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Sequence analysis of chloroplast *psb*A-*trn*H region in *Citrus* L. (Rutaceae) species from the Aegean region of Turkey

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Abstract

The aim of the study was to analyze the sequence of some citrus species found in the Aegean Sea region of Turkey based on the *psb*A-*trn*H cpDNA region. Genomic DNA was isolated from healthy and green leaves. Total genomic DNA was extracted using the GeneMark DNA isolation Plant Kit. The *psb*A-*trn*H region of chloroplast DNA was amplified using primers *psb*A and *trn*H. DNA sequences were edited using the Sequencher 5.4.6 and sequencing data were analyzed using the MEGA 6.0 software. The maximum likelihood (ML) tree was created to determine the relationships between *Citrus* taxa. Amplified cpDNA *psb*A-*trn*H sequences ranged between 426 and 470 nucleotides. The maximum likelihood phylogenetic tree was composed of two clades. The divergence values differed between 0.000 and 0.012. In addition, the sequences of some species belonging to the Rutaceae family were obtained from NCBI and a maximum likelihood tree was constructed. The phylogenetic relationship between the sequence data of some species belonging to the Rutaceae family taken from NCBI and *Citrus* species was revealed.

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Introduction

Rutaceae Juss. is a large tree, bush and grass family comprising around 150 – 170 genera and nearly 2.040 species (Morton and Telmer 2014). The genus *Citrus* L. belongs to the Aurantioideae subfamily of the Rutaceae family. *Citrus* includes economically and commercially important fruit crops, major fruit plants of the world such as limons, mandarins, sour oranges, sweet oranges, squash, grapefruits, kumquats and others. (Groppo *et al*. 2008; Baig *et al*. 2009; Kumar *et al*. 2013). It is believed that especially the region extending from the north eastern India to eastward, from primary main origins of the genus *Citrus* are in South and Southeast Asia from Malaya Archipelago to China and Japan, and from south to Australia (Jena *et al*. 2009). The fruits and leaves of *Citrus* species contain a variety of essential oils containing biologically active compounds such as vitamin C, folic acid, potassium, flavonoid glycosides, coumarins, pectin, which are important for various nutrients and human diets (Othman *et al*. 2016; Bozkurt *et al*. 2017; Elkhatim *et al*. 2018; Liu *et al*. 2018).

Molecular analysis to be conducted on genetic variation among the individuals of a population

may offer a tool to monitor the genetic variability of a diminishing population and evaluate the genetic consequences of fragmentation on the remaining populations (Al-Qurainy *et al.* 2014). Chloroplast DNA (cpDNA) particularly includes non-coding regions which are maternally hereditary in angiosperms and have rich variability. These regions are significantly applied in various studies in molecular phylogenetic, population genetics and protection with a more comprehensive perspective (Chen *et al.* 2013; Gutiérrez-López *et al.* 2016). The *psb*A-*trn*H intergenic region is among the most variable regions of the chloroplast genome. This region is suitable for DNA barcoding studies, consists of two parts in evolutionary conservation (Štorchová and Olson 2007; Filiz and Koç 2012; Yılmaz 2020). The aim of the study was to determine the sequence analysis of selected *Citrus* L. taxa using the *psb*A-*trn*H cp (DNA) sequence in order to explain the phylogenetic relationships between them.

Experimental

Collection of plant materials and isolation of genomic DNA

The fresh green leaves from 11 populations of five species of the genus *Citrus*: *C. aurantium* L., *C. limon* (L.) Burm. f., *C. paradisi* Macfad., *C. reticulata* Blanco, *C. sinensis* (L.) Osbeck, were collected from Aegean region (Aydın, Muğla, İzmir, Manisa-Salihli) in Turkey (Fig.1) and brought to the plant biotechnology laboratory at Aydın Adnan Menderes University. Genomic DNA was isolated using the DNAeasy Plant Kit (GeneMark) based on the manufactures protocol. Then the samples were stored at -20 °C.

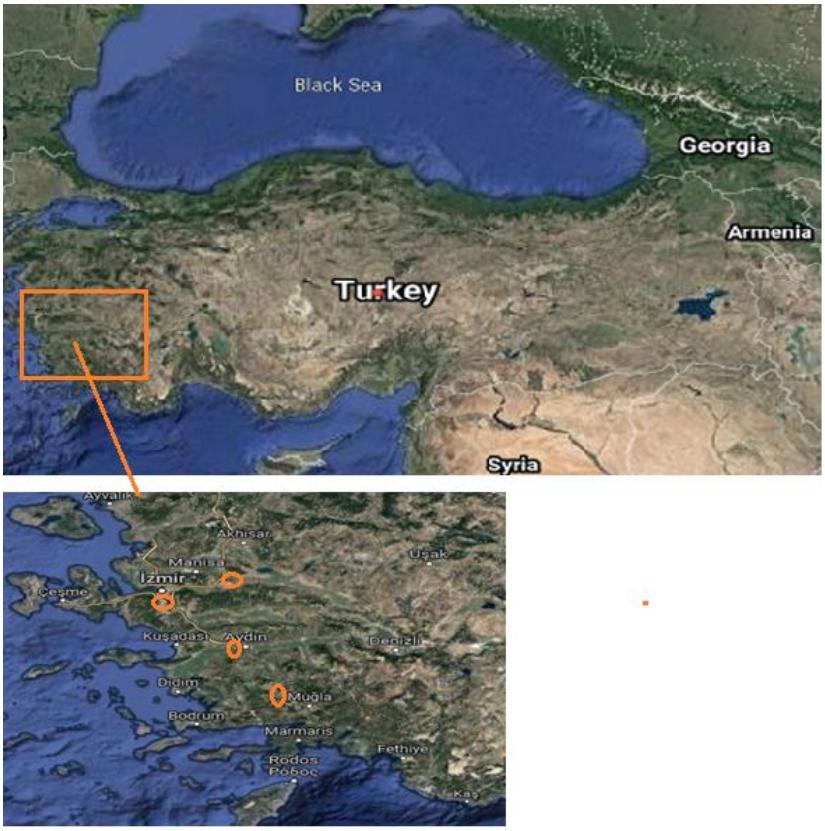

Fig. 1. Location of collecting places in the Aegean region, Turkey (https://www.google.com/maps).

PCR amplification and sequencing

The entire *psb*A-*trn*H region was amplified using the Biometra Personal Thermal Cycler. The PCR reactions were performed using *psb*A and *trn*H primers designed by Sang *et al.* (1997) and Tate and Simpson (2003) (Table 1) for amplification of the *psb*A-*trn*H region of cpDNA. The

amplification process was carried out in 25 µL of PCR reaction volume, which contained 5.0 µL master mix (0.75 U of Taq DNA polymerase, reaction buffer, 2 mM $MgCl_2$, 250 µM dNTP), 1 µL for *psb*A and 1 µL for *trn*H primers, approximately 2.0 µL of total genomic DNA, and 17 µL of ddH_2O. Gel electrophoresis in 0.8 % agarose gel run in 1.0X Tris-Borate-EDTA buffer was used to separate amplicons that were stained with ethidium bromide and visualized using a UV transilluminator (Fig. 2). Purified PCR products were sequenced at Labbiotek (İzmir, Turkey) using an ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA, USA) with a BigDye cycle sequencing kit (Applied Biosystems). DNA sequences were edited both manually and by the Sequencher 5.4.6 programs. Sequences for individual samples were obtained through at least 3 independent sequencing runs whereas sequences for each taxon were based on at least 3 independent specimens. When all the sequences of a taxon were identical, only one sequence (based on one specimen) was included in the phylogenetic analysis.

Table 1. cpDNA *psb*A-*trn*H primers and PCR amplification.

Primers	Sequences (5'-3')	PCR amplification (35 cycles)
*psb*A	5'-GTTATGCATGAACGTAATGCTC-3'	94 ºC/5 min
	(Sang *et al.* 1997)	94 ºC/45 s
*trn*H	5'-CGCGCATGGTGGATTCACAATCC-3'	50 ºC/45 s
	(Tate and Simpson 2003)	72 ºC/1 min
		72 ºC/10 min

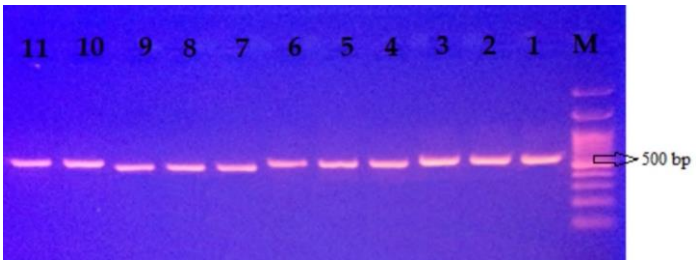

Fig. 2. Amplified cpDNA *psb*A-*trn*H region.

Aligment and phylogenetic analysis

cpDNA *psb*A-*trn*H sequences were aligned using the MEGA 6.0 alignment software (Tamura *et al.* 2013). Sequences distance values and maximum likelihood (ML) trees were created using MEGA 6.0 (Tamura *et al*. 2013). To evaluate the degree of support for given clades, a bootstrap analysis (1000 replicates) was applied (Felsenstein 1985). Additionally, *Tetradium austrosinense* (KR533457.1), *Tetradium ruticarpum* (MK419238.1), *Melicope degeneri* (EU493198.1), *Melicope polybotrya* (EU493202.1), *Clausena anisate* (MK260868.1), *Clausena lenis* (KR533453.1), *Murraya exotica* (GQ435449.1), *Murraya paniculata* (GU135341.2), *Glycosmis lucida* (KR533450.1), *Glycosmis pentaphylla* (GQ435452.1), *Zanthoxylum molle* (MF070225.1), and *Zanthoxylum bungeanum* (MF070227.1) *psb*A-*trn*H sequences were obtained from NCBI and were used to reveal phylogenetic relationships among *Citrus* species.

Results and Discussion

Phylogenetics aims to reveal the evolutionary relationship between all groups of organisms in the form of ancestor-lineage relationships and has become a powerful tool and starting point in many areas of biology such as taxonomy, biogeography, and developmental genetics (Wei *et al.* 2014; Sarıçam and Müştak 2015; Sevindik and Okan 2020). Recently, phylogenetic has get into a rapidly expanding area thanks to major improvements in nucleic acid and protein sequencing techniques and analyses (Patwardhan *et al.* 2014). The sequence analyses are very useful for phylogenetic analysis in cases when the morphological characters are insufficient for information. Sequence analysis methods are used in a variety of

fields ranging from detecting the geographic origins of the living organisms to finding molecular evidence of the phylogenies (Inal *et al.* 2017). There are many studies in *Citrus* using various molecular markers, e.g. RAPD (Coletta Filho *et al.* 1998; Nicolosi *et al.* 2000), ISSR (Fang and Roose 1997), AFLP (Al-Nadabi *et al.* 2018), SSR (Jannati *et al.* 2009), as well as chloroplast DNA (Nicolosi *et al.* 2000; Penjor *et al.* 2010; Wali *et al.* 2013; Oueslati *et al.* 2016; Uchoi *et al.* 2016; Sevindik and Yalçın 2018) and nuclear ribosomal DNA (nrDNA) markers (Amar *et al.* 2014; Sun *et al.* 2015).

In this study, we obtained *psb*A-*trn*H sequences ranging from 426 to 470 nucleotides for 11 specimens. The highest number of nucleotides was determined for *Citrus sinensis* (Aydın) (470 bp) while the lowest for *Citrus sinensis* (Bergama) (426 bp). Average nucleotide composition of *psbA-trnH* was 42.3 % T, 11.7 % C, 28.8 % A, and 17.2 % G. (Table 2).

Table 2. Length and A, T, G and C contents of cpDNA *psb*A-*trn*H sequences of *Citrus* taxa.

Taxa	***psb*A-*trn*H**	A [%]	T [%]	G [%]	C [%]
C. paradisi (Aydın)	455.0	28.8	42.0	16.9	12.3
C. limon (Aydın)	452.0	29.0	42.7	17.5	10.8
C. sinensis (Salihli)	450.0	28.7	42.4	17.1	11.8
C. sinensis (Muğla)	464.0	28.4	41.8	17.9	11.9
C. sinensis (Aydın)	470.0	28.5	41.5	17.9	12.1
C. reticulata (Bergama)	466.0	29.2	41.0	17.6	12.2
C. reticulata (Aydın)	448.0	29.5	41.5	17.6	11.4
C. aurantium (Aydın)	453.0	28.9	41.5	17.2	12.4
C. paradisi (İzmir)	434.0	28.3	43.1	16.4	12.2
C. limon (Muğla)	430.0	28.6	44.2	16.7	10.5
C. sinensis (Bergama)	426.0	28.6	43.7	16.7	11.0
Avg.	449.8	28.8	42.3	17.2	11.7

The total length of the aligned *psb*A-*trn*H sequence matrix was 483 nucleotides. The genetic distance method based on *psb*A-*trn*H set was performed with MEGA 6.0 software. The lowest sequence divergence among the ingroup taxa was 0.000 while the highest was 0.012 (Table 3).

Table 3. Pairwise sequence distances among some *Citrus* taxa for cpDNA *psb*A-*trn*H sequences using MEGA 6.0 software distance matrix.

Species	1	2	3	4	5	6	7	8	9	10	11
C. paradisi (Aydın)	-										
C. limon (Aydın)	0.007										
C. sinensis (Salihli)	0.005	0.002									
C. sinensis (Muğla)	0.005	0.002	0.000								
C. sinensis (Aydın)	0.005	0.002	0.000	0.000							
C. reticulata (Bergama)	0.012	0.010	0.007	0.007	0.007						
C. reticulata (Aydın)	0.012	0.010	0.007	0.007	0.007	0.000					
C. aurantium (Aydın)	0.012	0.010	0.007	0.007	0.007	0.000	0.000				
C. paradisi (İzmir)	0.002	0.005	0.002	0.002	0.002	0.010	0.010	0.010			
C. limon (Muğla)	0.010	0.002	0.005	0.005	0.005	0.012	0.012	0.012	0.007		
C. sinensis (Bergama)	0.005	0.002	0.000	0.000	0.000	0.007	0.007	0.007	0.002	0.005	-

Using the MEGA 6 program, Tajma's Neutrality Test (Tajima 1989) was calculated based on *psb*A-*trn*H sequences of *Citrus* species. Numbers of sequences (m) gave one segregation site (S) revealing very low nucleotide diversity (π) of 0.005690 (Table 4). Maximum likelihood (ML) tree was generated using *psb*A-*trn*H sequences of certain *Citrus* species distributed in Turkey. It consists of two large clades. Clade 1 is divided into two subclades, A and B. Subclade A consists of *C. paradisi* (Aydın), *C. paradisi* (İzmir), *C. limon* (Muğla), *C. limon* (Aydın) species, whereas all *C. sinensis* populations are placed in subclade B. Clade 2 consists of *C. reticulata* (Aydın), *C. aurantium* (Aydın) and *C. reticulata* (Bergama) species (Fig. 3).

Table 4. Tajima's Neutrality Test Values based on cpDNA *psb*A-*trn*H of date *Citrus* species.

No. of sequences "m"	No. of segregating sites "S"	*P*s=S/n	$\Theta = p_s/a_1$	nucleotide diversity '' π ''	Tajima test statistic ''D''
11	7	0.017115	0.005843	0.005690	-0.106371

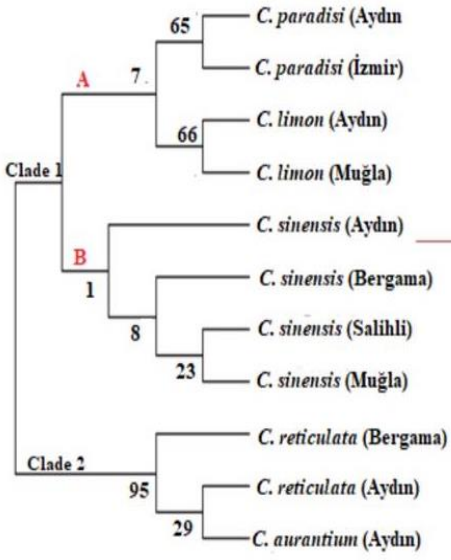

Fig. 3. The maximum likelihood tree generated using cpDNA *psb*A-*trn*H sequences.

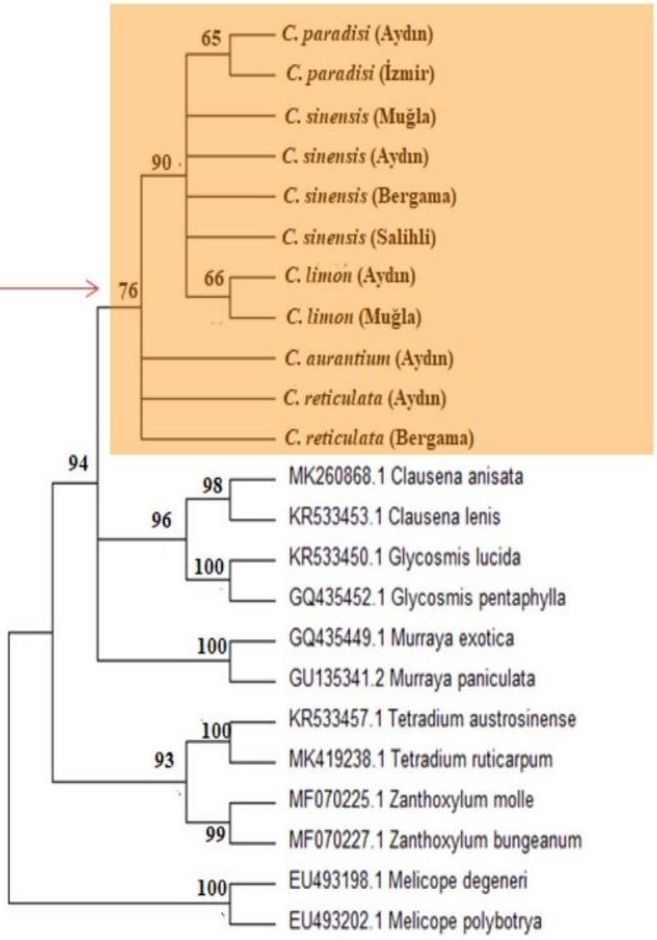

Fig. 4. The maximum likelihood tree generated using cpDNA *psb*A-*trn*H sequences and other species sequences retrieved from NCBI.

So far, many studies have been done on *Citrus* using a wide range of gene regions and markers. Wali *et al*. (2013) carried out the phylogenetic analysis of *Citrus* species using cpDNA *rps*14 genes. They situated *C. reticulata* and *C. aurantium C. sinensis* var. *malta*, and *C. sinensis* var. *fruiter* species in one branch, and *C. limon* and *C. sinensis* var. *mousami* species in another branch.

In our study, *C. reticulata* and *C. aurantium* were detected in Clade 2, whereas populations of *C. sinensis* and *C. limon* in Clade 1. Wali *et al*. (2013) concluded that *rps*14 gene sequence was highly protected in *Citrus* species, and it did not provide much information to form the phylogeny of *Citrus* species. Sevindik and Yalçın (2018) performed phylogenetic analysis of some *Citrus* species widespread in the Aegean Region based on cp(DNA) *trn*L intron and *trn*L-F sequences and they located *C. aurantium* (Aydın), *C. reticulata* (Bergama) and *C. reticulata* (Aydın) species in the same branch of the phylogenetic tree (Fig. 3). These three species were also situated in the same branch of our phylogenetic tree formed on the basis of *psb*A-*trn*H sequences. Analysis of *trn*L intron and *trn*L-F sequences revealed that *C. limon* (Aydın) and *C. paradisi* (Aydın) belong to a

subsidiary group, whereas *C. limon* (Muğla) is situated in a separate branch. Generally, according to our results, populations of the same species are found to be together. The study by Sevindik and Yalçın (2018) showed that, based on *trn*L intron analysis, *C. paradisi* (Aydın) and *C. paradisi* (İzmir) populations are located in different branches, however the results of *trn*L-F analysis indicated that these populations are in the same branch. Our analysis of *psb*A-*trn*H sequence showed that both populations are in Clade 1. In case of populations of *C. sinensis* species, the above mentioned authors reported that they belong to different branches, both in the *trn*L intron tree and in the *trn*L-F tree. In *psb*A-*trn*H tree, all populations of all *C. sinensis* species were collected in one group. According to *psb*A-*trn*H results, populations belonging to the same species form a group, while this did not happen with *trn*L intron and *trn*L-F sequences (Sevindik and Yalçın, 2018). Uchoi *et al*. (2016) analysed phylogenetically *Citrus* species distributed in India using *rbc*L and *mat*K sequences. They applied the maximum parsimony (MP) and neighbor-joining (NJ) methods. In the NJ bootstrap consensus tree formed based on *rbc*L sequence, *C. reticulata* and *C. sinensis* were grouped together, while *C. limon* and *C. aurantium* species were in different groups. In turn, in the NJ phylogenetic tree based on the *mat*K sequences, *C. aurantium* and *C. reticulate* were in one group and the species *C. sinensis* and *C. limon* were in different groups. Our research has shown that *C. reticulata* and *C. aurantium* species are located in Clade 2, which is partially consistent with the NJ tree formed with the matK sequence. Sun *et al.* (2015) investigated the taxonomy and phylogeny of 26 Citrus materials of 22 Citrus species using sequence analysis of the ITS region of nrDNA. In their research, *C. aurantium* and *C. sinensis* were found in one group, while *C. limon* belonged to a separate group. According to our results, *C. aurantium* populations belong to Clade 2 and *C. sinensis* and *C. limon* populations to Clade 1. However, the ITS sequences results obtained by Sun *et al.* (2015) are not consistent with ours. Penjor *et al*. (2010) determined the phylogenetic relations of *Citrus* and its relatives using maximum parsimony and neighbour-joining methods and cpDNA *rbc*L gene sequence. They placed *C. aurantium, C. limon, C. sinensis* and *C. paradisi* in one group. We found that *C. limon, C. sinensis* and *C. paradise* populations belong to Clade 1, while *C. aurantium* to Clade 2. The phylogenetic relationship of some species of Rutacee family (taken from NCBI) with five *Citrus* species investigated in this study is given in Fig. 4. As it is seen from the tree, *Citrus* species are separated from *Tetradium ruticarpum, Melicope degeneri, Melicope polybotrya, Clausena anisate, Clausena lenis, Murraya exotica, Murraya paniculata, Glycosmis lucida, Glycosmis pentaphylla, Zanthoxylum molle* and *Zanthoxylum bungeanum* species. Penjor *et al.* (2010) grouped *Citrus* species with *Fortunella, Poncirus, Microcitrus, Eremocitrus,* and *Clymenia*. Groppo *et al.* (2008) determined cpDNA in *rps*16 intron analysis and situated *Citrus, Poncirus* and *Microcitrus* in one group, whereas *trn*L-F analysis revealed that *Balsamocitrus, Afraegle*, *Citrus* and *Clausena* form one group.

Conclusion

In this study, phylogenetic analysis of Turkish *Citrus* L. taxa using cpDNA *psb*A-*trn*H sequences was performed to elucidate phylogenetic relationships. According to the results of the study, the separation of *Citrus* species in the phylogenetic tree obtained with *psb*A-*trn*H sequence data was realized. However, it has been found that cpDNA *psb*A-*trn*H sequence populations of species belong together. The data obtained from this study will guide phylogenetic studies on both *Citrus* and different plant species.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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