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Determination of an SSR Marker Set to Distinguish Genotypes of Different Tobacco Classes

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Abstract: Tobacco is produced as cultivated old varieties or local varieties in Turkey. Seed exchanges among farmers who produce their own seeds lead to different names for the same genotypes in different regions or the same name for different genotypes over time. Thus, an efficient cultivar identification system is required in tobaccos grown in Turkey. This study was carried out to determine a marker set to differentiate different tobacco classes grown in Turkey. A total of 14 genotypes from different tobacco classes, eight local varieties and six registered cultivars, were screened using 21 simple sequence repeats (SSR) markers. Seventeen SSR markers produced polymorphic bands while four markers were monomorphic. Average allele number of the markers was 3.38 and PIC values of the markers ranged from 0.000 to 0.741. In dendrogram established using the marker data, genotypes were separated in four distinct cluster. Clusters were made of Virginia or semi-oriental, tombac, oriental and rustica type genotypes. Genetic fingerprinting analyses grouped the genotypes of similar use classes together. According to the results obtained a marker set of eight SSR markers could efficiently and reliably differentiated the tobacco genotypes of different classes.

Keywords: Fingerprint analysis, Microsatellite, *Nicotiana tabacum*, Polymorphism,

Farklı Tütün Tiplerinin Genotiplerini Ayırt Etmek İçin Bir SSR Markör Setinin Belirlenmesi

Öz: Türkiye'de tütün üretimi eski çeşitler veya yerel çeşitlerle yapılmaktadır. Kendi tohumlarını üreten çiftçiler arasındaki tohumluk değişimleri, aynı genotiplerin farklı bölgelerde farklı isimlerle ya da farklı genotiplerin zamanla aynı isimlerle kullanılmasına neden olmuştur. Bu nedenle, Türkiye'de yetiştirilen tütünlerde etkili bir çeşit belirleme sistemine ihtiyaç duyulmaktadır. Bu çalışma, Türkiye'de üretilen farklı tütün sınıflarını ayırt etmek için bir markör seti belirlemek amacıyla yapılmıştır. Farklı tütün sınıflarından sekizi yerel çeşit ve altısı tescilli olmak üzere toplam 14 genotip 21 basit dizi tekrarları (SSR) markörü kullanılarak incelenmiştir. On yedi SSR markörü polimorfik, dört markör monomorfik bantlar üretmiştir. Markörlerin ortalama allel sayısı 3,38 olurken ve PIC değerleri 0,000 ile 0,741 arasında değişmiştir. Markör verileri kullanılarak oluşturulan dendogram genotipleri dört gruba ayrılmıştır. Gruplar Virginia veya yarı-oryantal, tömbeki, oryantal ve rustica tipi genotiplerden oluşmuştur. Genetik parmak izi analizleri, benzer kullanım sınıflarının genotiplerini birlikte gruplandırmıştır. Elde edilen sonuçlara göre sekiz SSR marköründen oluşturulan bir marker seti, farklı sınıfların tütün genotiplerini verimli ve güvenilir bir şekilde ayırt edebilmiştir.

Anahtar kelimeler: Mikrosatellit, *Nicotiana tabacum*, Parmak izi analizi, Polimorfizm,

1. Introduction

Tobacco is one of the most important non-food agricultural products. Turkish tobaccos are used in cigarette blends all over the world because of their rich aroma and other desired quality characteristics. Tobacco production in Turkey is carried out mostly using local cultivars and seeds of these cultivars have been maintained by farmers (Kınay 2014). Seed exchanges among farmers have led to different naming of the same tobacco genotypes after years of production. Similarly, some different genotypes could be produced under the same cultivar name. Growing of different tobacco types in a given area makes it difficult to identify tobacco types to be used in blends.

Tobaccos that belong to *Nicotiana tabacum* (oriental, Virginia, tombac and semi-oriental tobaccos) and *Nicotiana rustica* (Deli tütün and Maraş otu) species are produced in Turkey. Although different types of a tobacco species could be identified morphologically, they could have similar morphological characteristics (color, form, length and width of leaves, plant height, etc.) under the same growing conditions (Peksüslü et al. 2012). Some semi-oriental tobaccos such as tobaccos grown in East and Southeast Regions of Turkey resemble Virginia-Burley type tobaccos morphologically (Arslan and Okumuş 2006). In addition, although Deli tütün and Maraş otu tobaccos of *Nicotiana rustica* species are morphologically similar to katerini type tobaccos, they have quite different quality characteristics. Thus, identification of these tobacco types based on only morphological characters could become inadequate and erroneous.

DNA markers are reliable tools to identify different genetic materials. Simple Sequence Repeats (SSR) markers have advantages for this purpose because of their high polymorphism rates, reliability and reproducibility (Filiz and Koç 2011). SSR markers have been used intensely to determine genetic variation in tobacco (Moon et al. 2009). The first tobacco SSR linkage map was established using 282 polymorphic SSRs out of 637 (PT markers) developed for tobacco (Bindler et al. 2007). Of 5119 subsequently developed SSR markers, 2415 (PT markers) were determined to be polymorphic among 16 different tobacco genotypes of different classes (Bindler et al. 2011). Tong et al. (2012) developed 4886 new SSR markers (TM and TME markers) and found that 892 of them were polymorphic among eight different tobacco types. Besides, Tong et al. (2016) determined that 5 867 of 13 645 SSR markers (TM and TME markers) they developed were polymorphic among 18 tobacco genotypes. Thus, a total of 9 217 SSR markers revealed to be polymorphic among different tobacco types are sufficient for DNA marker studies in tobacco. Therefore, SSR markers are useful tools to distinguish genotypes of different tobacco types.

There are many local tobacco cultivars known by the names where they are grown. Lack of genetic characterization of these genotypes make it difficult to distinguish tobacco types produced. The aim of the present study was to develop an SSR marker set to distinguish tobacco genotypes grown in Turkey in an efficient and reliable manner. This set would be useful both in allowing true-to-name production of tobaccos and use of correct tobacco types in different tobacco blends.

2. Material and Method

Five oriental tobaccos, one Virginia, one tombac, four rustica and three semi-oriental tobacco genotypes used in production in Turkey were used in the present study. Tobacco types used are given in Table 1.

Table 1. Tobacco genotypes from different types used

Çizelge 1. *Araştırmada kullanılan farklı tiplerden tütün genotipleri*

Code	Genotype	Species	Type
1	Sarıbağlar	*N. tabacum*	Oriental
2	Karabağlar	*N. tabacum*	Oriental
3	Xanthi-81	*N. tabacum*	Oriental
4	Nail	*N. tabacum*	Oriental
5	Canik	*N. tabacum*	Oriental
6	Gurs	*N. tabacum*	Semi-oriental
7	Kultik	*N. tabacum*	Semi-oriental
8	Çelikhan	*N. tabacum*	Semi-oriental
9	NC-55	*N. tabacum*	Virginia
10	Tömbeki	*N. tabacum*	Tombac
11	Deli tütün 1	*N. rustica*	Mahorka
12	Deli tütün 2	*N. rustica*	Mahorka
13	Deli tütün 3	*N. rustica*	Mahorka
14	Deli tütün 4	*N. rustica*	Mahorka

Seeds of NC-55 and Xanthi-2A cultivars were obtained from tobacco producing firms in Turkey, while seeds of other genotypes were collected from production fields in Karadeniz, Ege, Akdeniz, Doğu and Güneydoğu regions of Turkey.

DNA studies were conducted in Agricultural Biotechnology Laboratory in Agricultural Faculty of Tokat Gaziosmanpaşa University. Seeds were grown in viols. DNA was isolated from young seedlings using Turkuaz DNA isolation kit (Keskin et al. 2014). Amount and quality of DNA were checked in a 1% agarose gel and spectrophotometer, and DNA

concentrations were adjusted to 50 ng/µl. Polymerase chain reaction (PCR) was performed in a total 20 µl volume. Each reactions contained 0.25 µM of two primers, 0.2 µM dNTP mix, 2.5 mM $MgSO_4$, 10X PCR buffer, 50-100 ng genomic DNA and 0.5 units of *Taq* DNA polymerase (Biobasic). PCR conditions were as follows: after five minutes of hot start at 94 ºC, 30 cycles of 94 ºC for denaturation, 55-60 ºC for primer annealing and 72 ºC for DNA synthesis, each for 30 seconds, and a final extension at 72 ºC for five minutes. Amplicons produced by SSR markers were run on 3% metaphor agarose gels (Lonza cat no: 50180) and visualized by ethidium bromide using a gel imaging system (Vilber Lourmat CN-08). DNA bands were scored using BioCapt v.11.02 software.

PT markers used in the study were selected from markers with high PIC values reported by Moon et al. (2009), Bindler et al. (2007) or Bindler et al. (2011) while TM and TME markers were from Tong et al. (2012). Some information regarding the SSR markers used are given in Table 2.

Table 2. Some general characteristics of SSR markers used

Çizelge 2. *Kullanılan SSR markörlerinin genel özellikleri*

SSR marker	AT (˚C)	Length (bp)	RM	LG
PT20172	55	203	CTT	3
PT20242	55	200	AGG	12
PT30034	55	216	TAA	22
PT30137	55	219	TAA	13
PT30274	55	213	GGA	17
PT30364	58	173	TAA	22
PT30375	55	230	TAA	2
PT30449	55	143	TA	6
PT40005	55	250	GAA	24
PT50182	55	150	TA	1
PT53303	55	200	GA	7
PT61056	55	200	TA	3
TM10013	60	141	ATA	-
TM10181	60	118	AGA	-
TM10211	60	115	ACA	-
TM10654	60	145	TA	18
TM10821	60	132	TTA	8
TM10976	57	139	AAT	13
TM11110	60	123	AAC	2
TM11359	60	137	ACA	-
TME0293	60	127	TCA	11

AT: Annealing temperature, LG: Linkage group, RM: Repeat motif

Genetic distances of tobacco genotypes were determined using Nei's coefficient (Nei 1972) using POPGENE v.1.31 software (Yeh et al. 1997). Genetic distance values were evaluated with UPGMA algorithm and a dendrogram was prepared using POPGENE v.1.31. PIC values of the markers were calculated based on the following formulae: $PIC = 1 - \sum Pi2$, where Pi is the frequency of ith allele (Anderson et al. 1993). An allele less than 5% of all alleles of a locus is considered rare allele (Fricano et al. 2012). An allele found in only one genotype out of the total genotype number of 14 examined genotypes in the present study was considered a rare allele.

3. Results and Discussion

DNA profiling results of 14 tobacco genotypes of different types using 21 SSR markers are given in Table 3. PT30375 produced two bands which were evaluated as two different markers. All other SSR markers had single bands. Gel picture of a marker (TM10181) are given in Figure 1.

Eighteen of the 22 markers (81.8%) (except for TM1135, T30364, PT30449 and TME0293) were polymorphic. Some of them had up to five alleles. These were PT30137, PT30375B, TM10976 and TM10181. PT61056, TM10013, PT30034, PT30274 and PT40005 had four alleles while TM10211, TM10654 and TM10821 had three and PT20172, PT20242, PT50182, PT53303, PT30375A and TM11110 had two alleles. Average number of alleles of polymorphic markers was 3.38 (Table 4). Marker polymorphism rate of 81.8% in the present study was similar to 80% polymorphism rate found by Davalieva et al. (2010) who studied genotypes of different tobacco classes grown in Macedonia. Average allele number of 3.38 in the present study was higher than 3.00 reported by Davalieva et al. (2010), but lower than 6.80 reported by Fricano et al. (2012) studying 312 tobacco genotypes with 49 SSR markers. Allele number is closely associated with number of different tobacco types and genotypes studied. Allele number of 3.38 obtained from only 14 tobacco genotypes in the present study showed high variability among the genotypes used.

Table 3. SSR marker profiles of tobacco genotypes
Çizelge 3*. Tütün genotiplerinin SSR markör profilleri*

Markers	Genotypes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PT20172	B	A	B	A	B	A	A	A	A	B	*	*	*	B
PT20242	B	A	A	A	A	B	B	B	A	A	*	*	*	*
PT30034	C	D	D	D	D	C	C	C	B	A	A	D	D	B
PT30137	D	B	C	E	D	C	D	E	D	E	A	A	A	A
PT30274	D	D	D	B	C	D	C	B	C	B	A	A	A	A
PT30375A	B	B	B	B	B	B	B	B	B	B	A	A	A	A
PT30375B	C	E	C	B	C	D	D	D	E	B	A	B	*	*
PT40005	C	C	C	C	C	A	A	B	D	A	C	C	C	*
PT50182	A	A	A	A	A	A	A	A	A	B	A	A	A	A
PT53303	B	B	B	B	B	A	B	B	A	B	B	B	B	B
PT61056	C	C	C	D	B	A	B	D	D	C	C	C	C	C
TM10013	C	D	B	B	B	B	B	B	D	C	A	A	A	C
TM10181	B	B	E	B	D	C	C	C	D	A	D	B	D	D
TM10211	B	B	A	B	C	B	B	B	B	B	B	B	B	B
TM10654	B	B	B	B	B	A	B	A	B	C	*	*	*	*
TM10821	C	C	C	C	C	B	A	C	B	C	A	A	A	C
TM10976	A	B	E	D	D	D	C	B	A	D	D	C	C	C
TM11110	B	B	A	B	A	A	A	A	A	A	A	A	A	A

***** Marker did not produce an amplicon.

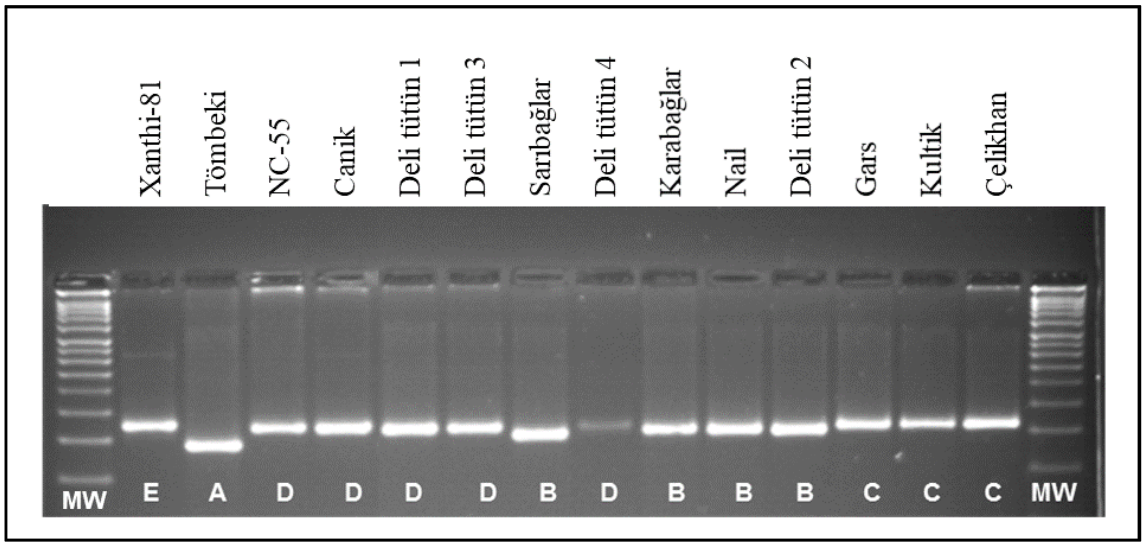

Figure 1. TM10181 SSR marker profiles of the genotypes. The first and last lanes are 50 bp molecular weight marker. Calculated lengths of the produced bands were: A, 91 bp; B, 100 bp; C, 121 bp; D, 136 bp and E, 151 bp.
Şekil 1. *Genotiplerin TM10181 SSR markör profilleri. İlk ve son kulvardakiler 50 bp moleküler ağırlık markörleridir (MW). Üretilen bantların hesaplanan uzunlukları A, 91 bç; B, 100 bç; C, 121 bç; D, 136 bç ve E, 151 bç'dir.*

Polymorphic information content (PIC) value is a measure of marker informativeness. Polymorphic markers in the present study had PIC values varying from 0.124 to 0.741, and the average was 0.509. PT30375B had the highest PIC value (0.741) followed by PT30137, TM10976, PT30274, TM1081, TM10013 and PT30034 markers. Ten of the 22 markers used in

the present study had PIC values over 0.5 (Table 4). Davalieva et al. (2010) found that 10 of the 30 markers they studied had PIC levels over 0.5 and seven markers over 0.65 and an average PIC value of 0.39 in 10 different tobacco types. On the other hand, studying 100 tobacco genotypes with 13 SSR markers, Darvishzadeh et al. (2013) found PIC values over 0.5 in seven markers. Thus, PIC values in the present study appeared to be higher than those in the literature. This finding could primarily be due to the fact that the markers used in our study were selected among the ones with high PIC values based on the previous studies. The fact that genotypes of different tobacco classes from two different species could also have contributed high PIC values in the present study. Such high PIC values reveal high distinguishing power of the markers used.

Table 4. SSR marker results of tobacco genotypes
Çizelge 4. *Tütün genotiplerinin SSR markör sonuçları*

Marker	Polymorphism	Number of total alleles	Number of rare alleles	PIC
PT20172	Polymorphic	2	0	0.365
PT20242	Polymorphic	2	0	0.372
PT30034	Polymorphic	4	0	0.641
PT30137	Polymorphic	5	1	0.726
PT30274	Polymorphic	4	0	0.697
PT30375A	Polymorphic	2	0	0.325
PT30375B	Polymorphic	5	1	0.741
PT40005	Polymorphic	4	2	0.506
PT50182	Polymorphic	2	1	0.124
PT53303	Polymorphic	2	0	0.215
PT61056	Polymorphic	4	1	0.576
TM10013	Polymorphic	4	0	0.655
TM10181	Polymorphic	5	1	0.690
TM10211	Polymorphic	3	2	0.240
TM10654	Polymorphic	3	1	0.468
TM10821	Polymorphic	3	0	0.640
TM10976	Polymorphic	5	1	0.704
TM11110	Polymorphic	2	0	0.280
TM11359	Monomorphic	1	0	0
PT30364	Monomorphic	1	0	0
PT30449	Monomorphic	1	0	0
TME0293	Monomorphic	1	0	0

PIC: Polymorphic information content

A dendrogram showing the relationships of genotypes based on marker data is shown in Figure 2. Basically, four clusters appeared in the dendrogram. The first cluster consisted of NC-55, Gurs, Kultik and Çelikhan cultivars. The second cluster was constituted by tombac genotype. The third cluster comprised oriental tobaccos while the fourth cluster had Deli Tütün 1, Deli Tütün 2, Deli Tütün 3 and Deli Tütün 4 genotypes. Thus, clusters reflected tobacco types investigated. Tobaccos in the first cluster were Virginia and semi-oriental tobaccos. It is thought that semi-oriental tobaccos have developed from crosses between oriental and Virginia type tobaccos. Therefore, close clustering of these two types was an expected situation. The second cluster had tombac tobacco. The third cluster had oriental tobaccos. The fourth cluster included tobaccos of *Nicotiana rustica* species. Bindler et al. (2007) studied different tobacco types using SSR markers and found that known tobacco groups (Burley, Flue-cured, Oriental and Dark) formed different clusters among them. Similar results were reported by Tong et al. (2016) and Fricano et al. (2012).

Only PT30375A marker could easily distinguish rustica type tobaccos (Deli tütün) from other tobacco classes (Table 3). PT30375B marker differentiated semi-oriental tobaccos (Gurs, Kultik and Çelikhan) from other genotypes while PT30034 and PT30375B could identify Virginia type tobacco (NC55). An allele of PT40005 marker was present in only NC-55 and therefore distinguished it from other tobaccos. TM10181 marker could distinguish both tombac and semi-oriental tobaccos from other tobacco types. PT53303 and TM10654 differentiated tombac type tobacco genotypes from others, while a

combined use of TM10281 and TM11110 identified oriental type tobaccos. Davalieva et al. (2010) found that a set of 13 SSR markers could identify large-leaved tobacco genotypes and another set of 14 markers could identify two tobacco types. Relatively low number of markers needed to distinguish all tobacco genotypes could be a result of highly polymorphic SSR markers used in the present study.

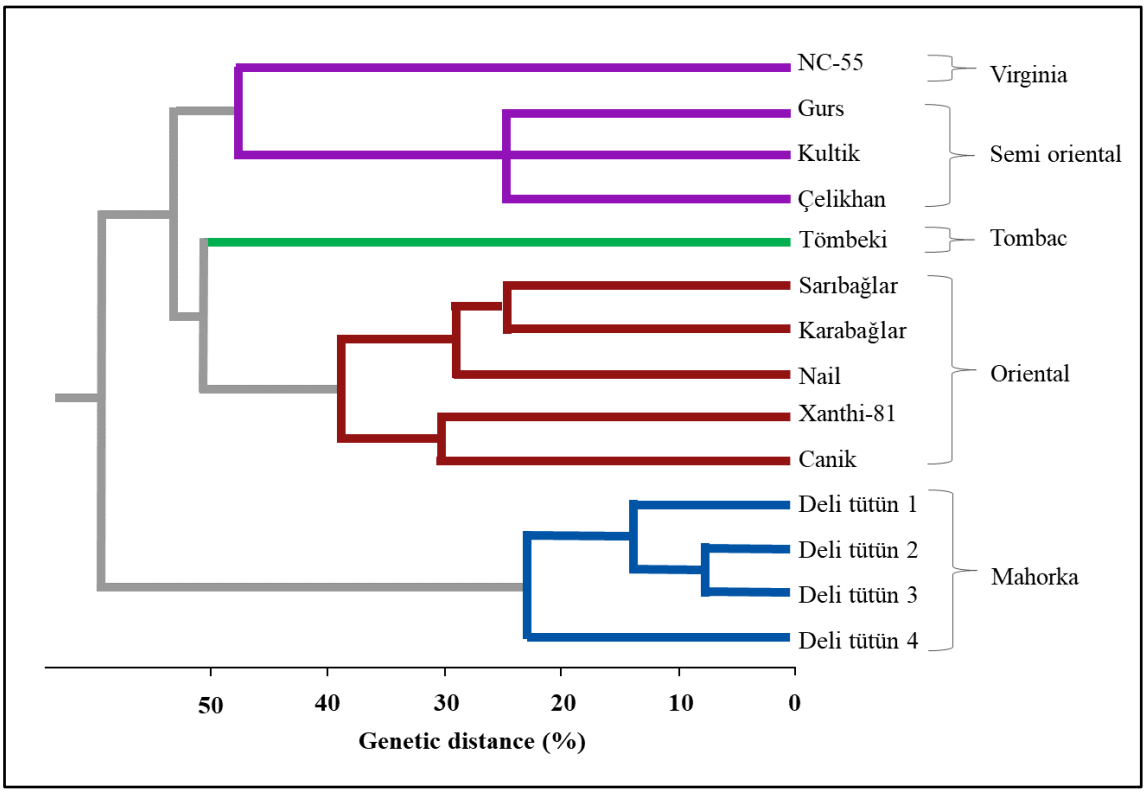

Figure 2. Dendrogram of tobacco genotypes based on SSR marker data. The colors indicate clusters
Şekil 2. *Tütün genotiplerinin SSR markör verilerine dayalı dendrogramı. Renkler kümeleri göstermektedi.*

4. Conclusion

According to the results, SSR marker analyses clustered the genotypes of similar use classes together, indicating powerful and easy use of SSR markers for detection of different classes of tobacco genotypes. A marker set of eight SSR markers (TM10821, TM10013, TM10181, TM10976, PT30034, PT30137, PT30274 and PT30375) was sufficient to differentiate genotypes of all tobacco types grown in Turkey. These markers could identify tobacco genotypes of different classes without using morphological, quality or agronomical characters. Results of the present study indicated that SSR markers are highly efficient tools for DNA fingerprint analysis.

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