica* (Fabaceae; Papilionoideae)

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ARTICLE INFO

Research Article

Received : 26/02/2019
Accepted : 16/04/2019

Keywords:
V. turcica
rbcL
trnL
Sequencing
Phylogeny

ABSTRACT

Vuralia turcica is endemic to Turkey and currently endangered. Little molecular information is available for this plant. Previous characterization and classification of *V. turcica* have been based on the DNA sequences of the ITS region. Molecular markers are essential for studying of genotyping and biogeography, but any of each marker is not enough to characterize a plant species in its use alone. In this study, the chloroplast *rbcL* and *trnL* regions were amplified in *V. turcica* using the primers that have been published in the previous studies. Successfully amplified DNA fragments were extracted and commercially sequenced. The partial *rbcL* and *trnL* sequences were submitted to the NCBI database (accession number KX164510, KX164511, respectively). Amplified both DNA of two regions of *rbcL* and *trnL* were used to construct a phylogenetic tree.

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Introduction

Turkey is in the intersection area of 3 different gene centers (Mediterranean, Iran-Turan, and Europe-Siberia), is surrounded by the sea on 3 sides, and is rich in water resources. For these reasons, Turkey has a rich flora (Dölarslan and Gül, 2015). In addition, Turkey's endemism rate (34%) is higher than in many European countries such as Spain (18.6%), Greece (14.9%), and France (2.9%). It is known that Turkey hosts 3.700 endemic plants (Dilaver, 2013). One of the most important gene sources in Turkey is *Vuralia turcica* (Kit Tan et al. 1983) Uysal et al. (2014) (Figure 1) known as 'piyan' among the local people. *V. turcica* belongs to the Papilionoideae subfamily of the Fabaceae family and is endemic to Turkey. This flowering plant distributed in a limited area in the Central Anatolia of Turkey (Cenkci et al. 2012).

Deforestation and local collectors are the main problems to cause a reduction in the natural populations of this species. Since 2000, *V. turcica* has been in the red list of Turkey's species threatened with extinction. According to a literature review regarding with *V. turcica*, studies were implemented mostly about its micropropagation. Few studies focused on the subject different from the micropropagation of the species. Previous work has reported that the dominant bacteria at the rhizomes of *V. turcica* is *Bacillus megaterium* (Çiftçi, 2018) and the researcher assumed that *B. megaterium* has the potential growth-promoting effects on *V. turcica*. Yildiz et al. (2017) studied the proteomic analysis in *V. turcica* and proteins involved in flower development were identified. There has been only one study existed on the genetic classification of *V. turcica*, and it has been based on the nucleotide sequence of the nuclear ribosomal internal transcribed spacer region (ITS) (Uysal et al., 2014). Hence, evaluation of genetic diversity of *V. turcica* using the genetic markers is very important to advance the knowledge of its biology and genome structure. Chloroplast and nuclear DNA regions are currently employed as a barcode DNA region to discriminate plant species from each other (Hollingsworth et al., 2011). Recently, nucleotide sequences of ribulose-

1,5-bisphosphate carboxylase/oxygenase (*rbcL*) and its combination with different nucleotide sequences of barcode DNA regions have been tested to discriminate species from each other (Hollingsworth et al., 2009). The nucleotide sequences of *rbcL* and *tRNA-Leu* (*trnL*) were commonly used DNA regions in phylogeny analysis. Using the nucleotide sequences of *rbcL* and *trnL* as well as other barcode DNAs have many advantages such as having easy experimental procedures and being more cost-effective (Lahaye et al., 2008; Hollingsworth et al., 2009) The aim of the current study was to characterize plastid *rbcL* and *trnL* DNA sequences in *V. turcica* for ecological interest. In this study, the utility of *rbcL* and *trnL* sequences was also tested to their utility for the phylogeny of *V. turcica*.

Material and Methods

Plant Material

The fresh leaves of *V. turcica* were used in this study and obtained from the Nezahat Gökyiğit Botanical Garden in Istanbul, Turkey.

Genomic DNA Isolation

Total genomic DNA was isolated from fresh young leaf tissue collected from a single plant of *V. turcica* following the MiniPrep DNA isolation procedure described by Edwards et al. (1998). Concentration and purity of the gDNA isolated were determined NanoDrop® ND-1000 Spectrophotometer.

PCR Analysis and Agarose Gel Electrophoresis

DNA barcoding markers (*rbcL* and *trnL*) were used in this study (Table 1). PCR amplifications were performed in a reaction volume of 25 µL reaction mixture containing 5 ng DNA, 0.8 µM of each primer (forward and reverse), 0.2 mM dNTP (Fermentas), 0.125 unit Taq DNA Polymerase (Fermentas, Germany), 1X Taq DNA Polymerase buffer (Fermentas, Germany), including 2.5 mM $MgCl_2$ (Fermentas, Germany). The PCR conditions had an initial cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30 seconds at 50°C and 30 seconds at 72°C with a final extension at 72°C for 7 min. Amplifications were performed in a thermocycler (Eppendorf, Hamburg, Germany). PCR products were separated and visualized on agarose gels (2%). DNA samples were mixed with DNA loading dye were loaded on the gel, which was run at 100V and 70 mA for 45 min in 0.5X TBE. DNA bands were visualized using UV light on a Biorad Imager (Bio-Rad Laboratories, Segrate (Milan), Italy). DNA bands of approximately the expected size were excised from the agarose gel and extracted using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, USA) according to the manufacturer's protocol.

Sequencing and Sequence Analysis

Extracted DNA bands from the agarose gel were sent for Sanger sequencing (Sanger et al., 1977) with the same primers as those used for PCR amplification. Three independent PCR products amplified using the same primers were sequenced. PCR products were sequenced in both directions. Sequencing service was commercially provided by BMLabosis**,** Ankara, Turkey (BMLABOSIS, 2019). Raw DNA sequence data were checked manually and then forward and reverse complement sequences were aligned using the Pairwise Sequence Alignment (Nucleotide) Tool constructed by EMBL-EBI (EMBL-EBI, 2019).

Phylogenetic Analysis

The consensus sequence was used to search for similar sequences in GenBank at the National Center for Biotechnology Information (NCBI), Bethesda, USA, using the BLAST (2019) search program (Altschul et al., 1990). Phylogenetic analysis was performed by the MEGA7 (2019) program. Aligned *rbcL* and *trnL* sequences were evaluated with bootstrap analysis (1000 replicates) (Felsenstein, 1985). The evolutionary history of the *rbcL* gene in *V. turcica* was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1346.25) is shown. There were a total of 487 positions in the final dataset. The evolutionary history of the *trnL* gene in *V. turcica* was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-1068.62) is shown. There were a total of 434 positions in the final dataset.

NCBI GenBank Data Transfer

The determined *V. turcica rbcL* and *trnL* partial sequences were deposited in the NCBI GeneBank using the Sequin program (NCBI, 2019)

Figure 1 General view of *V. turcica* during fruit ripening at Nezahat Gökyiğit Botanical Garden

Results and Discussion

Genomic DNAs were isolated from the leaves of *V. turcica* which were obtained from the Nezahat Gökyiğit Botanical Garden, Istanbul, Turkey. The quality of isolated DNAs was determined using agarose gel (1%) electrophoresis (Figure 2.) and was found to be of high quality, with a 260/280 ratio of 1.8-2.0.

PCR products were obtained using gDNAs of *V. turcica*'s leaves. To identify the putative orthologues of *rbcL* and *trnL* in *V. turcica*, purified PCR products of gDNAs were amplified by conventional PCR with the primers given in Table 1 (Figure 3.).

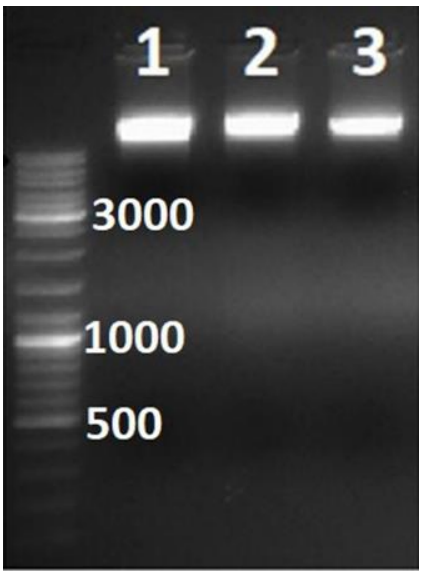

Figure 2 1% Agarose gel electrophoresis results of gDNAs isolated from the leaf samples of V. turcica (DNA Ladder: Thermo Scientific DNA Ladder SM0333). Three independent samples were used (1: V. turcica-1; 2: V. turcica-2; 3: V. turcica–3)

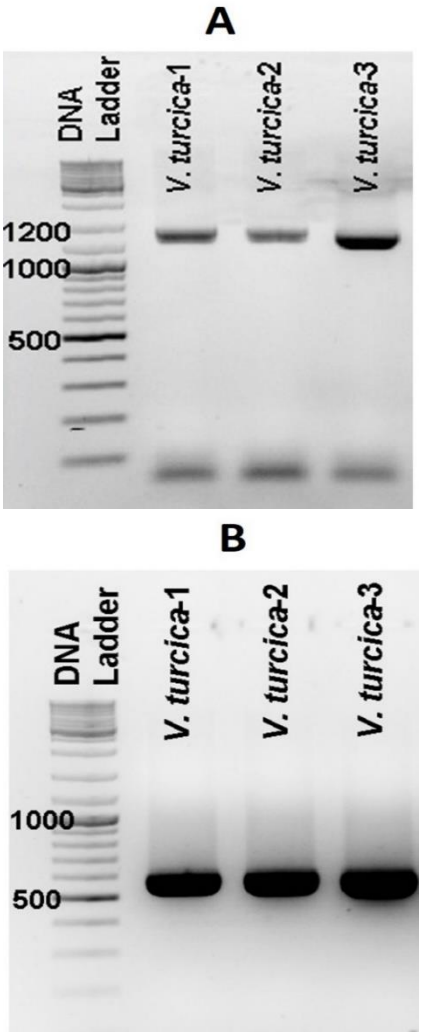

Figure 3 2% agarose gel electrophoresis of PCR analysis of isolated leaf samples of *V. turcica* using *rbcL* (A) and *trnL* (B) primers

Isolated and purified PCR bands were directly sequenced by the Sanger method (Sanger et al. 1977). A 516 bp putative *rbcL* homolog for *V. turcica* was obtained and deposited the NCBI GenBank with the following accession number: KX164510. Similarly, a verified 553 bp sequence was derived from PCR product in which *trnL* used as a primer pair and was considered to be a putative homolog of the *t*RNA-Leu gene. The partial *trnL* sequence of 553 base pairs was submitted to the NCBI GenBank with the following accession number: KX164511. The amino acid sequence of isolated *rbcL* sequence of *V. turcica* was:

GGFTANTSLAHYCRDNGLLLHIHRAMHAVIDR QKNHGMHFRVLAKALRMSGGDHIHAGTVVGKLE GEREITLGFVDLLRDDFIEKDRSRGIYFTQDWVSLP GVLPVASGGIHVWHMPALTEIFGDDSVLQFGGGTL GHPWGNAPGAVANRVALEACVQARNEGRDLARE GN

The *rbcL* isolated in this study contained a predicted Rubisco binding domain according to the NCBI conserved domain search (Figure 4).

The genome structure of *V. turcica* can be identified using several molecular approaches. Some DNA region of the chloroplast are proposed for barcoding plant species, and nucleotide sequences of chloroplast such as *rbcL* and *trnL* are recently one of the most popular types of genetic markers for molecular studies. The Maximum Likelihood (ML) tree was generated using Kimura (1980) distances based on nucleotide sequences of the *rbcL* and *trnL* region shown in Figure 5 and 6. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). While phylogenetic trees were constructed, to eliminate errors that may occur in the comparison of different sequence lengths, only sections of the homologous sequences in the NCBI database that cover the gene identified in this study were used. For phylogenetic tree construction based on *rbcL* and *trnL*, selected species were given in Table 2.

The phylogenetic tree of the *rbcL* gene showed that *V. turcica* is most closely to the other legumes and was well separated from *Arabidopsis* species (*A. thaliana* and *A. petraea*) (Figure 5). The *rbcL* region despite the low variability of this region has been used successfully in the previous studies for species identification (Meier et al., 2008; Bafeel et al., 2011, 2012). In this study, this region was used for phylogenetic analysis and found to be suitable for systematic molecular analysis of *V. turcica*. ML analysis of the region of *trnL* (Figure 6) revealed a cluster of the genus *Swartzia* and *Arabidopsis* at distinct nodes.

Table 1 The list of the primers including the forward (F) and reverse (R) sequences employed in this study. Primer names, their sequences, and their product sizes are given

Target	Name	Primer sequence (5'- 3')	Definition	Product size (bp)	Reference
rbcL	rbcLF	TGTCACCAAAAACAGAGACT	rbcLF/sense primer	516	Parani et al. (2000)
	rbcLR	TTCCATACTTCACAAGCAGC	rbcLR/antisense primer		
trnL	trnL1	TCGGTAGACCGCTACGGAC	trnL1/sense primer	553	Guo and Ge (2005)
	trnL2	GGATAGAGGGACTTGAACC	trnL2/antisense primer		

Table 2 Sequence similarities according to the BLAST search against NCBI database based on identified partial *rbcL* and *trnL* sequences in *V. turcica*

The BLAST search based on identified partial rbcL		
gDNA	Species	Accession No
Chloroplast rbcL	*Thermopsis fabacea*	Z70121.1
Chloroplast rbcL	*Baptisia tinctoria*	Z70120.1
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Thermopsis rhombifolia*	JX848468.1
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Thermopsis gracilis*	MF963121.1
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Baptisia alba*	J773304.1
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Arabidopsis petraea*	C481979.1
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Arabidopsis thaliana*	U91966.1
The BLAST search based on identified partial *trnL*		
tRNA-Leu	*Thermopsis rhombifolia*	HM590355.1
tRNA-Leu	*Baptisia australis*	AF309831.1
tRNA-Leu	*Thermopsis montana*	AF385411.1
tRNA-Leu	*Thermopsis villosa*	AF311384.1
tRNA-Leu (UAA)	*Baptisia tinctoria*	AJ890964.1
tRNA-Leu(UAA)	*Swartzia panacoco*	FJ039315.1
tRNA-Leu	*Arabidopsis kamchatica*	GQ303524.1

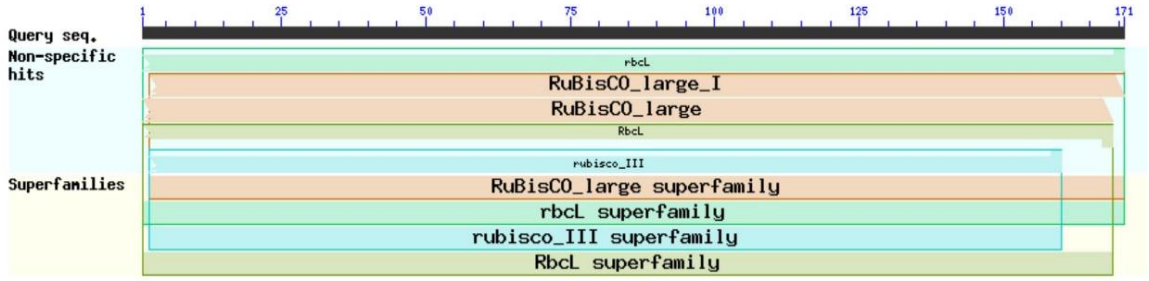

Figure 4. Putative conserved domains in Rubisco protein in *V. turcica*

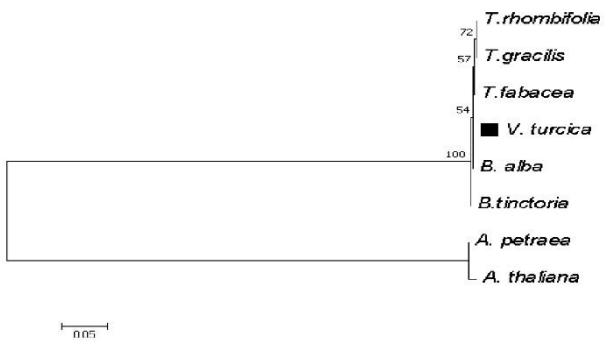

Figure 5 Molecular Phylogenetic analysis of *rbcL* sequence by Maximum Likelihood method

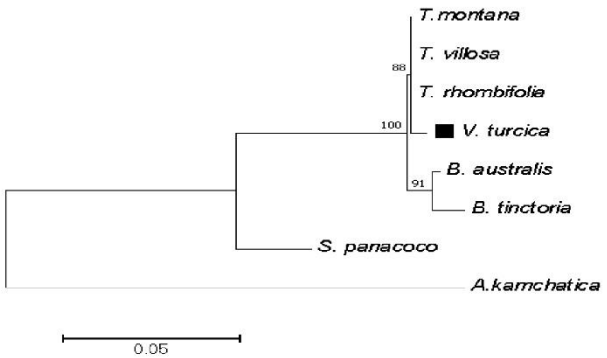

Figure 6 Molecular Phylogenetic analysis of *trnL* sequence by Maximum Likelihood method

Although the characterization of the nucleotide sequences of the *rbcL* and *trnL* regions were easier to recover, the full-length sequences of the *trnL and rbcL* genes in *V. turcica* were not determined; however, the sequencing data obtained were used for the construction of the phylogenetic tree and have moved the molecular information regarding *V. turcica* a step forward. High-quality sequence data is one of the critical steps for the efficient recovery of any desired region (CBOL Plant Working Group, 2009; Dong et al., 2014). In this study, rbcL and trnL regions were amplified successfully.

In conclusion, based on *rbcL* and *trnL* analyses, phylogenetic relationships of *V. turcica* with other closely (*Baptisia* and *Thermopsis*) and distantly related genera (*Arabidopsis*) analyzed in this study agreed with its previous taxonomic classification described by Uysal et al.

(2014). *V. turcica* was differentiated from the genus of *Arabidopsis.* The topology of phylogenetic trees based on *rbcL* and *trnL* analyses were found to be similar in this study. Both *rbcL* and *trnL* have served well for the construction of phylogenetic tree for *V. turcica*.

Accession Numbers

The sequences were deposited in the GenBank database with the following accession numbers: KX164510 and KX164511.

Acknowledgments

The author expresses herself appreciation to the Ali Nihat Gökyiğit Foundation, Nezahat Gökyiğit Botanical Garden for providing research materials for the study. The author is grateful to Prof. Dr. Selim Çetiner to provide laboratory facilities and would like to thank Dr. Stuart James Lucas for help with sequence analysis

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