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SSR marker-based genetic characterization of Turkish Oriental Tobaccos

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Abstract

Background Turkey is one of the traditional oriental tobacco producing countries. Oriental tobaccos produced in Turkey are ecotypes specific to certain regions, local varieties and landraces, and they have unique characteristics. The present study was conducted to reveal the general and interregional genetic diversity levels of tobacco genotypes collected from different regions of Turkey based on DNA markers.

Methods and Results 319 ecotypes/lines of Turkish Oriental Tobaccos collected from different regions (Aegean Region, Marmara Region, Karadeniz Region, and East and Southeast Anatolia Regions) of Turkey and cultivars Xanthi 81, Xanthi 2A, Canik Sitmasuyu, Katerini, Canik 190-5 and NC 55 were subjected to SSR marker analysis. A total of 89 alleles were obtained from 21 markers examined, and the average number of alleles per marker was 4.05. Nineteen polymorphic SSR markers find outed 314 unique genotypes among 319 tobacco plants evaluated. In the dendrogram constructed SSR marker data, genotypes were divided into six clades in account geographic regions and levels of genetic diversity.

Conclusions The fact that only five of these genotypes were the same as another, in other words, the presence of 314 unique genotypes among 319 tobacco plants evaluated was another finding indicating the high level of genetic diversity in Turkish tobaccos. These genotypes could be tobaccos of different origins, and they merit further agronomic and technological characterizations.

Introduction

Production and curing processes of oriental tobaccos require intensive labor and expertise. Thus, certain countries in the world are concentrated on tobacco production. Turkey is one of the traditional oriental tobacco producing countries. Oriental tobaccos produced in Turkey are ecotypes specific to certain regions, and they have unique characteristics. Genetic characterization of Turkish oriental tobacco ecotypes may contribute to the development of better varieties, as well as to the initiation of detailed agricultural and technological research.

Significant differences in soil and climatic conditions in tobacco production regions have led to the emergence of various tobaccos with different agronomic and quality characteristics . There is a close relationship between the quality characteristics of oriental tobaccos and the ecological conditions of the regions where they are produced. These tobaccos are usually identified by the names of the regions where they are produced. Although oriental tobaccos can preserve some of their morphological and biological characteristics when produced outside the regions they are adapted to, they cannot exhibit their characteristic commercial qualities such as leaf color, size and aroma [1].

Almost all of Turkish tobaccos (98%) is cigarette type belonging to the *Nicotiana tabacum* species. It was reported that tobacco production areas of Turkey are divided into four regions: Aegean, Black Sea, Marmara and East-Southeast Anatolia [2]. Tobaccos in these regions have characteristic leaf size and shape, stem shape, texture, color, taste strength, aroma, chemical content, etc. Tobaccos of Aegean Region have the characteristics of fine-smoking and aroma, and they are used to soften strong blends [3]. Tobaccos of Black Sea Region are used in blends due to their fine color, filling capacity, aroma and cigarette yields [2]. Tobaccos of Marmara Region are among the tobaccos with the highest combustibility and cigarette yields in Turkey. Eastern and Southeastern tobaccos are different from other regional tobaccos in terms of physical, chemicals and blending characteristics. Some of them are similar to Virginia type tobaccos for their aroma and smoking characteristics [2]. There are also productions of tombac and Hasankeyf tobaccos in this region [4].

Microsatellite markers are effectively used in genetic fingerprint analysis. These are PCR markers with high polymorphism rates, highly informative and ease of use [5]. In tobacco, Bindler et al. [6] mapped 282 SSR markers, most of which produced single locus DNA bands. Later, Bindler et al. [7] developed more than 5,000 microsatellit markers, and mapped 2317 of them using an F_2 population. Tong et al. [8] developed a total of 4886 SSR markers from genomic and expressed sequences. Using previously known SSR markers and a DH population, they created a tobacco genetic map consisting of 611 markers in 24 linkage groups. Moon et al. [9] screened 702 tobacco materials of the US *Nicotiana tabacum* germplasm collection, including oriental type tobaccos, with 70 SSR markers, and revealed that polymorphism was found for all the examined markers. The authors identified an average of 14.7 alleles per marker studied. Similarly, Darvishzadeh et al. [10] examined 70 oriental tobacco genotypes from the Middle East and the Balkans with 26 SSR markers, and found polymorphism in all the markers studied, with an average of 2.53 alleles per marker. Examining 30 markers in 10 oriental tobacco varieties from Macedonia, Davalieva et al. [11] observed polymorphism for 80% of the markers with an average of three alleles per

marker. These results indicate that there is a high level of genetic variation in *Nicotiana tabacum* in general and in oriental tobaccos in specific, which is a group within this species.

There is a considerable level of morphological and phenological diversity in oriental tobacco production areas of Turkey. In addition to the differences in environment and agricultural practices, the fact that the oriental tobaccos produced in Turkey are mainly ecotypes, local varieties and landraces contribute this diversity. The aim of the present study was to reveal the general and interregional genetic diversity levels of 319 tobacco genotypes collected from different regions of Turkey based on DNA markers.

Materials And Methods

Seeds belonging to 319 ecotypes/lines collected from different regions (seven provinces in Aegean Region, four provinces in Marmara Region, six provinces in Karadeniz Region and ten provinces in East and Southeast Anatolia Regions) of Turkey (Supplementary Table 1a, b, c, d) and cultivars Xanthi 81, Xanthi 2A, Canik Sitmasuyu, Katerini, Canik 190-5 and NC 55 were subjected to SSR marker (Table 1) analysis. The collected seeds were planted in pots in a greenhouse and DNA was obtained from the leaves of single plants in early seedling stage using Turkuaz DNA isolation kit [12]. These DNAs were run on the gel, analyzed in a spectrophotometer, and their quality and quantity were determined. DNA concentration was adjusted to 50 ng/µl.

Table 1
SSR markers used in the study and their characteristics

SSR marker name	Chromosome	Forward	Reverse	Primer annealing temperature (°C)	Product size (bp)	Microsatellite repeat
PT20172	3	acacctccttcttcctgc	ccaaaatggttcactgga	55	203	CTT
PT20242	12	tccaaagttggaccagaa	gtcctacatggggctctt	55	200	AGG
PT30034	22	gacgaaactgaggatattccaaa	tggaaacaaagccattaccc	55	216	TAA
PT30137	13	tttggtgaggtgttacgataaaga	tccacaccaaacatcaacttt	55	219	TAA
PT30274	17	tgacagctaagctaataacagtaaatg	ggactttggagtgtcaaatgc	55	213	GGA
PT30364	22	cactttcaagttcgtcacgc	atatgttgacgacgacccgt	58	173	TAA
PT30375	2	tcctctacccaacgtcaagaa	ggcaaaccagctagcacat	55	230	TAA
PT30449	6	cgagaatctatgagcgagcc	gctcacatgctgatattctctca	55	143	TA
PT40005	24	tgatcacacttgatagcctaaagaa	cgcacgacctatacccattt	55	250	GAA
PT50182	1	tgctttggtataatttatttcttacg	gctggtcaaagagaggtgtca	55	150	TA
PT53303	7	gtaaggtgtccggagctgaa	acataaatgcaacgcatgga	55	200	GA
PT61056	3	tccaatctttacacaattagtcgttc	tggcttctctgtctagggagg	55	200	TA
TM10013	-	tggaattccggttatgtcct	ttgaaatagcgcgtaccctaa	60	141	ATA
TM10181	-	gtggtttgatcttcttcccatt	ggaattaaccaccaccatgc	60	118	AGA
TM10211	-	atccggacgaggctatctct	gcaggggtaaggtctgcat	60	115	ACA
TM10654	18	atggggcccacatagtgtat	ggtcttggatcatgagagaacc	60	145	TA
TM10821	8	gcaaacatctcaggattccac	ggcctctggatctggtatga	60	132	TTA
TM10976	13	cccgtcccattagataacca	tatgccttggcgtttttagg	57	139	AAT
TM11110	2	aaacccgttggcaattgat	gtcagcctccccacctatg	60	123	AAC
TM11359	-	cgaacgctacggtcagattt	catatttccctccccagacc	60	137	ACA
TME0293	11	aaggaggagcaggaccaact	tggagccatttattgtcaagc	60	127	TCA
*Primers starting with PT are from Moon et al. [9], Bindler et al. [6] or Bindler et al. [7]						
Primers starting with TM and TME from Tong et al. [8].						

Table 1. SSR markers used in the study and their characteristics

PCR reactions were performed in a total volume of 40 µl. In the PCR reaction, 250 nM of each primer and 0.2 mM of each nucleotide were used. The reactions also had 1.5 mM $MgCl_2$, 50–100 ng genomic DNA and 0.5 units of Taq-DNA polymerase. A typical PCR cycle was as follows: after a five-minute hot start at 94 °C, 32 cycles of 94 °C for 30 seconds, 55–60 °C for 30 seconds and 72 °C for 30 seconds [5]. A five-minute primer extension at 72 °C was performed after the cycles.

Amplicon lengths obtained after the PCR reaction were determined using a 3% MetaPhor Agarose gel system containing ethidium bromide. Gel image of PT20242 marker is given in Fig. 1. DNA band profiles were determined by a gel image system (Vilber Lourmat CN-08) and scored using a gel analysis software (Biocapt Version: 11.02). The relationships of the genotypes were determined by dendrogram created using the unweighted pair group method using arithmetic mean (UPGMA) algorithm specified by Sneath and Sokal [13]. PopGene version 1.31 software was used to construct the dendrogram (https://sites.ualberta.ca/~fyeh/).

Polymorphic information content values (PIC) were calculated using the following formula to determine the informativeness of the markers used:

PIC = 1 − $\sum P_i^2$ where P_i is the frequency of i^{th} allele [14].

Results

Genetic similarities were analyzed using 21 SSR markers in the study. Three of these primers produced monomorphic bands in the studied genotypes (Table 2). One of the 18 polymorphic markers (PT30375) produced two scorable bands, and they were evaluated as two separate markers. Most of the remaining 17 primers gave single band, but weak secondary bands were observed in some primers which were not taken into account for scoring purposes. Among the 19 loci from 18 SSR primers showing polymorphism in the studied genotypes, the highest number of alleles was obtained from TM10976 with eight alleles. This marker was followed by PT30137, PT30375B, PT61056, TM10181 and TM10654 with six alleles each. A total of 89 alleles were obtained from 21 markers examined (86 alleles from 19 polymorphic loci), and the average number of alleles per marker was 4.05.

Table 2
Results of SSR loci evaluated in the study

Locus	Polymorphism	Number of total alleles	Number of rare alleles	PIC
PT20172	Polymorphic	3	1	0.515
PT20242	Polymorphic	5	2	0.582
PT30034	Polymorphic	5	1	0.673
PT30137	Polymorphic	6	2	0.727
PT30274	Polymorphic	5	2	0.655
PT30375A	Polymorphic	3	2	0.054
PT30375B	Polymorphic	6	0	0.800
PT40005	Polymorphic	5	1	0.736
PT50182	Polymorphic	2	1	0.024
PT53303	Polymorphic	3	0	0.311
PT61056	Polymorphic	6	2	0.644
TM10013	Polymorphic	4	1	0.632
TM10181	Polymorphic	6	1	0.742
TM10211	Polymorphic	4	1	0.564
TM10654	Polymorphic	6	1	0.691
TM10821	Polymorphic	4	0	0.537
TM10976	Polymorphic	8	2	0.820
TM11110	Polymorphic	3	1	0.469
TM11359	Polymorphic	2	1	0.018
PT30364	Non-polymorphic	1	0	0
PT30449	Non-polymorphic	1	0	0
TME0293	Non-polymorphic	1	0	0
Average/locus		**4.05**	**1.00**	**0.463**

Figure 1. DNA profiling of some Turkish tobacco lines using SSR marker PT20242. MW: 50 bp molecular weight marker.

Alleles that constitute less than 5% of all alleles of a locus are called rare alleles [15]. Abundant rare alleles of a marker reduce its discriminating power. Among the polymorphic markers (with more than one allele) in the present study, the ratio of rare alleles to total alleles ranged from 0% (TM10821, PT53303 and PT30375B) to 67% (PT30375A with two rare alleles out of three alleles). A total of 36

heterozygous loci were identified in 319 genotypes evaluated with 19 markers in the study. Only four of the genotypes (M27, B33, B82 and E7) were found to have two heterozygous loci, while 32 genotypes had only one heterozygous locus, and none of them had more than two. The highest PIC value, which is a measure of the informativeness of the markers used on genetic characterization, was obtained from the TM10976 marker as 0.820. This marker was followed by PT30375B as 0.800, TM10181 as 0.742, PT40005 as 0.736 and PT30137 as 0.727.

According to the dendrogram showing the genetic closeness of the genotypes based on marker profiles (Fig. 2), genotypes A95 and A96, A106 and A107, M1 and M2, M5 and M6, and B56 and B57 were identical. All others were unique genotypes. The dendrogram could be divided into six clades based on geographic regions and levels of genetic diversity. The first clade was the largest one and consisted of a total of 162 genotypes. Within this clade, there were three groups of Aegean tobaccos and one group each of Marmara and Black Sea tobaccos. The Clade II consisted of a cluster of 12 East-Southeast genotypes (between E33-E17) and a cluster of 8 Black Sea genotypes (between B2 and B48). There were also some scattered genotypes of all regions in this clade. Clade III consisted of an East-Southeast tobacco cluster of 16 genotypes (between E13 and E28) and also scattered tobaccos of all regions. Clade IV mostly consisted of Black Sea tobaccos. There were 27 Black Sea tobaccos in this clade (genotypes between B41 and B5). Clade V included six diverse genotypes (four Aegean and two Black Sea genotypes). Clade VI had *N. rustica* and some other genotypes only. Some genotypes in Clade VI (E9, E10, E11 and E23) were previously known to belong to *N. rustica* species. Also known as strong tobacco or Aztec tobacco, these tobaccos were easily separated from other genotypes in the dendrogram. Genotypes E54 and E55, named Kılıc/Kurucuova and Hasankeyf/Gaziantep, which were not known to belong to this species before the study, should also belong to *N. rustica* according to their dendrogram locations. Five genotypes (A13, A17, A40, A112 and B74) were remarkably distant from other *N. tabacum* genotypes and were in a place between *N. tabacum* and *N. rustica* genotypes. Genotype A40 was even closer to *N. rustica* genotypes than it was to other *N. tabacum* genotypes.

Table 2. Results of SSR loci evaluated in the study

Figure 2. Dendrogram based on SSR marker data of 319 Turkish Oriental tobaccos.

Aegean region tobaccos were in three clusters in Clade I. The first cluster contained 51 genotypes between Genotype A1 and A54 on the dendrogram (Aegean I). The second cluster was very close to the first one and consisted of eight genotypes between A14 and A117 genotypes (Aegean II). The third cluster consisted of 31 genotypes between genotypes A2 and A47 (Aegean III). Studied 19 polymorphic markers produced a total of 74 alleles in 123 genotypes (0.60 alleles/genotype) collected from the Aegean region (Table 3).

Table 3
Number of alleles produced by 19 polymorphic SSR loci in tobaccos of different regions in Turkey

SSR locus	Aegean (123 genotypes)	Marmara (50 genotypes)	Black Sea (89 genotypes)	East-Southeast (57 genotypes)	Number of Total alleles per marker
PT20172	3	2	2	2	3
PT20242	3	5	3	5	5
PT30034	4	4	4	5	5
PT30137	5	4	4	4*	6
PT30274	4	3	4	5*	5
PT30375A	2	1	2	3*	3
PT30375B	6	6	5	6	6
PT40005	5	4	4	3	5
PT50182	2	1	1	1	2
PT53303	3	3	3	2	3
PT61056	4	4	5	5	6
TM10013	3	3	3	4*	4
TM10181	5	4	5	6	6
TM10211	4	4	4	2	4
TM10654	6	5	6	6	6
TM10821	3	3	4	4	4
TM10976	7	5	7	5	8
TM11110	3	3	3	3	3
TM11359	2	1	1	1	2
Total	74	65	70	72	86
Allele/genotype	0.60	1.30	0.79	1.26	0.27

* One of the alleles is from *N. rustica* genotypes.

Marmara tobaccos constituted only one main cluster which was located in Clade I between the genotypes M35 and M18 on the dendrogram. Other tobaccos of Marmara region were dispersed in other clades. The 50 genotypes collected from the Marmara region had a total of 65 alleles for the 19 polymorphic markers examined, i.e., 1.30 alleles per genotype (Table 3).

Black Sea Region tobaccos were clustered as three groups on the dendrogram. The largest cluster was in Clade IV (Black Sea III) and consisted of 27 genotypes. The second cluster was in Clade I and consisted of 12 genotypes between B20 and B38 on the diagram (Black Sea I). The third cluster had eight genotypes in Clade II between B2 and B48 (Black Sea III). Other tobaccos of the region were scattered in other clades. The 89 genotypes collected from the Black Sea Region had a total of 70 alleles for the 19 polymorphic markers examined (0.79 alleles per genotype, Table 3).

Eastern and Southeastern tobaccos, on the other hand, featured a rather dispersed trend. The largest cluster included 16 genotypes located between genotypes E13 and E28 in Clade II on the dendrogram. The second cluster was in Clade II and included 12 genotypes from E33 and E17. The third cluster was made of *N. rustica* and similar genotypes in Clade VI. The 57 genotypes collected from this region produced a total of 72 alleles for the 19 polymorphic markers examined (1.26 alleles per genotype, Table 3). Four of these alleles came from six *N. rustica* genotypes (E9, E10, E11, E23, E54 and E55) and were not found in other genotypes of the region. The total 68 alleles from 51 *N. tabacum* genotypes collected from this region (1.33 alleles per genotype) were very close to 74 alleles produced by 123 tobaccos genotypes from Aegean region.

Table 3. Number of alleles produced by 19 polymorphic SSR loci in tobaccos of different regions in Turkey

Discussion

Tobacco producers in Turkey obtain their seeds from different sources. Certified seeds of registered varieties constitute only a small part of the seeds used. Producers usually get the seed from their own field or from their neighbors [16]. Sometimes it is procured from private companies under whose contract the production is made [17]. However, farmers consider the seeds given by the companies insufficient and mix them with the seeds from their own fields. The fact that producers obtain tobacco seeds from different sources and that there is constant exchange between the farmers as well as between the production areas causes great genetic diversity in tobacco production areas in Turkey.

The polymorphism rate of 85.7% for the markers observed in the present study (18 out of 21 markers), is similar to the 80% found by Davalieva et al. [11] who evaluated 10 oriental tobacco cultivars from Macedonia using 30 markers and the 100% found by Darvishzadeh et al. [10] who examined 70 oriental tobacco genotypes from the Middle East and the Balkans with 26 SSR markers. Evaluating 702 *Nicotiana tabacum* genotypes, including oriental tobaccos, with 70 SSR markers, Moon et al. [9] found polymorphism in all the markers examined. These results indicate the high level of genetic variation in *Nicotiana tabacum* in general and in oriental tobaccos in particular, which constitute a subgroup within this species.

The total number of alleles and the average number of alleles per marker in the present study are similar to the other studies performed on genotypes of N. *tabacum*. Davalieva et al. [11] found that the markers had 1–6 alleles (average 3.0) with 30 SSR markers in 10 oriental tobacco cultivars. Darvishzadeh et al. [10] examined 70 oriental tobacco genotypes with 26 SSR markers and found 2–3 alleles per marker (mean 2.53). Analyzing 135 genotypes from the Flue-cured Virginia seed collection with 25 SSR markers, Ganesh et al. [18] identified a total of 85 alleles (3.4 alleles per locus). However, examining 702 *Nicotiana tabacum* genotypes from all eight market classes (flue-cured, burley, dark air-cured, dark fire cured, Maryland, cigar filler, cigar wrapper and oriental) with 70 SSR markers, Moon et al. [9] identified between 2 and 41 alleles per marker (average 14.7). Although this number is considerably higher than the number determined in the present study, it is an expected result considering the fact that the genotypes examined by Moon et al. [9] included very different type of tobaccos.

All but four of the 325 genotypes examined can be distinguished by only nine of the markers used in the study (PT30375, TM10976, TM10181, PT30137, PT40005, TM10654, PT30034, PT61056 and TM10013). These nine markers form a discrimination set to distinguish oriental tobaccos. The mean PIC value for all the studied markers was 0.463. In another study on oriental tobaccos, Davalieva et al. [11] found an average PIC value of 0.39. Accordingly, based on PIC values, the genetic diversity level of SSR markers on Turkish oriental tobaccos can be considered high. However, our mean PIC value of 0.463 was considerably lower than 0.736 determined by Moon et al. [9]. Considering the fact that Moon et al. [9] evaluated tobaccos of very different origins, it could be stated that genetic diversity level among our material as revealed by SSR markers were considerably high. Nine markers (PT30375, TM10976, TM10181, PT30137, PT40005, TM10654, PT30034, PT61056 and TM10013) form a discrimination set to distinguish oriental tobaccos.

Considering the high polymorphism rates of the markers in the study, true heterozygotes would have been expected to be heterozygous at a greater number of loci for the 19 markers examined, if they had not been outcrossed several generations earlier. Observed heterozygosity of the loci could be due to the reading errors as a result of strong stutter bands, an inherent problem of SSR markers [19]. Therefore, it could be stated that all genotypes examined were homozygous. The fact that only five of these genotypes were the same as another, in other words, the presence of 314 unique genotypes among 319 tobacco plants evaluated was another finding indicating the high level of genetic diversity in Turkish tobaccos. These genotypes could be tobaccos of different origins, and they merit further agronomic and technological characterizations.

One hundred twenty-three Aegean genotypes had an average of 0.6 alleles per genotype studied, which means relatively low genetic diversity among Turkish oriental tobaccos. Aegean tobaccos constitute the main export tobaccos in Turkey. Tobacco seeds are mostly distributed by companies which carry out the exports. There are also some seeds obtained from Aegean Agricultural Research Institute. Only in some limited areas, producers use traditional seeds in production. Thus, the diversity is low among the tobaccos of Aegean region.

Black Sea tobaccos (89 genotype) have a moderate level of diversity with 0.79 alleles per genotype. The tobaccos of this region are also exported. Habit of producing their own seeds or seed exchange between them is more common among the farmers of this region compared to Aegean farmers. The Black Sea region of Turkey traditionally has Basma type tobaccos. However, starting from 2002,

tobaccos of Greece origin started to be grown in the region. Greek Basma tobacco is one of these tobaccos [20]. The producers preferred these genotypes over time, and genetic hybridizations took place between native and Greek Basma tobaccos, which increased the genetic diversity to some extent [21].

Tobaccos of Marmara (50 genotypes) and Eastern - Southeastern (57 genotypes) Regions have quite high levels of genetic diversity with an average of 1.30 and 1.26 alleles, respectively. Tobaccos of these regions are mostly used for domestic cigarette production. Tobaccos of Marmara region include sessile and petiolate types as well as the intermediate ones. Eastern and Southeastern Anatolia production areas of Turkey had tobacco introduction from both Europe and the Middle East [22]. While other regions have only *Nicotiana tabacum* L. genotypes, *N. rustica* types are also grown in this region [23]. The presence wide variety of ecologies in the region has led to the emergence of genetically distant ecotypes. Especially one group of tobaccos, called semi-oriental type, were reported to have high levels of variation for morphological and chemical properties [2, 24]. As a result, the region had the highest level of genetic diversity among Turkish tobaccos.

Conclusion

There is a considerable level of genetic diversity within tobaccos grown in Turkey. Although most of these tobaccos are of oriental type, there are some *N. rustica* tobaccos as well as some semi-oriental tobaccos. Based on DNA marker data, tobaccos of different regions in Turkey formed small groups which were dispersed in different clades of dendrogram. Tobaccos from Marmara and East-Southeast region had almost twice the genetic diversity levels of Aegean and Black Sea Region tobaccos based on the number of alleles produced by 19 polymorphic SSR loci.

Declarations

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Supplementary Information

The online version contains supplementary material available at (Supplementary Table 1).

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Data availability

The datasets generated in the present study are available from the authors upon request.

Authors' contributions

NK, SA, and AKA conceptualized, designed the research. AK collected the ecotypes/lines. IS and NK performed experiments, analyzed data, and wrote the manuscript.

Code availability

Not applicable.

Declarations

Conflict of interest

The authors declare no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate and publish

All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Figures

Figure 1

DNA profiling of some Turkish tobacco lines using SSR marker PT20242. MW: 50 bp molecular weight marker.

Figure 2

Dendrogram based on SSR marker data of 319 Turkish Oriental tobaccos.

Supplementary Files

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