

Sequence Analysis of nrDNA ITS Region in Paşa and Şah Apple (*Malus × domestica*) Genotypes

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RESEARCH ARTICLE

Abstract

In this study, phylogenetic analysis of Şah and Paşa apple genotypes was performed using nrDNA ITS sequences. After the plant leaves were brought to the laboratory, gDNAs were obtained by genomic DNA isolation method. PCR amplification was performed using primers ITS4 and ITS5A. ITS sequences of some apple and Rosaceae species were retrieved from NCBI, bioinformatics analyzes were made with Bioedit 7.2.3, Finch TV 1.4.0 and MEGA 6.0 programs and phylogenetic trees were constructed. In the study, ITS sequence length of the Paşa apple genotype was 656 bp, and the ITS sequence length of the Şah apple genotype was 649 bp. Only in the maximum likelihood phylogenetic tree constructed using sequences of apple genotypes, Paşa and Şah apple genotypes appeared in the same clade as *Malus domestica* cultivar="Casciana" and *Malus domestica* cultivar="Rotella". In the phylogenetic tree generated including other species belonging to the Rosaceae family; apple genotypes *Pyrus*, *Cotoneaster*, *Crataegus*, *Sorbus*, *Eriobotrya* and *Prunus* were detected in a same clade. Overall results clearly suggested that the ITS sequences were both suitable for differentiation between the selected genera and were compatible with previous phylogenetic studies.

Keywords: Apple, ITS, phylogenetic, sequences, Türkiye

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INTRODUCTION

Rosaceae family members are widely grown worldwide and include plants of economic importance, famous fresh and dried fruits with important varieties (Pan et al., 2020). Rosaceae family contains economically important fruit trees such as almonds, peaches, plums, apples, pears, roses, strawberries, and raspberries (Jiang et al., 2019; Costa and Mafra, 2022). The genus *Malus* belongs to the Rosaceae family and includes about 35 species all over the world (Li et al., 2020). Plants of this genus are of great economic importance, notably the domesticated apple (*Malus domestica* Borkh.) and some horticultural ornamental plants (eg *M. halliana* Koehne, *M. hupehensis* (Pamp.) Rehder and *M. micromalus* Makino) (Kalkman, 2004; Liu et al., 2022). Apple (*Malus × domestica*) is the main fruit crop of temperate regions all over the world. Türkiye, one of the leading apple producers is among the apple spreading areas (Ercisli, 2004; Balta et al., 2022; Mignard et al., 2022). World apple production is approximately 86.5 million tons and Türkiye ranks 3rd in the world with its production exceeding 4.3 million tons (FAO, 2020). According to the data of 2021, in Türkiye, 4.493,264 tons of apple production was realized with a total apple production of 2.670,318 tons, approximately 60% of this production being in Isparta (1.130,134 tons), Niğde (552.617 tons), Karaman (535.350 tons), and Antalya (451.927 tons) (TÜİK, 2021). Apple, has beneficial properties for human health as one of the frequently

consumed fruits, is known as a source of micronutrients such as dietary fiber, vitamin C, monosaccharides, organic acids, minerals, phenolic compounds and phytochemical compounds (Wu et al., 2007; Özdemir et al., 2009; Gokdogan and Baran, 2017; Mignard et al., 2022). Using molecular markers representing genome level differences has further advantages over the traditional phenotypic method that they are stable and can be detected in all tissues of the plant regardless of growth, development, differentiation or environment (Agarwal et al., 2008; Devi et al., 2022). nrDNA ITS (internal transcribed spacer) includes ITS1, 5.8S and ITS2 regions of plants. The rapid rate of evolution, high variability and bi-parental inheritances make it suitable for ITS region for genetic diversity analysis at species levels (Li et al., 2020). In addition, ITS genes are considered to be the most successful primers in plant DNA barcoding. In the past, ITS regions have been used in barcode studies of many plant species (Wang et al., 2013; Olivar et al., 2014; Castro et al., 2015; Liu et al., 2017; Hürkan, 2020; Ralte and Singh, 2021). In this study, using nrDNA ITS sequences, phylogenetic analysis of Şah and Paşa apple genotypes were conducted.

MATERIALS AND METHODS

Plant material and DNA extraction

In the study, Şah and Paşa apple genotypes were collected from certain regions in Ardahan/Türkiye. The green leaves of the collected samples were brought to the Plant Biotechnology laboratory and prepared for total genomic (gDNA) isolation. Total genomic DNA samples were extracted using the DNA plant kit (GeneMark, catalog no: DP022).

PCR and sequences analyses

PCR reactions were carried out using gDNAs with ITS4 (White et al., 1990) and ITS5A (Stanford et al., 2000) primers. Primer sequences, PCR components and PCR program are given in Table 1.

Table 1. nrDNA ITS primer sequences, PCR components and protocol

Primers	DNA Sequences (5'-3')	PCR components	PCR Amplification (35 Cycles except final extension step)
ITS5A (Forward)	5'-CCTTATCATTTAGAGGAAGGAG-3'	1 µL genomic DNA 1 µL primer (ITS4 and ITS5), 10 µL master mix (PCR buffer, 2 Mm MgCl ₂ , dNTP, 0.75 U Taq DNA polymerase) and 7 µL dH ₂ O	94 °C / 5 min 94 °C / 45 sec 50 °C / 45 sec 72 °C / 1 min 72 °C / 10 min (final extension: 1 cycle)
ITS4 (Reverse)	5'-TCCTCCGCTTATTGATATGC-3'		

PCR products were also run by electrophoresis on a 0.8% agarose gel. After the ITS regions were amplified, the PCR reactions were sent to Triogen biotechnology (Istanbul/Türkiye) with ITS4 and ITS5A primers for sequence analysis. Contigs were made and analyzed from forward and reverse sequences using Bioedit 7.2.3 (Hall, 1999) and Finch TV 1.4.0 programs. Subsequently, the contig sequence was blasted in NCBI. By using MEGA 6.0 (Tamura et al., 2013) software, both ITS sequences of apple genotypes and ITS sequences of some species belonging to Rosaceae family were taken from NCBI and phylogenetic trees were obtained with the maximum likelihood (Tamura Nei model) algorithm to evaluate the degree of support for given clades, a bootstrap analysis (1000 replicates) was also applied (Felsenstein, 1985). In addition, genetic distance matrices between other apple genotypes were calculated by MEGA 6.0 program.

RESULTS AND DISCUSSIONS

nrDNA ITS gene regions are frequently used in plant systematics to detect and document phylogenetic relationships (Yeşiltaş and Kolören, 2019). In the past, the ITS region has been used in phylogenetic studies of species belonging to the Rosaceae family (Potter et al., 2000; Bortiri et al., 2001; Yang and Pak, 2006; Faghir et al., 2014; Li et al., 2014; Liu et al., 2020). In this study, the ITS sequence length of the Paşa apple genotype was determined as 656 bp, and Şah apple genotype sequence length was determined 649 bp. Genotypes were uploaded to NCBI. Accession numbers: Paşa apple: OR704536, Şah apple: OR704543. To understand the phylogenetic relationship between Şah and Paşa genotypes and some apple genotypes, ITS sequences were retrieved from NCBI database for *Malus domestica* cultivar="Rotella (MH633851.1), *Malus domestica* cultivar="Casciana (MH633847.1), *Malus domestica* cultivar="Rotella (MH633854), *Malus domestica* cultivar="Casciana (MH633843.1), *Malus*

domestica cultivar="Casciana (MH633848.1), *Malus domestica* cultivar="Bramley's Seedling (AF186479.1), *Malus domestica* cultivar Leathercoat (AF186477.1), *Malus domestica* cultivar Reinette (AF186478.1) *Malus* × *domestica* cultivar Ralls (EU150074.1), and *Malus domestica* cultivar Autumn Pearmain (AF186481.1), and a maximum likelihood phylogenetic tree was constructed. The phylogenetic tree consists of 2 clades (Figure 1). Clade 1 consisted of Casciana, Rotella cultivars and Şah and Paşa apple genotypes, and this group received a bootstrap value of 99%. Clade 2 consists of the cultivars Bramleys, Ralls, Autumn Pearmain, Leathercoat, and Reinette.

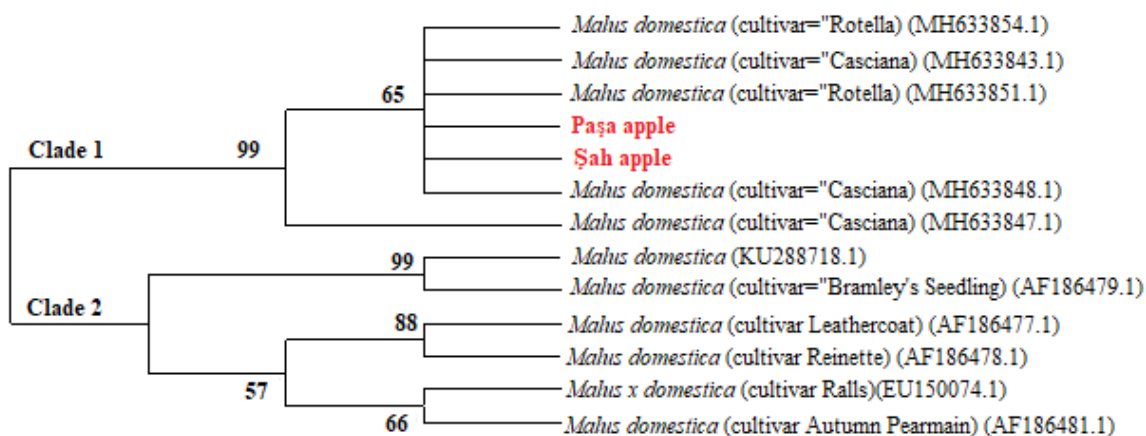


Figure 1. The maximum likelihood (Tamura-Nei model) tree with bootstrap values (1000 replicates) generated using ITS sequences of some apple genotypes

According to the genetic distance method based on ITS sequences, the smallest distance was determined as 0.000, while the highest distance was determined as 0.037 (Table 2). Uzun et al. (2016), examined the genetic relationships of Turkish apple genotypes using ISSR markers. In their study, it was determined that the Şah apple and Paşa apple genotypes were in the same group. Sevindik et al. (2018), revealed the genetic diversity of apple genotypes with the ISSR marker technique. In the UPGMA dendrogram, they determined that the Paşa and Şah apple genotypes were related to each other within the same clade. Sevindik et al. (2019) determined the molecular characterization of apple genotypes with cpDNA *trnL-F* sequences, and they found that the Paşa and Şah genotypes were in the same group.

Table 2. Pairwise genetic distance (Tamura-Nei model) matrix obtained from nuclear ribosomal DNA internal transcribed spacer sequences (together with other *Malus domestica* cultivars)

Paşa apple	-	-	-	-	-	-	-	-	-	-	-	-
Şah apple	0.000	-	-	-	-	-	-	-	-	-	-	-
<i>Malus domestica</i> (USA)	0.026	0.026	-	-	-	-	-	-	-	-	-	-
<i>Malus domestica</i> (cultivar Rotella)	0.002	0.002	0.028	-	-	-	-	-	-	-	-	-
<i>Malus domestica</i> (cultivar Casciana)	0.003	0.003	0.026	0.005	-	-	-	-	-	-	-	-
<i>Malus domestica</i> (cultivar Rotella)	0.005	0.005	0.032	0.007	0.009	-	-	-	-	-	-	-
<i>Malus domestica</i> (cultivar Casciana)	0.005	0.005	0.032	0.007	0.009	0.010	-	-	-	-	-	-
<i>Malus domestica</i> (cultivarCasciana)	0.007	0.007	0.033	0.009	0.010	0.012	0.012	-	-	-	-	-
<i>Malus domestica</i> (cultivar Bramleys)	0.026	0.026	0.000	0.028	0.026	0.032	0.032	0.033	-	-	-	-
<i>Malus domestica</i> (cultivar Leathercoat)	0.030	0.030	0.017	0.032	0.033	0.035	0.035	0.037	0.017	-	-	-
<i>Malus domestica</i> (cultivar Reinette)	0.028	0.028	0.016	0.030	0.032	0.033	0.033	0.035	0.016	0.012	-	-
<i>Malus x domestica</i> (cultivar Ralls)	0.024	0.024	0.014	0.026	0.028	0.030	0.030	0.031	0.014	0.016	0.014	-
<i>Malus domestica</i> (cultivar Autumn Pearmain)	0.021	0.021	0.010	0.023	0.025	0.026	0.026	0.028	0.010	0.012	0.010	0.007

In addition, in our study, ITS sequences of the taxa from Rosaceae family obtained from NCBI: *Pyrus pyrifolia* (KC895397.1), *Pyrus ussuriensis* (EU150070.1), *Sorbus americana* (FJ810037.1), *Sorbus aucuparia* (KY661717.1), *Cotoneaster melanocarpus* (JQ392405.1), *Cotoneaster salicifolius* (JQ392378.1), *Prunus spinosa* (EU669100.1), *Prunus armeniaca* (HF969271.1), *Eriobotrya japonica* (KJ170775.1), *Eriobotrya cavaleriei* (KJ170784.1), *Potentilla anatolica* (KT985669.1), *Potentilla kotschyana* (KT985720.1), *Rosa arvensis*

(KM353028.1), *Rosa canina* (KM353044.1), *Rubus saxatilis* (AF055747.1), *Rubus idaeus* (AF055755.1), *Crataegus maximowiczii* (EU683914.1), *Crataegus heldreichii* (EU500465.1), *Alchemilla faroensis* (EU072527.1), *Alchemilla vulgaris* (EU072573.1), *Fragaria vesca* (AF163510.1), *Fragaria* × *ananassa* (AF163494.1) were used to construct a maximum likelihood phylogenetic tree (Figure 2).

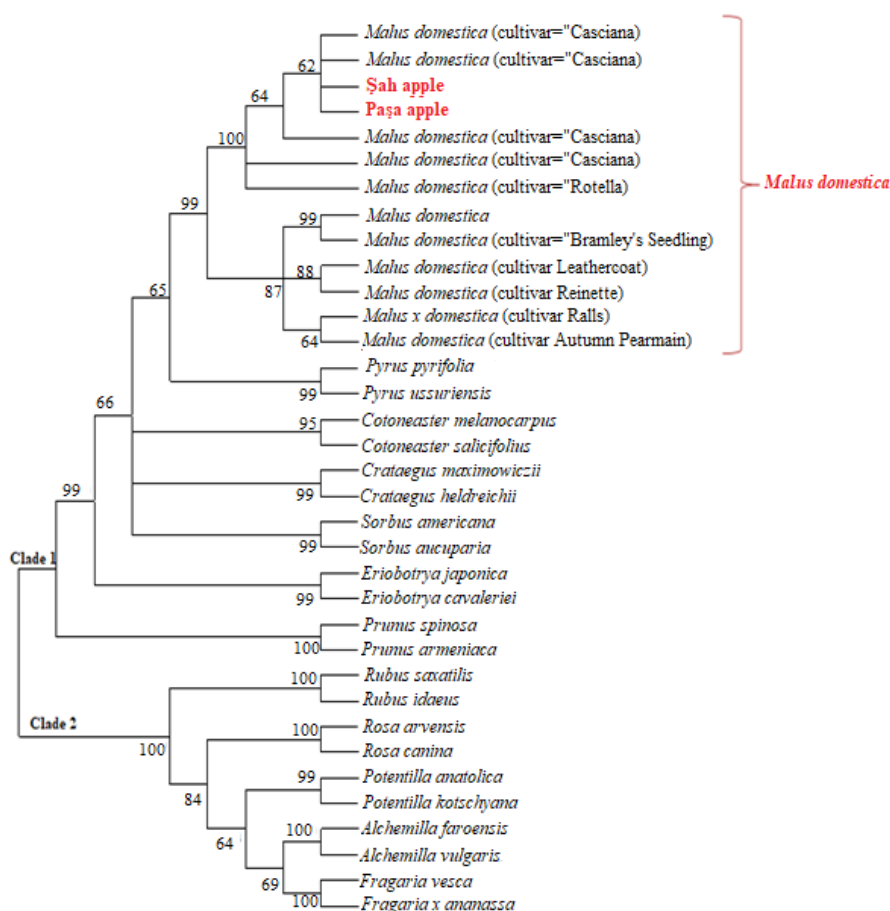


Figure 2. The maximum likelihood (Tamura-Nei model) tree generated using ITS sequences and other species sequences retrieved from NCBI

Apple genotypes, *Pyrus*, *Cotoneaster*, *Crataegus*, *Sorbus*, *Eriobotrya* and *Prunus* species detected in a same clade. The nrDNA ITS results showed discrimination at the genus level. Verbylaitė et al. (2006) determined *Malus* species together with *Pyrus*, *Kageneckia*, *Pyracantha*, *Cydonia*, *Sorbus*, and *Photinia* species using cpDNA *trnL-trnF* analyses. Sun et al. (2018), in their phylogenetic analysis, used 15 chloroplast gene regions, and *Malus*, *Docyniopsi*, *Docynia*, and *Eriolobus* were identified together. Sevindik and Murathan (2023) with cpDNA *rbcl* sequences, and Sevindik et al. (2023) with cpDNA *trnL* intron and *trnL-F* sequences detected *Malus* species together with *Cotoneaster*, *Sorbus*, *Prunus*, and *Pyrus*. Our results were consistent with previous cpDNA results. According to Judd et al. (1999), a combination of nuclear and chloroplast sequence data can provide complementary insights into the phylogenetic relationships of a genus.

CONCLUSIONS

As a result, in phylogenetic trees with ITS sequences, Paşa and Şah apple genotypes were detected together with *Malus domestica* cultivar="Casciana" and *Malus domestica* cultivar="Rotella", *Pyrus*, *Cotoneaster*, *Crataegus*, *Sorbus*, *Eriobotrya* and *Prunus* species in the same clade. ITS sequences were both suitable for differentiation between genera and were compatible with previous phylogenetic studies.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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