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MOLECULAR CHARACTERIZATION BASED ON CHLOROPLAST (*trnL-F*) DNA SEQUENCE OF THE APPLE GENOTYPES IN ARDAHAN/TURKEY

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Abstract

Genetic diversity among Turkish apple genotypes in Ardahan province was conducted based on cpDNA *trn*L-F sequences. Apple genotypes were plotted on a phylogenetic tree where *Pyrus x bretschneideri* was used as the outgroup. The plant samples were collected from different locations and genomic DNA was isolated from healthy and green leaves. For sequence in *trn*L-F region *trn*Le and *trn*Ff primers were used. Later obtained DNA sequences were edited using the BioEdit and FinchTV. Sequencing data were analyzed using MEGA 6.0 software. Neighbor joining and bootstrap trees were constructed in order to verify the relationships among the apple genotypes. Phylogenetic tree consisted of two clades. The divergence values of *trn*L-F sequences differed between 0.000 and 0.005. Average nucleotide composition was 38.3 T, 14.9 C, 31.9 A and 14.9% G. The phylogenetic tree constructed based on *trn*L-F region sequences was nearly parallel to prior phylogenetic studies on apple genotypes.

Introduction

Anatolia is accepted as the homeland of many wild, wild edible and cultivated fruit species in addition to harboring rich apple genetic resources including mostly historical cultivars selected by humans among wild populations for centuries (Çelik *et al.* 2018). Apple (*Malus* L.) belongs to Rosaceae is a widespread genus in the world with many species (Dziubiak 2004, Ercisli 2004). Four origin centers for apples were reported, including East Asia, Central Asia, East Asia-Europe and North America. Turkey belongs to East Asia-Europe origin center and has considerable diversity (Uzun *et al.* 2016). Apple can easily grow in many places due to the wide area and the climate tolerance (Bulantekin and Kuşcu 2017) and a good source of phenolic compounds and antioxidants for human diet and health (Wolfe *et al.* 2003, Wolfe and Liu 2003). The preference criteria that consumers pay the greatest attention are its bright and red colour. Under the light of previous studies, it was detected that red vegetables and fruits have more antioxidants compared to green ones. It was also detected that in addition to having more antioxidants, red fruits also have more phenolic compounds (Neill *et al.* 2002a,b, Neill and Gould 2003, Hughes *et al.* 2005, Shao *et al.* 2007).

Genetic markers give an insight in the amount and distribution of genetic variation within populations (Bolovic *et al.* 2013). By using DNA sequence data, differences and the genetic relationships of living things have been reported through the latest developments in molecular biology (Weiguo *et al.* 2005). Chloroplast DNA (cpDNA) sequence variations have been widely utilized for detecting species relationships between angiosperms and other plants. Hence, it has been widely used in phylogenetic assessments. Low rate of change of these molecules is restrictive in in-species degree (Taberlat *et al.* 1991, Türktaş *et al.* 2012). Chloroplast DNA (cpDNA) has many useful noncoding regions in terms of phylogenetics. Due to its phylogenetic

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value, the region of tRNA (*trn*T - *trn*F) has been extensively studied. Its intergenic space takes place between *trn*L (UAA) 3'exon and *trn*F (GAA) gene (Taberlat *et al.* 1991, Gielly and Taberlat 1994, Gielly and Taberlat 1996, Liu *et al*. 2005). There are two protected exon areas in *trn*L gene (Fig. 1).The aim of this study was to conduct molecular characterization of local apple cultivars, which are distributed in the ecological conditions of Ardahan, through the use of *trn*L-F region in chloroplast DNA.

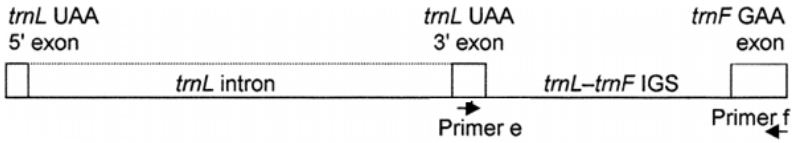

Fig. 1. cpDNA*trn*L-F region (Pirie *et.al.* 2007).

Materials and Methods

Apple genotypes used in the study were collected from certain regions in Ardahan/Turkey between July and August, 2015 (Fig. 2). Total genomic DNA samples were extracted using DNA Plant Kit (Gene Mark). The genomic DNA samples were stored at –20 °C.

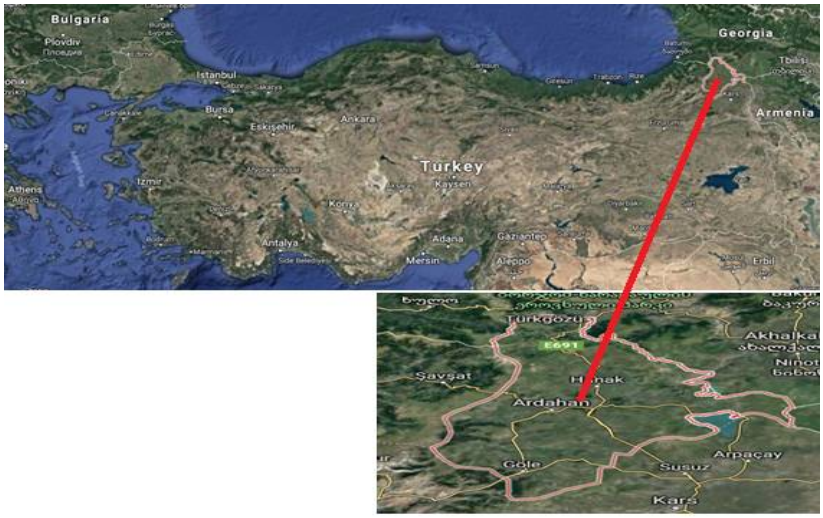

Fig. 2. Location of the Ardahan province. The upper map shows the location of Ardahan within Turkey while the enlarged region of Ardahan is shown in the lower map. The maps were obtained from Google Maps (maps.google.com).

Double-stranded DNA of the complete *trn*L-F regions in each genomic DNA sample were amplified. Amplification of the whole *trn*L-F molecular marker analyzed in this study belongs to the cpDNA. PCR amplifications of *trn*L-F cpDNA were performed using the primers designed by Taberlet *et al.* (1991) (Table 1) for all taxa included in this work. The amplification process was carried out in 25 µl of PCR reaction volume. Each PCR reaction contained 2.0 µl of total genomic DNA, 15 µl of dd H_2O, 5 µl master mix (PCR buffer, $MgCl_2$, dNTP, Taq DNA polymerase), 1 µl for *trn*Le and 1 µl for *trn*Ff primers and 1 µl of DMSO. Table 2 shows *trn*L-F PCR cycles with their respective conditions. Gel electrophoresis in 0.8% agarose gel run in 1.0X TBE (Tris-Boric EDTA) buffer was used to size fraction at amplicons. Subsequently gels were stained with ethidium bromide and visualized over a UV trans-illuminator. The *trn*Le and *trn*Ff primers were

used both for amplification and for sequencing which were conducted at Labbiotek (İzmir, Turkey) using an *ABI*3130XL genetic analyzer. For each sample, forward and reverse sequencing reactions were performed and the sequences were analyzed via GenBank (NCBI) through BLASTn search. Subsequently obtained DNA sequences were edited both manually and by using BioEdit (Hall 1999), and FinchTV programs.

Table 1. cpDNA *trn*L-F primers used in this study with their designers.

Primer name	5' to 3' primer sequence	Based on (the source publication)
Forward *trn*Le	GGTTCAAGTCCCTCTATCCC	Taberlet *et al.* 1991
Reverse *trn*Ff	ATTTGAACTGGTGACACGAG	Taberlet *et al.* 1991

Table 2. Cycles and conditions of *trn*L-F -PCR reactions.

Pre-heating	94°C	5 min	1 cycle
1. step	94°C	30 s	
2. step	50°C	30 s	
3. step	72°C	90 s	35 cycles
4. step	72°C	8 min	1 cycle
5. step	4°C	20 min	

cpDNA *trn*L-F sequences were aligned using ClustalW alignment software (Thompson *et al.* 1994). The phylogenetic tree was generated using the Neighbor joining tree (Saitou and Nei 1987) and constructed using MEGA 6.0 software (Tamura *et al.* 2013). The phylogenetic tree was evaluated with bootstrap test with 1000 resamplings (Felsenstein 1985). Beside *Pyrus* × *bretschneideri* (GenBank: JX122456.1) taxa was used out group in the tree obtained from NCBI.

Results and Discussion

*trn*L-F sequences ranged from 418 to 428 nucleotides among 13 genotypes (only apple genotypes and except for outgroup *Pyrus* × *bretschneideri*). The highest number of nucleotides for the *trn*L-F sequence was observed in Uruset apple and Kırmızı Safran (428 bases) while the lowest number of nucleotides for the *trn*L-F sequence was observed in Sobe apple (418 bases). Average nucleotide composition of *trn*L-F was 38.3 T, 14.9 C, 31.9 A and 14.9% G. The maximum GC content (30.2%) and the lowest AT content (69.9%) were observed in Kırmızı Safran and Uruset apple while the lowest GC content (29.3%) and the highest AT content (70.7%) were recorded in Limon apple (Table 3). Genetic distance method based on *trn*L-F set was performed with MEGA 6.0 software. The lowest distance was 0.000 while the highest distance was 0.005 (Table 4).

Neighbor joining tree was construted using *trn*L-F sequences of apple genotypes distributed in Ardahan province, and sequences of outgroup was retrieved from NCBI (GenBank). Neighbor joining tree generated based on *trn*L-F sequences of the samples consists of two large clades. Clade 1 consists of Sisli Uruset, Sobe apple, Sarı Safran, Paşa apple, Şah apple, Uruset apple, Kırmızı safran, Karpuz apple, and Limon apple genotypes. (Fig. 3). Sevindik *et al.* (2018) reported the genetic diversity of Ardahan apple genotypes using ISSR markers. In their study, Sobe apple, Kırmızı safran, Limon apple, Uruset apple, Şah apple and Sisli uruset apple genotypes were found to be in the same clade. Uzun *et al.* (2016) studied the genetic relationships of Turkish apple genotypes using ISSR markers. In their study, Karanfil apple, Şah apple and Paşa apple genotypes were found to be in the same group, while Kaba apple was found in a different group. In the present

study, these two genotypes (Şah apple and Paşa apple) were found in the clade 1, while Kaba apple and Karanfil apple were found in clade 2. Osmanoğlu (2008) revealed genetic relationships of apple genotypes collected from Ardahan/Posof region using RAPD markers. As a result of their study, Sobe apple, Sarı Safran, Kırmızı safran, Uruset apple, Kaba uruset and Paşa apple genotypes were grouped in the same clade, but Limon apple genotype was in a different clade. In clade 2 consist of

Table 3. Length and A+T and G+C contents of cpDNA *trn*L-F sequences of apple genotypes.

Genotypes	cpDNA *trn*L-F (bp)	A (%) content	T (%) content	G (%) content	C (%) content	A+T (%) content	G+C(%) content
Kaba apple	424	31.8	38.4	14.9	14.9	70.2	29.8
Kaba Uruset	423	31.7	38.5	14.7	15.1	70.2	29.8
Kaburga apple	427	32.6	37.9	14.5	15.0	70.5	29.5
Karanfil apple	426	31.9	38.0	15.0	15.0	69.9	30
Karpuz apple	427	31.6	38.4	15.0	15.0	70	30
Kırmızı Safran	428	31.8	38.1	15.0	15.2	69.9	30.2
Limon apple	420	31.9	38.8	14.5	14.8	70.7	29.3
Paşa apple	426	31.9	38.3	15.0	14.8	70.2	29.8
Şah apple	424	31.8	38.2	15.1	14.9	70	30
Sarı Safran	424	31.8	38.4	15.1	14.6	70.2	29.7
Sisli Uruset	426	31.9	38.3	15.0	14.8	70.2	29.8
Sobe apple	418	31.8	38.8	14.6	14.8	70.6	29.4
Uruset apple	428	31.8	38.1	15.0	15.2	69.9	30.2
Average	424.7	31.9	38.3	14.9	14.9	70.2	29.8

Karanfil apple, Kaburga apple, Kaba apple and Kaba Uruset genotypes formed a group, (Fig. 3). Sevindik *et al.* (2018) identified Kaburga apple and Kaba apple genotypes in a clade while Karanfil apple genotype was determined different clade. Osmanoğlu (2008) identified Kaburga apple, and Kaba Uruset genotypes in one clade, and Kaba apple genotype in another clade. In the phylogenetic tree, *Pyrus x bretschneideri* species selected as outgroup constituted a separate group from apple genotypes (Fig. 3). There are several molecular studies among the previous studies carried out with the use of Apple genotypes. Markers such as RAPD, SSR, nrDNA ITS, cpDNA *mat*K, *atp*B, and *atp*B-*rbc*L were used in these studies (Savolainen *et al.* 1995, Hokanson *et al.* 1998, Robinson *et al.* 2001, Kaya *et al.* 2015, Mahmood *et al.* 2016, Daler *et al.* 2017). Daler *et al.* (2017) specified the relationship degree of genotypes using RAPD molecular technique on 6 apple genotypes obtained from Isparta/Eğirdir Fruit Research Institute Directorate's collection. Kaya *et al.* (2015) carried out molecular analysis of Apple genotypes collected from Van province, through using RAPD markers. Creating a dendrogram, the similarity index between genotypes was revealed. Robinson *et al.* (2001) revealed the phylogenetic relationships between apple species by carrying out sequence analysis of ITS of seed DNA and *mat*K areas of chloroplast DNA 29 apple species. Mahmood *et al.* (2016) revealed genetic relationships of varieties by carrying out sequence analysis of apple varieties' *atp*B gene areas, which they chose from Pakistan. Savolainen *et al.* (1995) revealed chloroplast variations by carrying ou t *atp*B-*rbc*L

Table 4. Pairwise sequence distances among some apple genotypes for cpDNA *trn*L - F data using MEGA 6.0 software distance matrix.

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Kaba apple														
Kaba Uruset	0,000													
Kaburga apple	0,000	0,000												
Karanfil apple	0,000	0,000	0,000											
Karpuz apple	0,000	0,000	0,000	0,000										
Kırmız Safran	0,000	0,000	0,000	0,000	0,000									
Limon apple	0,000	0,000	0,000	0,000	0,000	0,000								
Paşa apple	0,000	0,000	0,000	0,000	0,000	0,000	0,000							
Şah apple	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000						
Sarı safran	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000					
Sisli Uruset	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000				
Sobe apple	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000			
Uruset apple	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000		
Pyrus × *bretschneideri*	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	-

sequence analysis of 55 apple cultivars, and 15 of them were wild apples. Hokanson *et al.* (1998) explained genetic relationships of *Malus* × *domestica* populations by using SSR (microsatellite) markers. In conclusion, this study has revealed molecular characterization and genetic relationships of apple genotypes distributed in Ardahan province by using chloroplast

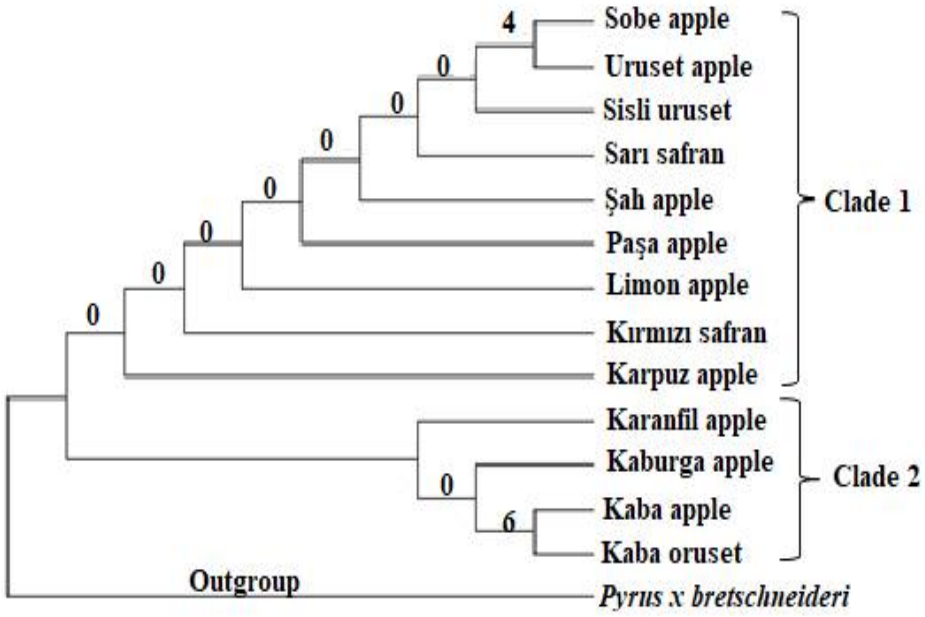

Fig. 3. The Neighbor joining tree with bootstrap values (1000 replicates) generated using cpDNA *trn*L-F sequences of apple genotypes and therelated sequence retrieved from NCBI GenBank (*Pyrus x bretschneideri* (GenBank: JX122456.1).

*trn*L-F sequences. The obtained findings can be a reference for molecular studies with different markers and different gene sequences to be carried out in the future. The results may also be utilized for the correct identification of the apple genotypes studied along with their close relatives.

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