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# Genetic diversity and phylogenetic analyses of *Vitex agnus-castus* L. populations using ISSR-PCR and chloroplast DNA *trn*L intron and *trn*L-F sequences

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**Abstract**

In this study, genetic diversity and phylogenetic analysis of some *Vitex agnus-castus* L. populations were conducted based on ISSR-PCR and chloroplast DNA *trn*L intron, and *trn*L-F sequence analyses. *Vitex agnus-castus* populations were detected in Aydın province, Turkey. Fresh leaf samples from the populations were collected and brought to the laboratory for genomic DNA isolation. 15 ISSR primers were used to determine the genetic diversity ofe *Vitex agnus-castus* populations. A total of 138 bands were obtained in ISSR analysis, 85 of which were polymorphic and 53 were monomorphic. Polymorphism rate was determined as 61.59 %. *trn*C, *trn*D, *trn*E, and *trn*F primers were used for PCR amplification of the chloroplast *trn*L intron and *trn*L-F region. A total of 138 bands were obtained by ISSR analysis. For *trn*L intron analyses, nucleotide lengths of 13 populations were between 508 and 516. The average nucleotide composition consisted of 38.5 % T, 18.3 % C, 27.5 % A and 15.7 % G. In *trn*L-F assays, the nucleotide lengths of the 13 populations ranged from 330 to 353. The average nucleotide composition consisted of 29.4 % T, 18.1 % C, 32.9 % A and 19.6 % G. The results of the phylogenetic trees constructed using some *trn*L intron and *trn*L-F sequences of *Vitex doniana*, *Vitex trifolia*, *Vitex triflora*, *Vitex turczaninowii*, *Vitex queenslandica*, *Vitex axillariflora*, *Vitex rotundifolia* and *Vitex negundo* species obtained from NCBI were compared. As a result of the study, polymorphisms were obtained at a rate of 61.59% from the ISSR analysis. In addition, the phylogenetic relationship between chloroplast *trn*L intron and *trn*L-F sequences of *Vitex agnus-castus* populations along with the other species was revealed.

## Introduction

Medical and aromatic plants have a long history of being used globally for medicinal purposes and have traditionally been an important component of health services throughout human history due to their health benefits (Eryigit *et al.* 2015; Chaachouay *et al.* 2019). The *Vitex* genus includes about 250 species and many of these have important medicinal effects (Yao *et al.* 2016). *Vitex agnus-castus* L., known as chasteberry, belongs to the Lamiaceae family (previously included in the Verbenaceae family), and it is a 1 – 3 m long highly branched small tree. Its trunk is covered with short, dense, soft and grey hairs. It is a deciduous shrub native to Europe and Central Asia (Stojković *et al.* 2011; Hürkul and Köroğlu 2018; Souto *et al.* 2020; Ilhan 2021). This plant is

naturally found in many cities throughout Turkey, including Amasya, Antalya, Bursa, Muğla, Trabzon and Çanakkale (Yilar *et al.* 2016). *Vitex agnus-castus*, used in conventional medicine, has a long tradition in folk medicine, and its use dates back to 2,500 years ago (Sorensen and Katsiotis 1999; Lataoui *et al.* 2014; Uçak Koç *et al.* 2017). It is widely used in Anatolian folk medicine as diuretics, appetizers, antifungals, antispasmodics, for preterm labor and for pain relief (Ilhan 2021). Its fruits, flowers and leaves have been reported to contain flavonoids, tannins, iridoids and diterpenoids (Sağlam *et al.* 2007; Sarikurkcu *et al.* 2009). *V. agnus-castus* fruits have been used as a flavor and spice component in meals, as a hormone-like remedy for menstrual problems and a mild sedative and digestive tool in Iranian traditional folk medicine (Ghannadi *et al.* 2012). The aromatic leaves are used as a spice (Stojković *et al.* 2011; Mari *et al.* 2015).

Genetic diversity is a prerequisite for the short and long-term survival of plant species in their natural environment (Verma *et al.* 2017). Morphological, biochemical, and DNA markers are used to determine the genetic diversity of plants (Sevindik *et al.* 2020). DNA-based molecular markers offer many advantages in determining genetic diversity. Among the polymerase chain reaction (PCR) based marker techniques, the ISSR technique is one of the simplest and most widely used markers. It is also a marker system utilized in the determination of genetic relationships and linkage mapping (Alansi *et al.* 2016; Atasagun *et. al.* 2018; Güngör *et al.* 2020). Chloroplast (cp) is an important organelle for green plants as it is the place where photosynthesis, carbon fixation and pigment synthesis take place. The Cp is an independent genome (cp genome) with a preserved circular structure and a low molecular weight (Zhou *et al.* 2019; Munyao *et al.* 2020; Zhu *et al.* 2021). Chloroplast DNA (cpDNA), which is inherited by the female in most angiosperm plants, is generally accepted as a single non-recombinant inheritance unit and is widely used to reveal plant phylogenies in interspecies studies (Taberlet *et al.* 1991; Gielly and Taberlet 1994; Dizkirici *et al.* 2019; Sun *et.al.* 2019). Non-coding regions of the chloroplast genome are used in molecular systematic and plant population genetic studies (Kalmer and Tekpınar 2017). Within this study the chloroplast regions with both variable and highly conserved primer sequences were used. The first of these regions is the *trn*L$^{UAA}$ intron; and the second is the *trn*L$^{(UAA)}$-F$^{(GAA)}$ intergenic spacer (IGS). Noncoding cpDNA *trn*L-F is located between *trn*L $^{(UAA)}$ 3' exon and *trn*F $^{(GAA)}$ gene. In addition, it is more variable towards the coding regions, and some studies have revealed that it shows higher variations and more frequent mutations than the coding regions (Taberlet *et al.* 1991; Hartana 2010). The *trn*L intron is the first group I intron identified in chloroplast DNA and is also the first intron identified to cut a tRNA gene (Bakker *et al.* 2000). In this study, we performed a genetic diversity analysis using ISSR and cpDNA *trn*L intron and *trn*L-F region markers for some *Vitex agnus-castus* populations grown in the Aydın region of Turkey.

## Experimental

*Plant materials, genomic DNA isolation and PCR*

Leaf samples belonging to the *Vitex agnus-castus* populations to be used in this study were collected from the center, Koçarlı, Çine, Germencik, İncirliova, and Köşk districts of Aydın province (Fig. 1).


**Fig. 1.** Location of collecting places in the Aydın/Turkey (https://www.google.com/maps).

Leaf samples of the collected *Vitex agnus-castus* plant were brought to the laboratory and prepared for genomic DNA isolation. A commercial kit (GeneMark) was used for the genomic DNA isolation from the plant specimens. The obtained gDNA samples were stored at -20 °C. The primers, PCR mix and protocol used for the amplification of

ISSR-PCR, cpDNA *trn*L intron and *trn*L-F regions are given in Table 1 and Table 2.

**Table 1.** Primers used in the ISSR-PCR reactions, PCR protocols and their Tm degrees.

| ISSR Primers | DNA Sequences (5'-3') | Tm [°C] | PCR Amplification (35 cycle) | Amplification |
|---|---|---|---|---|
| **UBC-831** | 5'-CTCTCTCTCTCTCTCTT-3' | 50 °C | | + |
| **UBC-830** | 5'-TGTGTGTGTGTGTGTGG-3' | 52 °C | | + |
| **UBC-807** | 5'-AGAGAGAGAGAGAGAGT-3' | 50 °C | | + |
| **UBC-808** | 5'-AGAGAGAGAGAGAGAGC-3' | 52 °C | | + |
| **UBC-836** | 5'-AGAGAGAGAGAGAGAGYA-3' | 52 °C | 94 °C/1min | + |
| **UBC-856** | 5'–ACACACACACACACACYA-3' | 52 °C | 94 °C/1min | + |
| **UBC-853** | 5'- TCTCTCTCTCTCTCTCRT-3' | 52 °C | 48-55 °C/1min | + |
| **UBC-892** | 5'- TAGATCTGATATCTGAAT-3' | 52 °C | 72 °C/1 min | - |
| **UBC-810** | 5' -GAGAGAGAGAGAGAGAT-3' | 50 °C | 72 °C/10min | + |
| **UBC-826** | 5'-ACACACACACACACACC-3' | 52 °C | | + |
| **UBC-811** | -GAGAGAGAGAGAGAGAC-3' | 53 °C | | + |
| **UBC-834** | 5'-AGAGAGAGAGAGAGAYT-3' | 52 °C | | + |
| **UBC-873** | 5'-GACAGACAGACAGACA-3' | 48 °C | | + |
| **UBC-855** | 5'-ACACACACACACACAC | 52 °C | | + |
| **UBC-880** | 5'-GGAGAGGAGAGGAGA-3' | 55 °C | | + |

**Table 2.** *trn*L intron and *trn*L-F primers used in this study with their designers and PCR protocols.

| Primer name | 5' to 3' Primer sequence | PCR Amplification (35 cycle) | Based on (the source publication) |
|---|---|---|---|
| **Forward *trnc*** | 5'-CGAAATCGGTAGACGCTACG-3' | 94 °C/4min. | Taberlet *et al.* 1991 |
| **Reverse *trnd*** | 5'-GGGGATAGAGGGACTTGAAC-3' | 94 °C/1min | Taberlet *et al.* 1991 |
| **Forward *trn*Le** | 5'-GGTTCAAGTCCCTCTATCCC-3' | 50 °C/1min. | Taberlet *et al.* 1991 |
| **Reverse *trn*Ff** | 5'-ATTTGAACTGGTGACACGAG-3' | 72 °C/1 min. | Taberlet *et al.* 1991 |
| | | 72 °C/10min. | |

1 % agarose gel 1X TBE buffer was used for electrophoresis of PCR products. 5 µl of the reaction mixture in PCR tubes was added to 1 µL loading buffer (Loading Dye Solution) and mixed, and 6 µL of this mixture was placed in the wells of the gel. After loading 3 kb DNA marker into the first well, the device was subjected to electrophoresis at 100 V for 90 min. Then, the DNA bands were imaged under UV light and their photos were taken (Fig. 2 – 4).

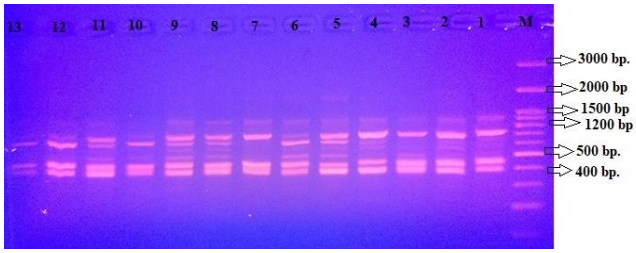


**Fig. 2.** Gel image of ISSR-PCR bands amplified with UBC-811 primer.

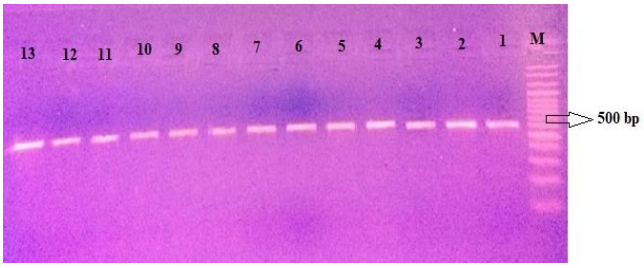


**Fig. 3.** Gel image of PCR bands amplified with cpDNA *trn*L intron.

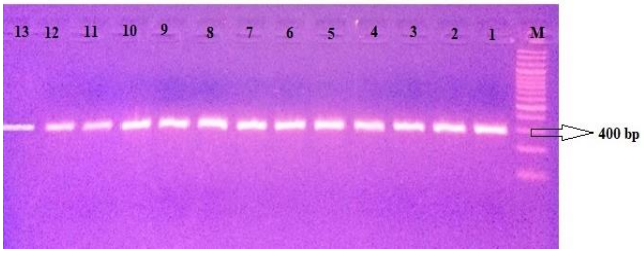


**Fig. 4.** Gel image of PCR bands amplified with cpDNA *trn*L-F region.

*ISSR-PCR 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. Using bands, the genetic relationships of *Vitex agnus-castus* populations used in the study were analysed using the PAUP 4.0b10 (Swofford 2001) program.

*cpDNA trnL intron and trnL-F sequences*

Service procurement from biotechnology company Triogen (Istanbul, Turkey) was obtained for PCR reactions and purification. To ensure healthy analyses, it was necessary to visually check the accuracy of DNA sequences one by one. To this end, professional computer programs BioEdit (Hall 1999) and Finch TV, which are frequently used in molecular systematic studies worldwide, were utilized. A maximum likelihood phylogenetic tree was constructed using the MEGA 6.0 (Tamura *et al.* 2013) to extract phylogenetic relationships among the sequenced *Vitex agnus-castus* populations. To evaluate the degree of support for given clades, a bootstrap analysis (1,000 replicates) was applied (Felsenstein 1985). In addition, the genetic distance matrix between populations was also performed using the same software.

## Results and Discussion

In recent years, some PCR-based molecular markers have been developed and tested in genetic studies of various organisms (Poyraz *et al*. 2016). In previous studies, genetic diversity, phylogenetic and molecular analyses of *Vitex* species have been conducted using RAPD (Sevindik *et al*. 2019), ISSR (Gıachıno and Avcı 2017; Saedyani *et al.* 2020), *rps*14 gene (Ayaz *et al.* 2020; Malik *et al.* 2021), nr*ITS* DNA, chloroplast *ndh*F, *trn*H-*psb*A, *trn*G-*trn*S sequences (Bramley *et al.* 2009; Sun *et al.* 2019), chloroplast *rbc*L and *ndh*F sequences (Wagstaff *et al.* 1998), and chloroplast *mat*K and *psb*A-*trn*H intergenic spacer (Phoolcharoen and Sukrong 2013).

*ISSR Analysis*

In recent years, PCR-based DNA marker systems such as AFLP, RFLP, RAPD, SSR and ISSR have been common for investigating the genetic makeup of populations, genetic characterization, genetic diversity, divergence and phylogenetic studies (Li and Ge 2001; Gajera *et al.* 2010; Chen *et al.* 2017; Hocaoglu-Ozyigit *et al.* 2020). ISSR markers have been proposed as a new source of genetic markers that are inherited in Mendelian fashion and are scored as dominant markers (Paul *et al*. 2020). These markers have the role of analyzing genetic diversity through the classification of varieties (Kiani and Siahchehreh 2018). In ISSR analysis 15 primers were used of which 14 yielded positive results while 1 primer did not (Table 1). A total of 138 bands were obtained in ISSR analysis, 85 of which were polymorphic and 53 were monomorphic. Polymorphism rate was determined as 61.59 %. The UPGMA tree generated based on the ISSR dataset consisted of two clades (Fig. 5).

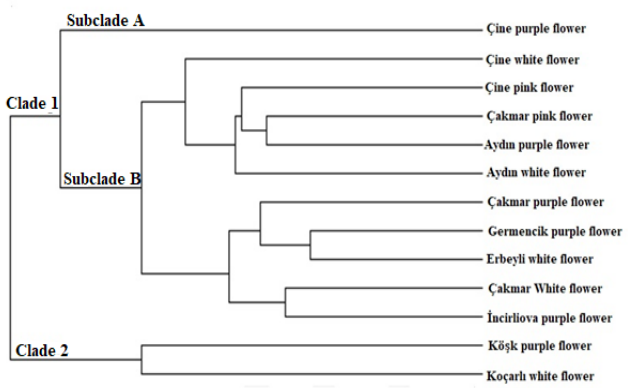


**Fig. 5.** The UPGMA tree generated using ISSR data.

Clade 1 was divided into 2 subclades. Subclade A consisted of only the Çine purple flowered population. Çine purple flowered population was found together with Koçarlı white flowered, Çakmar white flowered, Germencik purple flowered and Erbeyli white flowered populations in RAPD analysis (Sevindik *et al.* 2019). In the subclade B, Çine white flower, Çine pink flower, Çakmar pink flowered, Aydın purple flowered and Aydın white flowered appeared in the same group. According to Sevindik *et al.* (2019), these populations were found together according to the RAPD results. In subclade B, Çakmar purple flowered, Germencik purple flowered, Erbeyli white flowered, Çakmar white flowered and İncirliova purple flowered populations emerged together. According to Sevindik *et al.*

(2019), Germencik purple flowered, Çakmar white flowered and Erbeyli white flowered were found together, while İncirlova purple flowered and Çakmar purple flowered populations detected in separate groups. Clade 2 consisted of Köşk purple flower and Koçarlı white flower populations. According to Sevindik *et al.* (2019), both populations emerged in different groups. According to PAUP analysis, the closest genetic distances were between Germencik purple flowered and Erbeyli white flowered populations with a value of 0.10145; and the greatest genetic distance was between Çine purple flowered and Koçarlı white flowered populations with a value of 0.36957 (Table 3).

Giachino and Avci (2017) used 6 of 21 ISSR primers for *Vitex agnus-castus* populations collected from various parts of the Yunt Mountain of Manisa. The discrimination power of ISSR primers was evaluated by calculating various marker parameters such as percent polymorphism, polymorphism information content (PIC), resolving power (RP), and marker index (MI). In the study, 31 (65.95 %) of a total of 47 useable bands were found to be polymorphic. The polymorphic band ratios of the primers varied from 25 % to 85.7 %. Polymorphism information content (PIC) values of 6 primers ranged from 0.22 to 0.35, and the mean PIC value was calculated as 0.30. The resolution power (RP) values ranged between 0.5 and 3.25, and the marker index (MI) ranged between 0.11 and 1.72. Saedyani *et al*. (2019) determined genetic diversity for 19 *Vitex agnus-castus* genotypes using 13 ISSR primers. In their study, 74 bands were obtained and the polymorphism rate was 95% on average. They determined the genetic distance of the genotypes between 0.195 and 0.593 using the Dice coefficient.

*cpDNA trnL intron and trnL-F sequence analysis*

Phylogenetic analyses of molecular sequences are part of many molecular and phylogenetic biology studies (Anisimova *et al.* 2013). The cpDNA *trn*L-F region is located in the large single-copy region of the chloroplast genome and is widely used for phylogenetic analyses (Hocaoglu-Ozyigit *et al.* 2020). For the cpDNA *trn*L intron, the base lengths of *Vitex agnus-castus* populations ranged from 508 to 516. The genetic distance between populations was between 0.000 and 0.002 (Table 4). The mean nucleotide ratio for the *trn*L intron was 38.5 % T, 18.3 % C, 27.5 % A, and 15.7 % G. Using the MEGA 6.0 program, Tajma's Neutrality Test (Tajima 1989) was calculated based on cpDNA *trn*L intron sequences of *Vitex agnus-castus* populations. Numbers of sequences (m) yielded one segregation site (S) revealing very low nucleotide diversity ($\pi$) of 0.001422 (Table 5).

The maximum likelihood phylogenetic tree constructed using 13 populations consisted of two large groups (Fig. 6). The first clade is divided into two subgroups. Subclade A consisted of İncirliova purple flowered, Koçarlı white flowered, Germencik purple flowered, Çine purple flowered, Çakmar white flowered and Çakmar purple flowered, and this group was supported with a bootstrap value of 55 % (Fig. 6). In the study of Sevindik *et al.* (2019), Çakmar purple flowered and İncirliova purple flowered populations were found in a separate group, while Koçarlı white flowered, Germencik purple flowered, Çine purple flowered and Çakmar white flowered populations were found in the same group. Subclade B consisted of Çakmar pink flowered, Çine pink flowered, Erbeyli white flowered and Köşk purple flowered, and this group was supported with a 60 % bootstrap value (Fig. 6). Sevindik *et al*. (2019) found Erbeyli white flowered and Köşk purple flowered populations in separate groups, while Çakmar pink flowered and Çine pink flowered populations in the same group. Clade 2 consisted of Aydın white flowered, Aydın purple flowered and Çine white flowered populations. Within this group, Aydın white flowered and Aydın purple flowered populations were monophyletic with a bootstrap value of 63 % (Fig. 6).

**Table 3.** Pairwise genetic distance matrix obtained from PCR with ISSR primers.

| Populations | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Çine purple flower** | - | 0.19565 | 0.23915 | 0.22464 | 0.20455 | 0.24638 | 0.27536 | 0.30435 | 0.26087 | 0.26812 | 0.26812 | 0.31159 | **0.36957** |
| **Çine White flower** | 27 | - | 0.17391 | 0.15952 | 0.13636 | 0.23913 | 0.22464 | 0.21014 | 0.18116 | 0.18841 | 0.20290 | 0.26087 | 0.33333 |
| **Çine pink flower** | 33 | 24 | - | 0.13043 | 0.15152 | 0.15217 | 0.16667 | 0.19565 | 0.19565 | 0.18841 | 0.20290 | 0.27536 | 0.31884 |
| **Çakmar pink flower** | 31 | 22 | 18 | - | 0.12879 | 0.13768 | 0.18116 | 0.23913 | 0.21014 | 0.20290 | 0.20290 | 0.27536 | 0.36232 |
| **Aydın purple flower** | 27 | 18 | 20 | 17 | - | 0.15152 | 0.13636 | 0.20455 | 0.19697 | 0.19637 | 0.18939 | 0.28788 | 0.29545 |
| **Aydın white flower** | 34 | 33 | 21 | 19 | 20 | - | 0.17391 | 0.27536 | 0.24638 | 0.22464 | 0.23913 | 0.32609 | 0.35507 |
| **Çakmar purple flower** | 38 | 31 | 23 | 25 | 18 | 24 | - | 0.17391 | 0.17391 | 0.12319 | 0.13768 | 0.25362 | 0.25362 |
| **Çakmar White flower** | 42 | 29 | 27 | 33 | 27 | 38 | 24 | - | 0.11594 | 0.13768 | 0.13768 | 0.19565 | 0.29710 |
| **İncirliova purple flower** | 36 | 25 | 27 | 29 | 26 | 34 | 24 | 16 | - | 0.13768 | 0.13768 | 0.19565 | 0.32609 |
| **Germencik purple flower** | 37 | 26 | 26 | 28 | 26 | 31 | 17 | 19 | 19 | - | **0.10145** | 0.21739 | 0.26087 |
| **Erbeyli white flower** | 37 | 28 | 28 | 28 | 25 | 33 | 19 | 19 | 19 | 14 | - | 0.15942 | 0.23188 |
| **Köşk purple flower** | 43 | 36 | 38 | 38 | 38 | 45 | 35 | 27 | 27 | 30 | 22 | - | 0.20290 |
| **Koçarlı white flower** | 51 | 46 | 44 | 50 | 39 | 49 | 35 | 41 | 45 | 36 | 32 | 28 | - |

**Table 4.** Pairwise genetic distance matrix obtained from cpDNA *trn*L intron sequences.

| **Populations** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aydın purple flower** | - | | | | | | | | | | | | |
| **Aydın white flower** | 0.000 | | | | | | | | | | | | |
| **Cakmar pink flower** | 0.002 | 0.002 | | | | | | | | | | | |
| **Cakmar purple flower** | 0.002 | 0.002 | 0.002 | | | | | | | | | | |
| **Cakmar white flower** | 0.002 | 0.002 | 0.002 | 0.000 | | | | | | | | | |
| **Cine pink flower** | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | | | | | | | | |
| **Cine purple flower** | 0.002 | 0.002 | 0.002 | 0.000 | 0.000 | 0.002 | | | | | | | |
| **Cine white flower** | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | | | | | | |
| **Erbeyli white flower** | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | | | | | |
| **Germencik purple flower** | 0.002 | 0.002 | 0.002 | 0.000 | 0.000 | 0.002 | 0.000 | 0.002 | 0.002 | | | | |
| **Incirliova purple flower** | 0.002 | 0.002 | 0.002 | 0.000 | 0.000 | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 | | | |
| **Kocarlı white flower** | 0.002 | 0.002 | 0.002 | 0.000 | 0.000 | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 | 0.000 | | |
| **Kösk purple flower** | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 | 0.002 | 0.002 | 0.002 | - |

**Table 5.** Tajima's Neutrality Test Values based on cpDNA *trn*L intron of date *Vitex agnus-castus* populations.

| **No. of sequences "m"** | **No. of segregating sites "S"** | ***P*s=S/n** | **$\Theta = p_s/a_1$** | **nucleotide diversity '' $\pi$ ''** | **Tajima test statistic ''D''** |
|---|---|---|---|---|---|
| 13 | 1 | 0.001980 | 0.000638 | 0.001422 | 2.700233 |

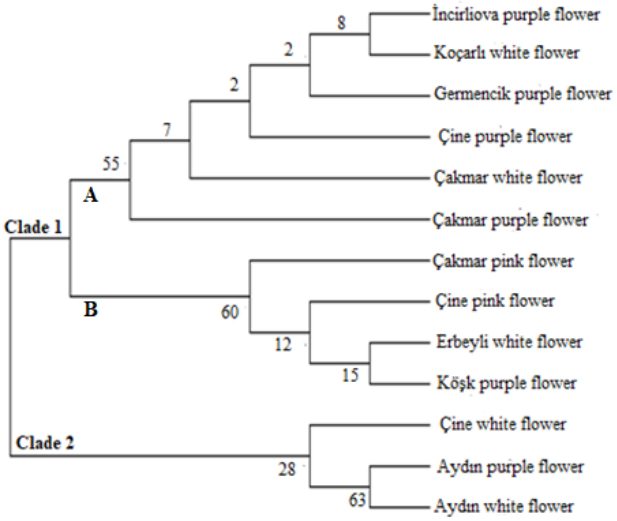


**Fig. 6.** The maximum likelihood tree generated using cpDNA *trn*L intron sequences.

According to Sevindik *et al.* (2019), these three populations were in the same group. There are both similarities and differences between the UPGMA dendrogram generated by RAPD analysis and the phylogenetic trees constructed with *trn*L intron sequences. Additionally, *Vitex doniana* (MK187249.1), *Vitex trifolia* (AJ505539.1), *Vitex triflora* (MK797715.1), *Vitex turczaninowii* (MG836415.1), *Vitex queenslandica* (MG836414.1), *Vitex axillariflora* (MG836413.1), *Vitex rotundifolia* (AB817427.1; AB817638.1; AB817574.1) and *Vitex negundo* (DQ304786.1; DQ304787.1) species were added to the analyses after obtaining their *trn*L intron sequences from NCBI. A maximum likelihood phylogenetic tree was generated and the relationship of *Vitex agnus-castus* populations with other species was revealed. The phylogenetic tree consists of 2 clades (Fig. 7). The first clade consists of *Vitex agnus-castus* populations, *Vitex axillariflora*, *Vitex trifolia*, *Vitex rotundifolia* and *Vitex negundo* species formed and is supported by a bootstrap value of 95 %. Clade 2 is divided into two subclades. Subclade A, consists of *Vitex triflora* and *Vitex doniana* species and is supported by a bootstrap value of 97 %, and subclade B, consists of *Vitex turczaninowii Vitex queenslandica* species and is supported by a 75 % bootstrap value (Fig. 7).

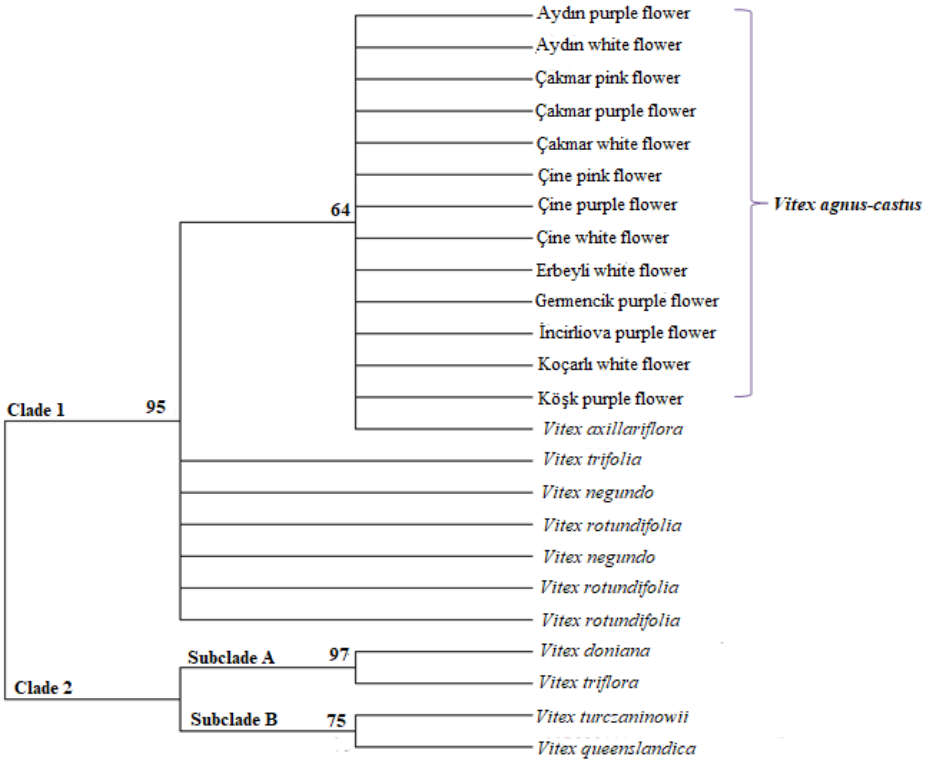


**Fig. 7.** The maximum likelihood tree generated using cpDNA *trn*L intron sequences and other species sequences retrieved from NCBI (Bootstrap values greater than 50 % are given above branches).

For the cpDNA *trn*L-F region, the base lengths of *Vitex agnus-castus* populations ranged from 330 to 353. The genetic distance matrix between populations turned out to be 0.000 (Table 6).

**Table 6.** Pairwise genetic distance matrix obtained from cpDNA *trn*L-F sequences.

| **Populations** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aydın purple flower** | | | | | | | | | | | | | |
| **Aydın white flower** | 0.000 | | | | | | | | | | | | |
| **Cakmar pink flower** | 0.000 | 0.000 | | | | | | | | | | | |
| **Cakmar purple flower** | 0.000 | 0.000 | 0.000 | | | | | | | | | | |
| **Cakmar white flower** | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | |
| **Cine pink flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | |
| **Cine purple flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | |
| **Cine white flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | |
| **Erbeyli white flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | |
| **Germencik purple flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | |
| **Incirliova purple flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | |
| **Kocarlı white flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | |
| **Kösk purple flower** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | - |

The mean nucleotide ratio for the *trn*L-F region was 29.4 % T, 18.1 % C, 32.9 % A, and 19.6 % G. Using the MEGA 6.0 program, Tajma's Neutrality Test (Tajima 1989) was calculated based on cpDNA *trn*L-F sequences of *Vitex agnus-castus* populations. Numbers of sequences (m) gave one segregation site (S) revealing very low nucleotide diversity ($\pi$) of 0.000000 (Table 7).

**Table 7.** Tajima's Neutrality Test Values based on cpDNA *trn*L –F of date *Vitex agnus-castus* populations.

| No. of sequences "m" | No. of segregating sites "S" | *P*s=S/n | $\Theta = p_s/a_1$ | nucleotide diversity '' $\pi$ '' | Tajima test statistic ''D'' |
|---|---|---|---|---|---|
| 13 | 0 | 0.000000 | 0.00000E+000 | 0.000000 | n/c |

The maximum likelihood tree constructed using *trn*L-F sequences consists of two clades (Fig. 8). Clade 1 is divided into 3 subclades in itself. Subclade A consists Aydın purple flowered, Aydın white flowered, Çakmar pink flowered, Çakmar purple flowered, Çine pink flowered and Çine purple flowered populations.

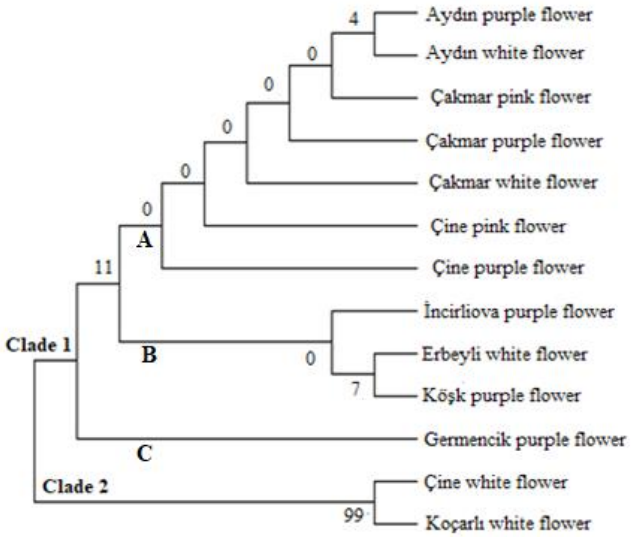


**Fig. 8.** The maximum likelihood tree generated using cpDNA *trn*L-F sequences.

Sevindik *et al*. (2019) detected the Çine purple flowered population in a different group, while all other populations were determined in the same group. In subclade B, Erbeyli white flowered and Köşk purple flowered populations were sister groups, while İncirliova purple flowered populations were close to these two populations. Sevindik *et al*. (2019) identified three populations in different groups. Subclade C consists of only the Germencik purple flowered population. Clade 2 consisted of Çine white flowered and Koçarlı white flowered populations, which was supported by a bootstrap value of 99 %. According to Sevindik *et al*. (2019), these two populations were included in separate groups. In this study, a maximum likelihood phylogenetic tree was generated using *trn*L-F sequences of *Vitex trifolia* (AJ505539.1), *Vitex turczaninowii* (MG836415.1), *Vitex queenslandica* (MG836414.1) and *Vitex axillariflora* (MG836413.1) from NCBI. The relationship of *Vitex agnus castus* populations with other species was revealed. The phylogenetic tree consists of 2 clades (Fig. 9). The first clade consists of *Vitex agnus castus* populations and *Vitex trifolia* species, and this group has a bootstrap value of 94 %. Clade 2 consists of *Vitex turczaninowii*, *Vitex queenslandica* and *Vitex axillariflora* species. In *trn*L intron analysis, *Vitex agnus-castus* populations coexisted with *Vitex axillariflora* and *Vitex trifolia* species. In addition, in *trn*L-F analysis, *Vitex turczaninowii*, *Vitex queenslandica* and *Vitex axillariflora* were found in one group. *Vitex axillariflora* and *Vitex trifolia* species were found together with *Vitex agnus-castus* populations, and *Vitex turczaninowii* and *Vitex queenslandica* species were found in a separate group in *trn*L intron analysis.

Malik *et al.* (2021) determined the phylogenetic relationship of some species belonging to the Lamiaceae family with the chloroplast *rps*14 gene. *Vitex agnus-castus* and *Vitex trifolia* species were found in the same group in the study. In our *trn*L intron and *trn*L-F sequence studies, *Vitex agnus-castus* and *Vitex trifolia* were found in the same group (Fig. 7 and Fig. 9). Ayaz *et al.* (2020) performed phylogenetic analysis of some species belonging to Lamiaceae in Pakistan using the *rps*14 gene. In their study, *Vitex agnus-castus* var *pseudo-negundo* and *Vitex negundo* species were found in the same group. In the ML tree constructed using *trn*L intron sequences and sequences taken from

NCBI, the *Vitex agnus-castus* populations were in the same clade with *Vitex axillariflora* and *Vitex negundo*. Wagstaff *et al*. (1998) used chloroplast *rbc*L and *ndh*F sequences to reveal the phylogenetic relationships of some Lamiaceae taxa. *Vitex agnus-castus* species formed a sister group with *Petitia domingensis* species in the strict consensus tree of *rbc*L and *ndh*F sequences.

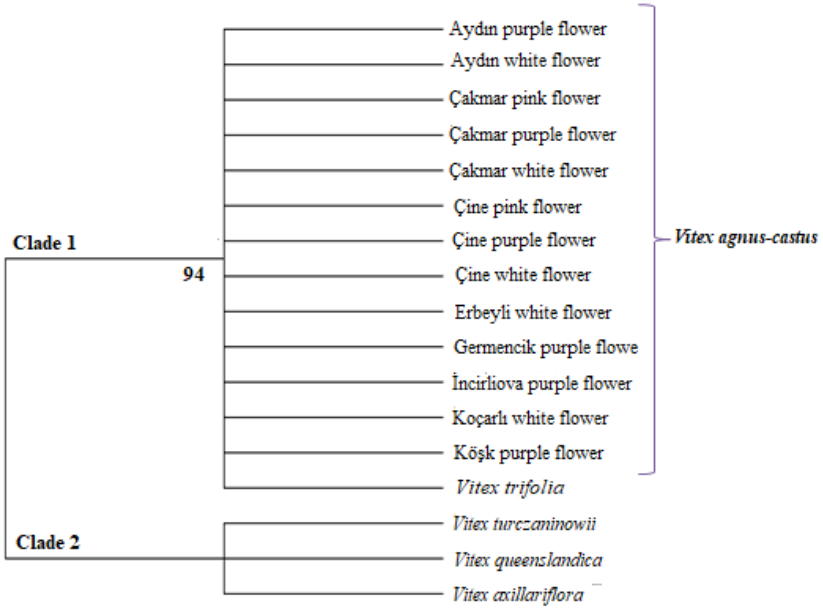


**Fig. 9.** The maximum likelihood tree generated using cpDNA *trn*L-F sequences and other species sequences retrieved from NCBI (Bootstrap values greater than 50 % are given above branches).

## Conclusion

As a result of the study, approximately 61.59 % polymorphism were detected among *Vitex agnus-castus* populations based on ISSR analyzes. In general, the results of this research aiming to analyze the genetic diversity and phylogenetic analysis of *Vitex agnus-castus* populations through ISSR, cpDNA *trn*L intron and *trn*L-F sequence comparisons will be used to determine the phylogenetic relationship between *Vitex agnus-castus* species and other species.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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