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Phylogenetic analysis using SCoT markers and chloroplast *trn*L intron in some *Eriobotrya japonica* (Thunb.) Lindl. (Rosaceae) populations from the Aegean region of Turkey

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Abstract

In this study, phylogenetic analyses of some *Eriobotrya japonica* (Thunb.) Lindl. populations based on SCoT marker technique and cpDNA *trn*L intron sequence analysis were performed. Specimens from the populations were collected from the Aegean region of Turkey and brought to the laboratory for genomic DNA isolation. To determine the phylogenetic analysis of *Eriobotrya japonica* populations, seven SCoT primers were used. In the SCoT analysis, a total of 54 bands were obtained, 49 of which were polymorphic and 5 were monomorphic. In chloroplast *trn*L intron region, *trn*C, *trn*D primers were used for PCR amplification. *Eriobotrya japonica trn*L intron sequence lengths were determined to be between 530 and 547 nucleotides. The average nucleotide rate detected as 38.9% for thymine, 18.1% for cytosine, 28.2% for adenine and 14.8% for guanine. Rosaceae family *trn*L intron sequences belonging to *Rhaphiolepis* Lindl., *Heteromeles* M.Roem., *Photinia* Lindl. *Cotoneaster* Medik, *Sorbus* L., *Malus* Miller, *Pyrus* L., *Prunus* L. and *Rosa* L. were retrieved from NCBI and their phylogenetic relationship with *Eriobotrya japonica* populations were revealed. As a result of the study, polymorphism rate was determined as 90.74% in SCoT analysis. According to cpDNA *trn*L intron results, *Eriobotrya japonica* populations were identified in the same group with *Rhaphiolepis indica*, a finding supported by past phylogenetic analyzes.

Keywords: *Eriobotrya japonica;* phylogenetic analysis; SCoT; *trn*L intron

Introduction

Fruits are rich sources of vitamins, bioactive compounds, minerals, fiber and secondary metabolites including various classes of antioxidants with potential health benefits, and have an important role in human nutrition (Sagdic *et al.*, 2022; Bozhuyuk, 2022; Karanjalker and Begane, 2016). Rosaceae family is economically important, among the dicotyledonous plant groups, includes various fruit and ornamental plants, and various members of the family have been used for a long time as food, medicinal and decorative plants (Liu *et al.*, 2020; Eroğul and Oğuz, 2018; Filyushin and Boris, 2017). This family consists of 2825-3500 species from 95-125 genera, mostly distributed in temperate regions of the northern hemisphere (Chen *et al.,* 2020a). *Eriobotrya*

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Lindl. belongs to the family Rosaceae (Xinaghui *et al*., 2012; Kottaimuthu and Basu, 2021). This genus, with about 30 species, consists of white-flowered evergreen shrubs and small to medium trees that blossom in autumn to early winter and yield mature fruits in spring (Chen *et al.,* 2020b; Idrees *et al.,* 2022). *Eriobotrya japonica* (Thunb.) Lindl. (Loquat) is a fruit tree found in subtropical, Asian, European, American and Mediterranean climate zones. Its fruits benefit human health due to their high nutritional value and medicinal applications and are preferred by many for their excellent taste (Shen *et al*., 2016; Lin *et al*., 2021). Also, its leaves and fruits are traditionally used to treat cough and phlegm, and its flowers are an excellent source of honey (He *et al*., 2011).

Traditional DNA markers have many applications in plant genetic diversity, taxonomy, conservation, phylogeny and ecological research. These markers include ISSR, AFLP, SNP, SSR and SRAP (Zhang *et al*., 2015; Ertuş *et al*., 2016; Agarwal *et al*., 2019). Start codon targeted (SCoT) marker was developed by Collard and Mackill (2009) recently and it has emerged as a promising marker for assessing the genetic diversity of plants (Singh *et al*., 2017). As a simple and new marker system, SCoT has been developed based on the short-conserved region surrounding the start codon (ATG) in plant genes (Collard and Mackill, 2009). SCoT technique is more directly used in constructing marker-assisted breeding programs than RAPD, ISSR, and SSR (Rayan and Osman, 2019). This technique is widely used in genetic diversity studies, phylogenetic analyzes and marker assisted breeding of many plants (Gao *et al*., 2014; Singh *et al*., 2017; Feng *et al*., 2018; Yue *et al*., 2019; Agarwal *et al*., 2019). The chloroplast has its own genome in plants (Deng *et al*., 2020). The chloroplast genome (cpDNA) consists of double-stranded DNA with a length ranging from 120 to 220 kb, encoding proteins that are key to photosynthesis and other metabolic processes. In chloroplast DNA, coding region and the non-coding region differences allow molecular systematic studies on plants at different levels (Tyagi *et al.,* 2020; Li *et al*., 2021). The cpDNA *trn*L intron was first used by Taberlet *et al.* (1991) for plant molecular studies, plant systematics, and phylogenetic analyzes at the intraspecies and interspecies levels (Drabkova *et al.,* 2004; Sarra *et al.,* 2015; Özdemir *et al.,* 2020).

In this study, phylogenetic analyzes of some *Eriobotrya japonica* populations based on the SCoT marker technique and chloroplast DNA *trn*L intron sequences were performed. In addition, *Rhaphiolepis indica* (DQ863246.1, MG202112.1), *Heteromeles arbutifolia* (DQ863236.1, JQ405573.1), *Photinia serratifolia* (MG202121.1), *Photinia glabra* (MG202117.1), *Photinia prunifolia* (JN676070.1), *Cotoneaster horizontalis* (JQ405545.1), *Cotoneaster silvestrii* (JQ405549.1), *Cotoneaster microphyllus* (JQ405555.1), *Cotoneaster salicifolius* (JQ405569.1), *Sorbus aria* (KF718328.1), *Sorbus chamaemespilus* (KF718341.1), *Malus baccata* (MG773083.1), *Malus sieversii* (LR588530.1), *Pyrus pyrifolia* (KF486723.1), *Pyrus betulifolia* (KF486746.1), *Prunus fasciculata* (JX414446.1), *Prunus armeniaca* (KF718336.1), *Prunus domestica* (GQ179673.1), *Rosa sericea* (KF851077.1) and *Rosa alba* (DQ778824.1) were compared to *Eriobotrya japonica* with respect to *trn*L intron sequences, after retrieving their sequence data from NCBI (*https://www.ncbi.nlm.nih.gov/*) nucleotide database.

Materials and Methods

Plant samples, genomic DNA isolation and Polymerase Chain Reaction

Eriobotrya japonica populations from Muğla, Aydın, Denizli, Manisa and Uşak provinces of the Aegean region were collected. For genomic DNA isolation, a commercial kit (GeneMark Catalog No: DP022) was used. The obtained gDNA samples were stored at −20 °C. PCR amplifications were performed with primers from selected SCoT and chloroplast (cpDNA) *trn*L intron region. Sequences of the primers and the PCR protocol are given in Table 1 and Table 2, respectdively. PCR applications were made in a gradient thermocycler device. For SCoT, 2 µL genomic DNA, 1.0 µL primer and 10 µL 2X master mix (Catalog No: G-

5000N) and 7 µL dH_2O were added into the PCR tube. For *trn*L intron, 2 µL genomic DNA, 1.0 µL primers (*trn*C and *trn*D), 5 µL 5X master mix [(Cat. No: RP02-II, 0.75 U of Taq DNA polymerase, reaction buffer, 2 mM $MgCl_2$) including 250 µM dNTPs and enzyme stabilizer] and 16 µL dH_2O were added into the PCR tube. For each primer sets, PCR programs are given in Table 1 and Table 2. PCR products were analyzed in 1.5% agarose gel. PCR gel images are shown in Figure 1 and Figure 2.

Table 1. SCoT primers and PCR components used for PCR amplification

SCoT primers	DNA Sequences(5'-3')	Tm °C	PCR components	PCR Amplification (35 cycle)
Primer 1	CAACAATGGCTACCACCA	54°C	2 µL genomic DNA 1 µL primer, 10 µL master mix (PCR buffer, 2 Mm $MgCl_2$, dNTP, 0.75 U Taq DNA polymerase) and 7 µL dH_2O	95°C/1min. 95°C/30 sec 54-56°C/30sec. 68°C/1 min. 68°C/5 min.
Primer 2	CAACAATGGCTACCACCC	56°C		
Primer 4	CAACAATGGCTACCACCT	54°C		
Primer 5	CAACAATGGCTACCACGA	54°C		
Primer 6	CAACAATGGCTACCACGC	56°C		
Primer 8	CAACAATGGCTACCACGT	54°C		
Primer 11	AAGCAATGGCTACCACCA	54°C		

Table 2. cpDNA *trn*L intron primers and PCR components used for PCR amplification

Primer name	5' to 3' Primer sequence	PCR components	PCR amplification (35 cycle)
Forward *trn*c (F)	5'-CGAAATCGGTAGACGCTACG-3'	2 µL genomic DNA 1 µL primer (F and R), 5 µL master mix (PCR buffer, 2 Mm $MgCl_2$, dNTP, 0.75 U Taq DNA polymerase) and 16 µL dH_2O	94 °C / 5 min 94 °C / 45 sec 50 °C / 45 sec 72 °C / 1 min 72 °C / 10 min.
Reverse *trn*d (R)	5'-GGGGATAGAGGGACTTGAAC-3'		

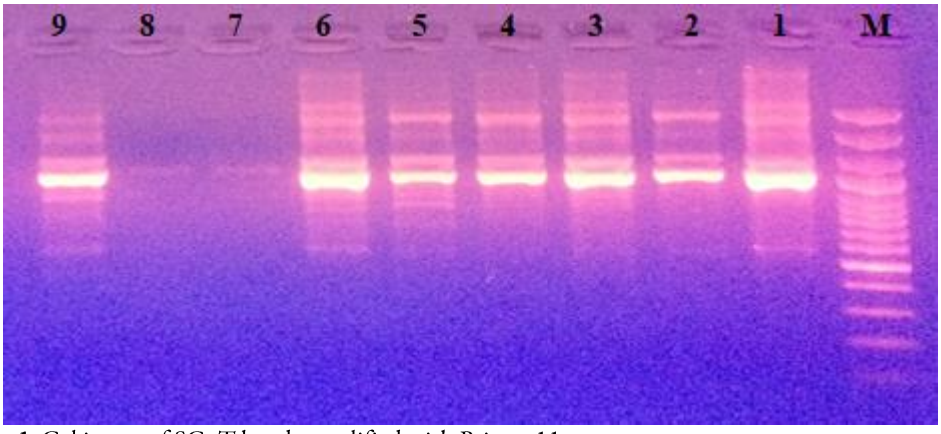

Figure 1. Gel image of SCoT bands amplified with Primer 11

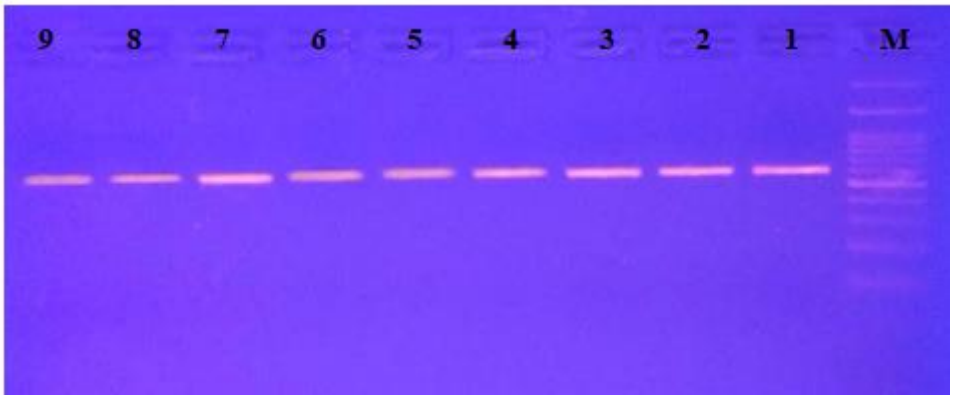

Figure 2. Gel image of cpDNA *trn*L intron bands

SCoT analyses

After the PCR analyses, DNA bands were scored as follows: "1" was given if there was DNA and "0" was given if there was no DNA in the bands. A "?" was given for missing data. Genetic relationship of *Eriobotrya japonıica* populations used in the study was analyzed using the PAUP 4.0b10 (Swofford, 2001) program. In addition, principal component analysis (PCA) was done among *Eriobotrya japonıica* populations using MVSP 3.22 software (Kovach, 2007), and spatial relationships were demonstrated.

cpDNA trnL intron analyses

Service procurement from biotechnology company Triogen (Istanbul, Turkey) was obtained for PCR reactions and purification. For the analysis of DNA sequences, professional computer programs BioEdit (Hall, 1999) and Finch TV were used and contig sequences were obtained. Using contig sequences, phylogenetic analysis was performed with the MEGA 6.0 program (Tamura *et al*., 2013). To evaluate the degree of support for given clades, a bootstrap analysis (1,000 replicates) was applied (Felsenstein, 1985). Also, genetic distance among populations and nucleotide contents were obtained. cpDNA trnL intron sequences were uploaded into NCBI and GenBank numbers; Aydın population (ON456311), Aydın samples (2)(ON456312), Denizli population (ON456313), Izmir population (ON456314), Mugla-Köyceğiz population (ON456315), Mugla population (ON456316), Aydın-Nazilli population (ON456317), Manisa-Salihli population (ON456318), Usak population (ON456319).

Results and Discussion

Nowdays, molecular markers are important tools and are used for both plants and animals. Spesificially for plants, DNA markers are used in germplasm characterization, genetic diversity, genome organization and phylogenetic analysis (Gupta *et al*., 1999; Gelotar *et al*., 2019). In the past, various molecular markers including nrDNA ITS (Li *et al.,* 2009), cpDNA *psb*A-*trn*H and *atp*B-*rbc*L (Idrees *et al*., 2021), cpDNA *rbc*L and *trn*L *trn*F (Li *et al.,* 2010), *Adh* (Xinaghui *et al.,* 2012), RAPD and ISSR (Sevindik *et al.,* 2020; Wang *et al.,* 2010; Yijie *et al.,* 2010), AFLP (Yang *et al*., 2009; Gisbert *et al*., 2009) and SSR (Soriano *et al*., 2005; Gisbert *et al*., 2009; He *et al*., 2011) were used to determine and document genetic relationship among *Eriobotrya s*pecies.

SCoT Analyses

In this study, seven SCoT primers were used for molecular analysis of *Eriobotrya japonica* populations. A total of 54 bands were obtained from primers, of which 5 were monomorphic and 49 were polymorphic. Total polymorphism rate was determined as 90.74%. The longest distance between populations was between

Manisa-Salihli and Muğla-Köyceğiz (0.76000), while the closest distance was between Muğla-Köyceğiz and Muğla populations (0.04000) (Table 3). According to SCoT analysis, UPGMA dendrogram divided into two clades (Figure 3). Clade 1 consisted of three subclades; in subclade A, Denizli and Uşak populations were in a group, Manisa-Salihli and Aydın-Nazilli populations were in another group; in subclade B, İzmir and Aydın populations were in a group and subclade C consisted of only Aydın population. Clade 2 consisted of Muğla and Muğla-Köyceğiz populations. Using MVSP 3.22 program, PCA image in Figure 4 was obtained. As a result of the analysis, three groups were obtained. Group one consisted of Manisa-Salihli and Aydın populations, group two consisted of Denizli, İzmir, Aydın-Nazilli, Uşak and Aydın populations (2), while group three consisted of Muğla- Köyceğiz and Muğla populations. The results obtained with the UPGMA tree are nearly in agreement with the results generated by the PCA analysis. Sevindik et al (2020) determined genetic diversity of *Eriobotrya japonica* populations distributed in the Aegean region using RAPD and ISSR markers. In RAPD analysis, 33 bands were obtained and 21 were polymorphic. In ISSR analysis, 43 bands were obtained and 18 were polymorphic. According to the RAPD analysis, Denizli, İzmir, Aydın and Aydın-Nazilli populations were in one group, and Manisa-Salihli populations in a separate group. In the SCoT results, Manisa-Salihli population was found in the same group with Denizli and Aydın-Nazilli populations. In ISSR analyses, Denizli, İzmir, Aydın and Manisa-Salihli populations were determined in one group and Aydın Nazilli population in a separate group. In the SCoT results, Manisa-Salihli population and Aydın-Nazilli population were found together. Also, polymorphism rate of SCoT results was higher than that of RAPD and ISSR. Yijie *et al.* (2010) identified 88 polymorphic loci out of 141 in 22 *Eriobotrya japonica* cultivars using ISSR technique with 11 primers. Cultivars between similarity coefficient varying from 0.560 to 0.838 were detected. Yang *et al.* (2009) determined the phylogenetic relationship of *Eriobotrya* species using the AFLP technique. In the study, they found that *Eriobotrya japonica* and *Eriobotrya malipoensis* species were in the same group, and the genetic similarity coefficient between *Eriobotrya japonica* and *Eriobotrya malipoensis* was 0.67. Soriano *et al.* (2005) determined the genetic relationship of 40 *Eriobotrya japonica* genotypes using 30 SSR primer pairs. Thirteen of the primers yielded polymorphic results revealing the differentiation in 34 genotypes. They identified a total of 39 alleles, 31 of which were polymorphic, ranging from 2 to 6 per locus with an average allele value of 2.40 per locus. Expected and observed heterozygosities averaged 0.46% and 51%, respectively, and allelic frequencies (p) ranged from 0.01 to 0.89, with a mean value of 0.41.

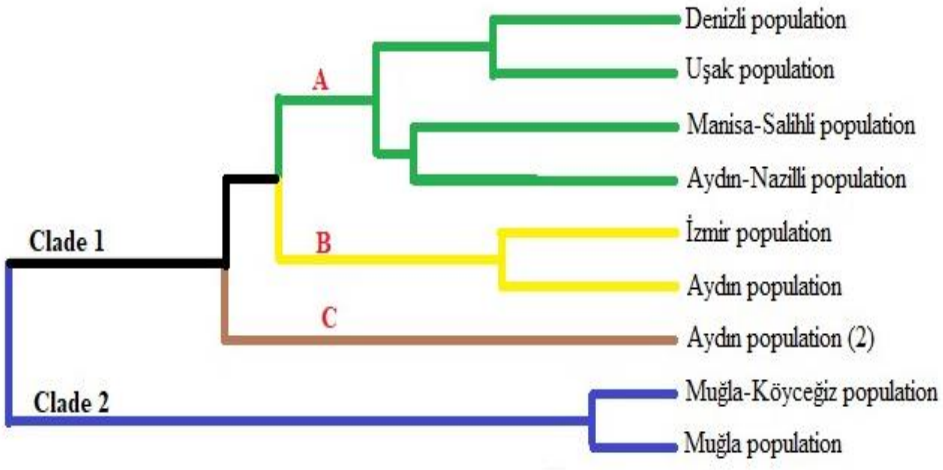

Figure 3. The UPGMA tree generated tree using SCoT data

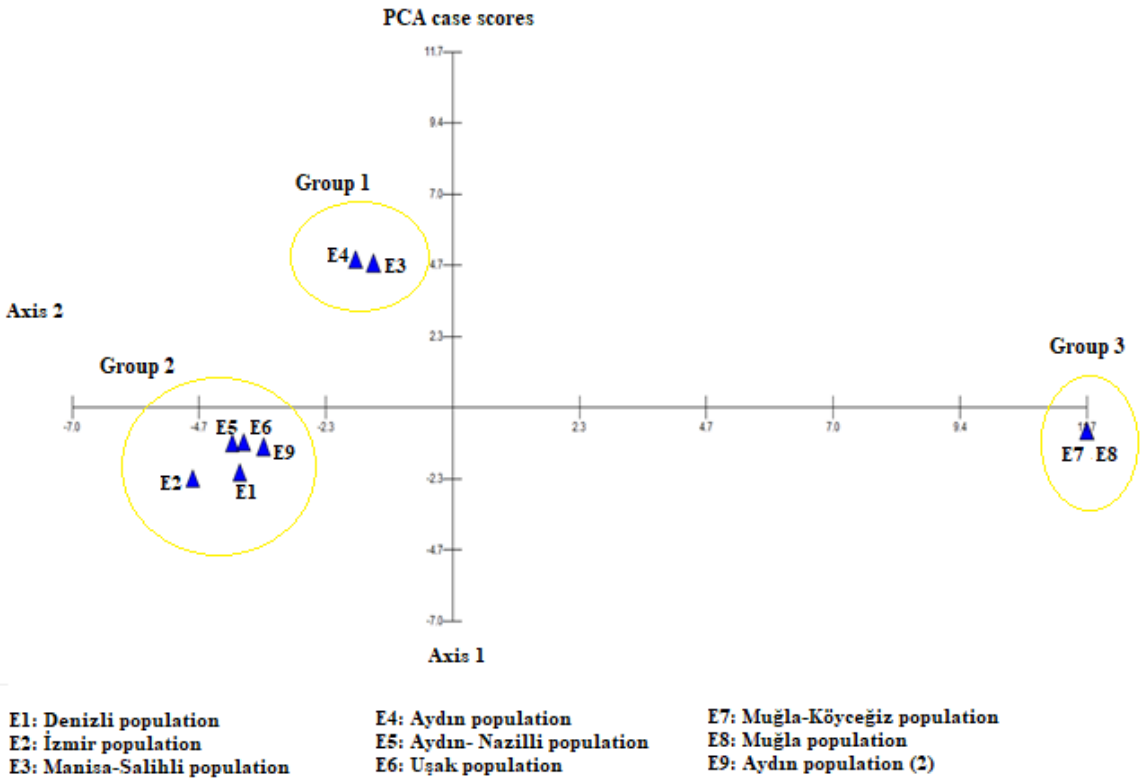

Figure 4. Principal component analyses of *Eriobotrya japonica* populations using MVSP 3.22 software

Table 3. Pairwise genetic distance matrix obtained from SCoT markers

Population	1	2	3	4	5	6	7	8	9
Denizli population	-	0.50000	0.29787	0.36170	0.36538	0.17308	0.60000	0.56000	0.42308
İzmir population	27	-	0.36170	0.17021	0.36538	0.48077	0.40000	0.44000	0.50000
Manisa-Salihli population	14	17	-	0.40426	0.25532	0.27660	**0.76000**	0.72000	0.51064
Aydın population	17	8	19	-	0.23404	0.38298	0.52000	0.56000	0.40426
Aydın-Nazilli population	19	19	12	11	-	0.23077	0.72000	0.68000	0.36538
Uşak population	9	25	13	18	12	-	0.60000	0.56000	0.44231
Muğla-Köyceğiz population	15	10	19	13	18	15	-	**0.04000**	0.60000
Muğla population	14	11	18	14	17	14	1	-	0.56000
Aydın population (2)	22	26	24	19	19	23	15	14	-

cpDNA trnL intron analyses

Contig sequences were aligned using MEGA 6.0 program. *Eriobotrya japonica* populations' *trn*L intron sequence lengths were determined between 530 and 547 nucleotides. The average nucleotide rate detected was 38.9% for Thymine, 18.1% for Cytosine, 28.2% for Adenine and 14.8% for Guanine (Table 4).

Table 4. Length and T, C, A and G contents of *trn*L intron sequences of *Eriobotrya japonica* populations

Populations	T(U)	C	A	G	Total
Aydın population	39.0	18.2	28.0	14.8	539.0
Aydın population (2)	38.7	18.5	28.1	14.7	545.0
Denizli population	39.1	17.9	28.0	15.0	535.0
Izmir population	38.9	18.0	28.3	14.8	540.0
Mugla-Köyceğiz population	39.0	17.9	28.4	14.7	536.0
Mugla population	38.9	18.1	28.1	14.9	530.0
Aydın-Nazilli population	39.0	18.0	28.3	14.8	534.0
Manisa-Salihli population	38.8	18.1	28.2	15.0	547.0
Usak population	39.1	17.8	28.2	14.8	539.0
Avg.	38.9	18.1	28.2	14.8	538.3

The maximum likelihood phylogenetic tree using *trn*L intron sequence data is given in Figure 5. *Prunus domestica* and *Rosa sericea* were used as outgroups in the study. This phylogenetic tree consisted of 2 large clades. Clade 1 consisted of Muğla, Muğla-Köyceğiz, Aydın-Nazilli, Aydın, Manisa-Salihli and Uşak populations. Clade 2 consisted of Denizli, Aydın (2) and İzmir populations. The genetic distance between populations was found to be 0.000 (excluding Outgroups) (Table 5).

Table 5. Pairwise sequence distances among *Eriobotrya japonica* populations for *trn*L intron data using MEGA 6.0 software distance matrix

Population	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Aydın population	-										
Aydın population (2)	0.000										
Denizli population	0.000	0.000									
Izmir population	0.000	0.000	0.000								
Mugla-Köyceğiz population	0.000	0.000	0.000	0.000							
Mugla population	0.000	0.000	0.000	0.000	0.000						
Aydın-Nazilli population	0.000	0.000	0.000	0.000	0.000	0.000					
Manisa-Salihli population	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
Usak population	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
Prunus domestica	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047		
Rosa sericea	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.078	-

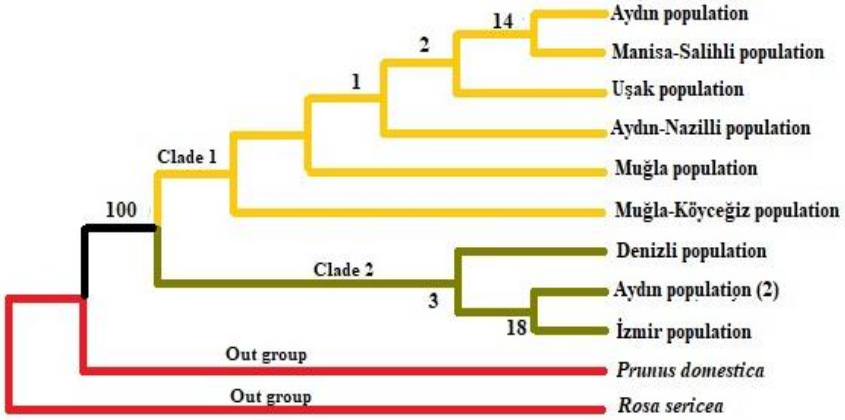

Figure 5. Phylogenetic tree of *Eriobotrya japonica* populations *trn*L intron sequences constructed using maximum likelihood method with MEGA 6.0

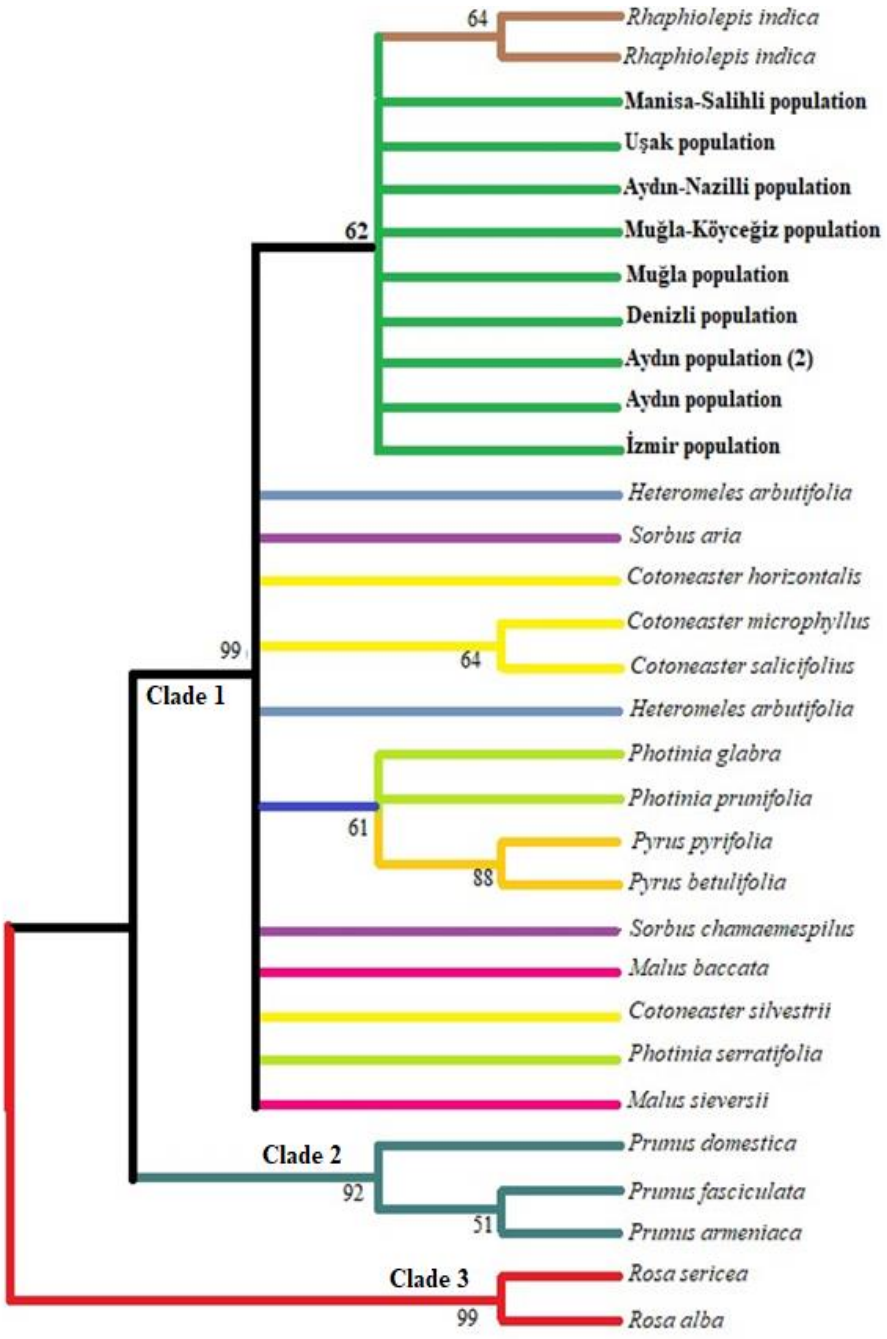

Figure 6. The maximum likelihood tree generated using cpDNA *tm*L intron sequences *Eriobotrya japonica* populations and other species sequences retrieved from NCBI

In their RAPD analysis, Sevindik *et al.* (2020) reported the Manisa-Salihli population in a separate group, while in the *trn*L intron analysis, it was found together with the Aydın and Aydın-Nazilli populations. In ISSR analysis, Aydın-Nazilli population was in a single group, whereas in *trn*L intron results, Aydın and Manisa-Salihli populations were found together. Xinaghui *et al.* (2012) investigated the phylogenetic relationships of the genus *Eriobotrya* on the basis of nrDNA *Adh* sequences. In their results; *E. prinoides* var. *daduheensis* and *E. japonica* were detected in the same group. It was concluded that despite the high genetic diversity of the *Adh* gene sequence in *Eriobotrya,* some species could not be separated. Idrees *et al.* (2021a) revealed the phylogenetic relationships of *Eriobotrya* species with the markers cpDNA *psb*A-*trn*H and *atp*B-

*rbc*L. In their studies, *E. japonica* species were found in the same group with *E. malipoensis* and *E. daduheensis.* Idrees *et al.* (2021b) applied morphometric analysis based on 40 morphological (quantitative and qualitative) characters to reveal the taxonomic relationship of the genus *Eriobotrya*. In data-based cluster analysis, *E. japonica, E. malipoensis,* and *E.* × *daduheensis* formed a subgroup, and morphological relationship among these species based on the following criteria: leaf blade abaxailly tomentose, leaf size 16-22 cm, leaf width 4-8 cm, 12-18 pairs of lateral veins, petiole sessile and tomentose, large inflorescence 7-10 cm and contracted, bract subulate, pedicel sessile, style free at base and style number 3-5. In our study, phylogenetic relationships of some taxa (belonging to Rosaceae family whose *trn*L intron sequences were retrieved from NCBI) with *E. japonica* populations have been revealed. The generated Maximum likelihood phylogenetic tree consisted of 3 clades (Figure 6).

In Clade 1, *Eriobotrya japonica* populations emerged together with *Rhaphiolepis indica* and received support with a bootstrap value of 62%. In addition, this group emerged in the same clade as *Heteromeles arbutifolia, Photinia serratifolia, Photinia glabra, Photinia prunifolia, Cotoneaster horizontalis, Cotoneaster silvestrii, Cotoneaster microphyllus, Cotoneaster salicifolius, Sorbus aria, Sorbus chamaemespilus, Malus baccata, Malus sieversii, Pyrus pyrifolia* and *Pyrus betulifolia* with a bootsrapt value of 99%. Clade 2, consisting of *Prunus fasciculata*, *Prunus armeniaca* and *Prunus domestica* species, received 92% support, while clade 3, consisting of *Rosa sericea* and *Rosa alba* species, received 99% support. Sun *et al.* (2018) identified the genus *Eriobotrya* in the same group with *Rhaphiolepis, Cotoneaster, Stranvaesia, Heteromeles* and *Photinia* in the phylogenetic analysis of Maleae (Rosaceae) based on multiple chloroplast regions. Liu *et al.* (2020) have revealed that nrDNA as well as plastome results in nuclear ribosomal DNA and chloroplast genome phylogeny support the paraphilia of *Eriobotrya* nesting in *Rhaphiolepis*, and that *Eriobotrya* and *Rhaphiolepis* should be combined into a single genus. They have also determined that the plastid genome tree supports the relationship of *Eriobotrya* and *Rhaphiolepis* with *Heteromeles.* However, nrDNA-based ML and BI analyzes failed to resolve the phylogenetic position of the *Rhaphiolepis* clade of *Eriobotrya.* The nrDNA ML result has revealed that the *Rhaphiolepis* clade of *Eriobotrya* is a sister clade with the major clade containing *Chaenomeles, Cydonia, Dichotomanthes, Docynia, Heteromeles, Malus, Phippsiomeles, Photinia, Pourthiaea, Pseudocydonia, Pyracantha, Pyrus,* and *Stranvaesia.* In their phylogenetic analysis with six chloroplast regions and five nuclear regions, Campbell *et al.* (2007) revealed that *Eriobotrya* and *Rhaphiolepis* coexisted based on both nucleus and chloroplast data.

Conclusions

As a result, in the SCoT analysis a high rate of 90.74% polymorphism was detected. Phylogenetic relationship of *Eriobotrya japonica* populations with *Rhaphiolepis, Heteromeles, Photinia, Cotoneaster, Sorbus, Malus, Pyrus, Prunus* and *Rosa* species was determined using cpDNA *trn*L intron sequences. *Eriobotrya japonica* populations were identified in the same group with *Rhaphiolepis indica*, a finding that is supported by previous phylogenetic analysis reports.

Authors' Contributions

E.S. plant samples collected. E.S and H.D. did the experimental work and E.S. wrote the article. Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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