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Genetic Characterization of Turkish Commercial Opium Poppy (*Papaver somniferum* L.) Cultivars Using ISSR and SSR Markers

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Abstract – Economically important plant opium poppy (*Papaver somniferum* L.) is a medicinal plant producing benzylisoquinoline alkaloids such as narcotic analgesic morphine and codeine, antimicrobial agent sanguinarine. In this study, it was aimed to utilize simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) markers to help breeding programs of opium poppy containing high level of morphine. 15 ISSR and 5 SSR primers were studied on registered 17 cultivars in Turkey. In ISSR, total 55 bands were obtained, of which 44 were polymorphic. Average genetic distance was found to be 0.27, and Shannon index was 0.38. The least number of bands belonging to primer AT3 was 1 and the highest was 6, belonging to primers AT19, AT8 and AT15. In SSR study, 5 SSR primers were studied and 39 polymorphic bands were obtained. Average genetic distance was found to be 0.47, and Shannon index was 0.46. As a result ISSR and SSR marker systems can be used for classification of the cultivars.

Keywords -
Papaver somniferum L., ISSR, SSR, genotyping, cultivar

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1. Introduction

Opium poppy is a member of *Papaveraceae* and has been recognized for centuries as an important medicinal plant. It is an annual herb that produces benzylisoquinoline alkaloids (BIA) like narcotic analgesic morphine, thebaine and codeine, as well as muscle relaxant papaverine. In addition to being a medicinal plant, it is commercially important because of its seeds and seed oil, which are used in food industry [1, 2]. Turkey is the world's largest opium poppy producer with the %54 of cultivation area. Because of its commercial importance, cultivation is under control in Turkey and is only permitted in restricted areas in 13 towns [3].

The aim of the characterization of plant genetic resources is to reveal the genetic variation among seed samples or populations and to lead to marker-assisted selection. Various molecular marker systems are useful tools for identifying the genetic differences among populations or accessions. Inter simple sequence repeat (ISSR) molecular marker is a PCR-based technique, which uses a single amplification primer composed of a microsatellite motif to target a subset of simple sequence repeat (SSR) or microsatellites [4]. Advantages of SSR over other molecular marker systems are the availability of SSR technique sequences for oligonucleotide synthesis, the protocol of easy implementation that produces reliable and highly detectable amplification products, and their co-dominance and single genome location [5]. SSR and ISSR molecular marker systems have been successfully employed for the analysis of genetic variation, population genetic analysis, and other purposes in various species [6-10] and genus *Papaver* section *Oxytona* [11, 12]. ISSR and Random Amplified Polymorphic DNA (RAPD) molecular markers were studied to detect the genetic diversity and heterozygosity among 24 opium poppy cultivars localized in Rajasthan [13]. In addition, amplified restriction fragment length polymorphic (AFLP) DNA analysis was also studied in various poppy lines [14, 15]. Therefore, these marker systems are useful in identifying the genetic diversity among opium poppy accessions.

However, to date these marker systems have never been used in registered *Papaver somniferum* L. for genetic characterization in Turkey. The primary objective of this study was to use ISSR and SSR marker systems to examine the genetic diversity of 17 registered Turkish cultivars and to find out the correlation among molecular marker data, alkaloid content and morphology.

2. Materials and Methods

2.1. Plant materials

17 registered poppy cultivar seeds (Table 1) were obtained from Prof. Dr. Neşet Arslan, Department of Field Crops, Faculty of Agriculture, Ankara University. The plants were grown in the growth chamber in a 12h photoperiod in Gaziosmanpaşa University Biotechnology laboratory.

2.2. DNA Isolation

Total DNA was isolated from fresh leaves using the Fermentas Genomic DNA Isolation Kit (Fermentas, USA) according to the manufacturers instruction. DNA samples were

quantified by using a spectrophotometer and %1 agarose gel electrophoresis. Isolated DNA with a final concentration of 100 ng mL^{-1} was used for PCR analysis.

2.3. SSR and ISSR analysis

In this study 5 SSR primers were used. Each 25 ml PCR reaction contained 30 ng of genomic DNA template, 10X buffer Mg^{2+}free (Biobasic, CA), 20 mM $MgSO_4$, 10 mM of dNTP, 1 unit of Taq DNA polymerase (Promega, Madison WI, USA), and 0.4 µM Forward and Reverse primer. PCR amplifications were performed in Apollo Instrumentation ATC401 Gradient Thermo cycler. The PCR amplification procedure was performed at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 55-65°C for 30 s, and at 72°C for 30 s. Finally the procedure was extended at 72°C for 5 min. PCR products were separated on %8 denaturing polyacrylamide gel. Electrophoresis was conducted at 300 V, 50 W, 50 mA, and 20°C using a BIO-RAD Protean xi Cell 165-1801.

Table 1. List of poppy accessions used in this study and their morphine contents and origins. *According to this study.

Code*	Cultivars name	Origin	Petal color	Seed color	Capsule morphine content (%)
1	**Kocatepe-96**	Turkey	White	White	High (0.60-0.85)
2	**Ankara-94**	Turkey	White	White	Medium (0.40-0.60)
3	**Ofis-4**	Turkey	White	Yellow	High (0.60-0.85)
4	**Şuhut-94**	Turkey	Light Violet	Blue	High (0.50-0.90)
5	**Anayurt-95**	Turkey	White	Yellow	Medium (0.40-0.60)
6	**TMO-1**	Turkey	White	Yellow	High (0.60-0.85)
7	**TMO-3**	Turkey	Medium Violet	Pink	High (0.60-0.85)
8	**TMO-2**	Turkey	Dark Violet	Grey	High (0.60-0.85)
9	**Afyon-95**	Turkey	White	Yellow	Medium (0.55-0.75)
10	**Ofis-8**	Turkey	White	White	High (0.60-0.85)
11	**Camcı-95**	Turkey	Light Violet	Blue	High (0.58-0.85)
12	**Ofis-95**	Turkey	White	Yellow	Medium (0.55-0.75)
13	**Kemerkaya-95**	Turkey	White	Yellow	Medium (0.50-0.70)
14	**Ofis-96**	Turkey	White	Yellow	Medium (0.55-0.75)
15	**Karahisar-96**	Turkey	White	Yellow	High (0.55-0.85)
16	**Afyon Kalesi**	Turkey	White	Yellow	High (0.55-0.80)
17	**Ofis-3**	Turkey	Light Violet	Grey	High (0.60-0.85)

In the same way, 15 selected ISSR primers used for ISSR analysis. Each 25 ml PCR reaction contained 20 ng of genomic DNA template, 10X buffer Mg^{2+} free (Biobasic, CA), 20 mM $MgSO_4$, 10 mM of dNTP, 1 unit of Taq DNA polymerase (Promega, Madison WI,

USA), and 0.4 μM of each ISSR primer. PCR amplifications were performed in Apollo Instrumentation ATC401 Gradient Thermo cycler. The PCR amplification procedure was performed at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, at 42 – 63 °C for 60 s, and at 72 °C for 2 min. Finally, the procedure was extended at 72 °C for 7 min. Amplification products were characterized on %1 (w/v) agarose gels (immersed) at 120 V for 2 hours.

Amplification products were visualized with ethidium bromide (0.5 g/ml) under UV light and photographed using the Gel-Logic 200 image system (Eastman-Kodak, NY, USA). The sizes of SSR and ISSR fragments were estimated by using 1 kb DNA ladder (SibEnzyme 125 M11, USA) as standards.

2.4. Scoring of the SSR and ISSR products

In ISSR and SSR analysis, the band patterns were scored as present (1) or absent (0) for each primer pair. Only clearly distinguished bands were scored in the analysis. The percentage of polymorphism and Shannon's index were determined for both SSR and ISSR by POPGENE version 1.32 (Population Genetic Analysis) and MEGA 4.0 (Molecular Evolutionary Genetic Analysis) as described [16-20].

3. Results

3.1. ISSR analysis

15 ISSR primers were used to detect the genetic variation among 17 cultivars. All primers generated reproducible bands. A total of 55 bands were obtained, 44 of which were polymorphic. Average genetic distance was found to be 0.27, and Shannon index was 0.38. The least number of bands was 1, belonging to primer AT3 and the highest was 6, belonging to primers AT19, AT8 and AT15. According to data, closest genetic distance was 0.08 and farthest genetic distance was 0.64 between two cultivars. The average genetic distance was found 0.35 while the polymorphism rate was %80.

In this study, two major groups (A and B) were distinguished by ISSR dendrogram (Figure 1). Group A included Kocatepe-96, Ankara-94, Camcı-95, Şuhut-94, Ofis-4 and TMO-1 cultivars while Anayurt-95, Ofis-95, Kemerkaya-95, Karahisar-96, Ofis-96, TMO-3, Ofis-8, Afyon-95, TMO-2, Afyon Kalesi and Ofis-3 clustered in group B. Within these groups, the nearest genotypes were Kemerkaya-95 and Karahisar-96 with 0.0183 genetic distances. Ankara-94 and Ofis-3 were the farthest genotypes with 0.64 genetic distances.

The closest cultivars within the group A are between Camcı-95 and Ankara-94 with 0.157 genetic distances. The farthest cultivars are between Kocatepe-96 and TMO-1 with 0.369. The closest cultivars within the group B are between Kemerkaya-95 and Karahisar-96 with 0.0183 genetic distances while the farthest cultivars are Ofis-3 and Anayurt-95 with 0.452.

3.2. SSR analysis

Analyzing the genetic diversity with 5 SSR primers, a total of 47 bands were obtained, 44 of which were polymorphic. The SSR results showed that the Nei's gene diversity (*H*) was 0.31 and Shannon's information index (*I*) was 0.46. Average genetic distance was found to be 0.47 and polymorphism ratio was %82.98. According to data, nearest

genetic distance was 0.089 between two cultivars and farthest genetic distance was 0.95 among four cultivars.

According to the dendrogram constructed from SSR data, samples were divided into two major groups A and B as shown in Figure 2. Group A consisted of Kocatepe-96, Ankara-94, Anayurt-95, Ofis-3, Ofis-8 and Kemerkaya-95 cultivars and group B consisted of Ofis-4, Şuhut-94, TMO-2, TMO-3, Afyon-95, Afyon Kalesi, Ofis-96, TMO-1, Camcı-95, Ofis-95 and Karahisar-96 cultivars. In these groups, the nearest genetic distance was 0.0889 between genotypes Ofis-4 and Şuhut-94, the farthest genetic distance was 1.21 between Anayurt-95 and Ofis-95.

The closest cultivars within the group A are between Anayurt-95 and Ofis-3 with 0.1611 genetic distances. The farthest cultivars are between Kocatepe-96 and Kemerkaya-95 with 0. 67. The closest cultivars within the group B are between Ofis-4 and Şuhut-94 with 0.089 genetic distances while the farthest cultivars are Ofis-4 and Afyon Kalesi with 0.449.

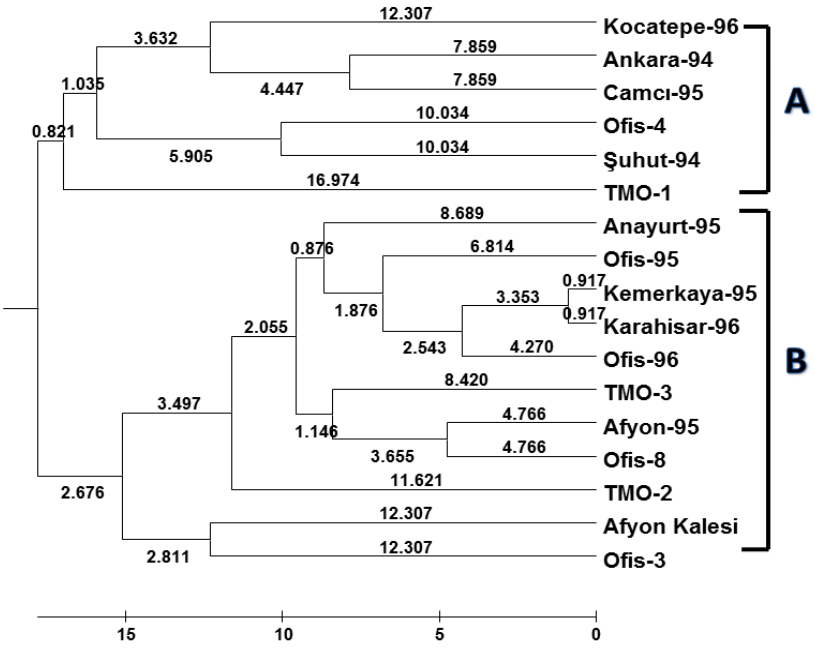

Figure 1. UPGMA dendrogram of seventeen poppy cultivars based on ISSR data.

3.3. Dendrogram from ISSR, SSR and Combined Data

Combination of SSR and ISSR data constructed a new dendrogram (Figure 3). As in others, the dendrogram displayed two groups: group A (Kocatepe-96, Ankara-94, Anayurt-95 and Ofis-3) and group B (Ofis-4, Şuhut-94, TMO-3, TMO-2, Afyon-95, Ofis-8, TMO-1, Camcı-95, Ofis-95, Afyon Kalesi, Kemerkaya-95, Ofis-96 and Karahisar-96). The farthest cultivars were Ankara-94 and Ofis-95, the nearest cultivars were Ofis-4 and Şuhut-94 with the genetic distance 0.6360 and 0.1476, respectively. As a result of this dendrogram, SSR data and SSR + ISSR data showed more correlation than ISSR data.

The closest cultivars within the group A are between Kocatepe-96 and Ankara-94 with 0.281 genetic distances. The farthest cultivars are between Kocatepe-96 and Ofis-3 with 0.405. The closest cultivars within the group B are between Ofis-4 and Şuhut-94 with 0.147 genetic distances while the farthest cultivars are Ofis-4 and Karahisar-96 with 0.42.

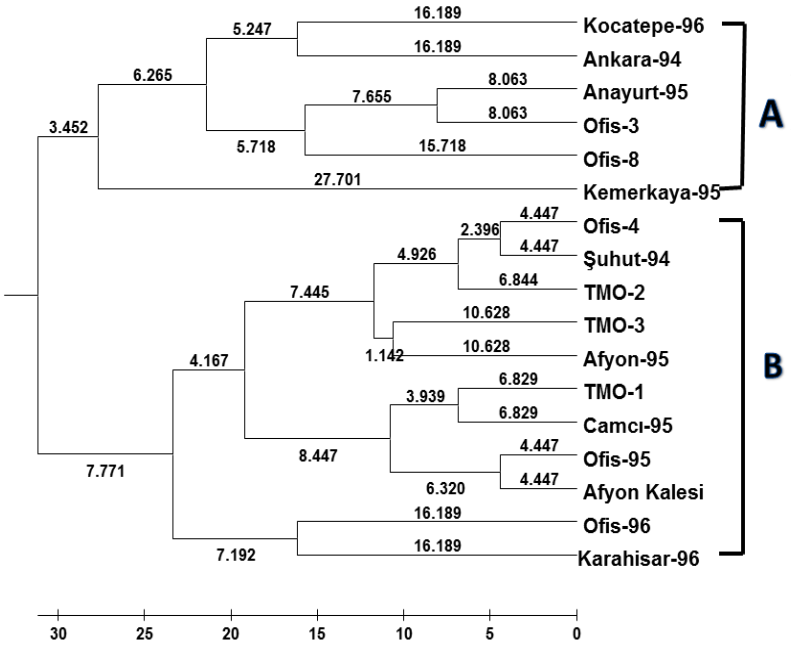

Figure 2. UPGMA dendrogram of seventeen poppy cultivars based on SSR data.

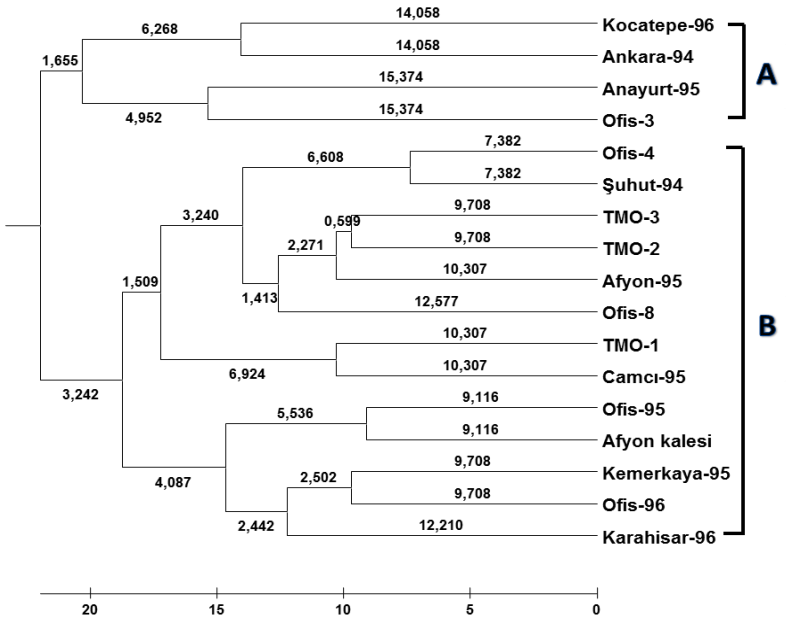

Figure 3. UPGMA dendrogram of seventeen poppy cultivars based on ISSR and SSR mixed data.

4. Discussion

A breeding collection of 17 registered opium poppy used for commercial alkaloid production in Turkey was assessed for genetic variation by SSR and ISSR analysis. Between two marker systems employed, 15 ISSR primers generated a total of 55 bands, 44 of which were polymorphic, whereas 5 SSR primers produced 47 bands, 44 of which were polymorphic. The number of bands produced with SSR primer pairs are more than the ISSR primers, which could be due to the resolving power of SSR primers and the gel electrophoresis system. It has been reported that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system [21].

We found considerably high percentage of polymorphism in both SSR (%82.98) and ISSR (%80) marker systems. The high level of polymorphism at SSR and ISSR loci indicates considerably high genetic variation among these cultivars. The high polymorphism ratio found in our cultivated plants could be due to the cross-pollination and the molecular marker system that we utilised.

This finding is consistent with reports of other studies using molecular markers ISSRs, AFLPs [14]. In the other study reported that the level of average polymorphism in *P. somniferum* L. germplasms was %69.56 with average 2.6 polymorphic bands per primer with RAPDs and that combined RAPD-ISSR analysis showed a cluster of 15 genotypes, indicating that these genotypes have low genetic diversity. These results show that ISSR primers provide more resolving power than RAPD markers [13] and ISSR marker system is more effective than RAPD [22]. On the other hand, it could also be explained by genetic stability of these germplasms.

The combination of these two marker system has been used effectively for the identification of plants, including yard long bean, morus, fig, sugarcane, Ruthenia medic and olive [23-28]. We combined the SSR and ISSR data, because the combined data allow a more thorough analysis of the genetic variations [25, 27, 29]. In Chinese-grown pecan cultivars, ISSR and SSR primers showed highly polymorphism and the dendrogram constructed with combined data was very similar [30]. Our results in combined data showed correlation with the polymorphism rate of %81.37.

The accessions were also evaluated according to their morphological traits and morphine contents because these features are used in registration. Our phylogenetic results showed that, although some cultivars like Afyon-95 and TMO-3 have different morphological traits, they clustered together in the dendrogram. Their petal color showed variation, as seen in the Table 1. Nevertheless, the petal color of TMO-2 is darker than Ofis-3, the cultivars that showed nearest genetic distance. Moreover, the seed color in Afyon-95 is yellow but it is pink in TMO-3.

Kocatepe-96, Afyon Kalesi Şuhut-94 and Ofis-4 cultivars have high morphine contents and their seed and petal colors are particularly different (Table 1). However, Kocatepe-96 and Afyon Kalesi were the farthest accessions; Şuhut-94 and Ofis-4 were nearest accessions in all dendrograms (Figure 1, 2, 3). If we consider the different environmental conditions and

different habitats they grow in, it is not surprising that the accessions cluster in the same group but show different morphology and alkaloid content.

Comparison of dendrograms showed that most of the cultivars in groups generated via three dendrograms have almost similar clustering (Figure 1, 2, 3). The fact that the polymorphism rate of cultivars of the same origin and species is high shows that the genetic stability within the species has not been fully realized. Thus, it is necessary to continue with the breeding programs.

As a result, if cultivars with interrelated morphogenetic properties and high morphine content are crossbred, it will be possible to obtain new cultivars containing higher morphine content. The data obtained from registered 17 varieties of *Papaver somniferum* L. with ISSR and SSR marker systems have revealed high genetic diversity. This study provides useful information for not only germplasm description but also for current breeding programs in Turkey.

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