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Genetic variation in oriental tobacco (*Nicotiana tabacum* L.) by agro-morphological traits and simple sequence repeat markers[1]

Variação genética em tabaco oriental (*Nicotiana tabacum* L.) por marcadores agro-morfológicos e traços simples de repetição de sequência

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ABSTRACT - The objectives of this study were to assess genetic diversity and determine differences between several oriental tobacco genotypes by examining both agro-morphological traits and molecular markers. Simple lattice design with two replications was used to evaluate 100 oriental tobacco genotypes. Analysis of variance manifested that there is high level of genetic diversity in oriental-type tobaccos based on morphological traits including number of leaf, days to 50% flowering, leaf length, leaf width, leaf fresh weight, leaf dry weight, stem height and stem girth. Classification of genotypes using agro-morphological data by means of un-weighted pair-group method using arithmetic average (UPGMA) algorithm based on squared standardized Euclidean distances resulted four distinguishable groups that pursuit own geographical distribution. In the molecular marker investigations, a total of 13 simple sequence repeats (SSR) primer pairs were used to determine polymorphism of the test germplasm. Thirty five alleles were scored at 13 SSR loci. The average number of alleles per locus (na) and the effective allele number (Ae) were 2.69 and 2.34, respectively. By using SSR data, pair wise Jaccard's similarity coefficients were produced. Grouping of genotypes via Jaccard's similarity coefficients and using UPGMA clustering method lead to three groups that had not any accommodated with own origins. Results reveled that there is not completely agreement for classification based on agro-morphological and SSR loci in oriental-type tobaccos. Because of non influence of environmental effects on molecular marker, heterotic groups based on SSR markers could be closer to reality.

Key words: Cluster analysis. Euclidean distance. Genetic diversity. Jaccard's similarity. Oriental tobacco.

RESUMO - Os objetivos deste estudo foram avaliar a diversidade genética e determinar as diferenças entre diversos genótipos de tabaco oriental através tratos morfológicos e marcadores moleculares. O delineamento utilizado foi o látice simples com duas repetições e foram avaliados 100 genótipos de tabaco simples. As análises de variância mostarram que há pouca alto nível de diversidade genética nos tabacos to tipo oriental baseado nos tratamentos morfológicos incluindo número de folhas, dias para os 50% do florescimento, comprimento da folha, largura da folha, peso fresco da folha, peso seco da folha e maior comprimento e espessura do caule. A classificação dos genótipos usando o método de dados morfológicos por médias de grupos pareados não ponderados usando média aritmética (UPGMA) algoritmo baseado nas distâncias Euclidianas quadradas padronizadas resultou quatro grupos distintos de acordo co a própria distribuição geográfica. Nas investigações dos marcadores moleculares, um total de 13 repetições de sequências simples (RSS) de iniciadores pares foram utilizados para determinar o polimorfismo do germoplasma de teste. Trinta e cinco alelos foram marcados em 13 locos RSS. O numero médio de alelos por loco (na) e o número de alelo efetivo (Ae) forma 2,69 e 2,34, respectivamente. Utilizando os dados RSS, coeficientes de similaridade de Jaccard foram produzidos. O agrupamento de genótipos através dos coeficientes de similaridade de Jaccard e usando o método de agrupamento UPGMA que considera três grupos que não tiveram algum alojamento com suas próprias origens. Os resultados revelaram que revelaram que não estão completamente de acordo para classificação baseada em agro-morfológicas por loco e RSS em tabacos do tipo oriental. Por causa da não influencia dos efeitos ambientais no marcador molecular, grupos heteróticos com base em marcadores SSR poderiam estar mais próximos da realidade.

Palavras-chave: Análise de agrupamento. Diversidade genética. Distância Euclidiana. Similaridade de Jaccard. Tabaco oriental.

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INTRODUCTION

Nicotiana spp. is one of the most important nonfood crops that are widely cultivated worldwide (MOON *et al*., 2009). It belongs to family *Solanaceae* which has more than 64 species, being *Nicotiana tabaccum* one of the most cultivated species among them (REN; TIMKO, 2001). It was proven that *N. tabaccum* is natural amphidiploid (2n = 4x = 48) arisen by hybridization of wild progenitor species (*N. sylvestris* × *N. tomentosiformis*). Numerous types of tobacco are defined by different criteria such as region of production, intended use in cigar (i.e., filler, binder and wrapper) and cigarette manufacturing, method of curing (flue-, air-, sun- and fire-cured tobacco) as well as morphological and biochemical characteristics (i.e., aromatic fire-cured, bright leaf tobacco, Burley tobacco, Turkish or oriental tobacco) (REN; TIMKO, 2001). Turkish or oriental tobacco has a much milder flavor and contains less nicotine and fewer carcinogens than other varieties (DAVIS; NIELSEN, 1999). In order to get an American Blend type of cigarette, it is mixed with more robust tobacco such as Virginia and Burley tobacco.

The study of genetic diversity of tobacco is of interest for the conservation of genetic resources, broadening of the genetic base and practical applications in breeding programs. Several traits such as agro-morphological (WENPING *et al*., 2009; ZHANG, 1994; ZEBA; ISBAT, 2011), chemical and cytological traits (DARVISHZADEH *et al*., 2011; EL-MORSY *et al*., 2009; OKUMUS; GULUMSER, 2001) have already been used to study the genetic variation of tobacco germplasm. This is noticeable that agro-morphological traits usually vary with environment and the number of chromosomal characters is limited (LU, 1997). With the emergence of molecular markers such as amplified fragment length polymorphism (VOS *et al*., 1995), simple sequence repeat (THOMAS; SCOTT, 1993), and inter simple sequence repeat (PRADEEP REDDY *et al*., 2002), this is possible to evaluate genetic divergence of plant germplasm in greater detail. In this sense, several studies were used molecular markers (DAVALIEVA *et al*., 2010; JULIO *et al*., 2006; REN; TIMKO, 2001; YANG *et al*. 2007; YAO ZHANG *et al*., 2008) to reveal genetic diversity of *N. tabaccum*. Molecular markers are stable and detectable in all tissues, regardless of growth, differentiation and development or stage of the cell. They are not subject to environmental, pleiotropic or epistatic effects (AGARWAL *et al*., 2008; MOOSE; MUMM, 2008). With the advent of high-density SSR maps for tobacco it is feasible to estimate genetic variation with a large number of markers that are well distributed across the tobacco genome (BINDLER *et al*., 2007). Recently, SSR as a reproducible, codominant, wide genome coverage and multi allelic marker has been successfully employed to reveal genetic variation of chewing tobacco genotypes (SIVA RAJU, 2011). Davalieva *et al*. (2010) could classify 10 tobacco genotypes into three groups using 24 microsattelite markers. The aim of this research was to employed SSR technique and agro-morphological traits simultaneously to assess the genetic variation of different local and exotic oriental tobacco genotypes belong to Urmia Tobacco Research Center of Iran.

MATERIAL AND METHODS

Field experiment

One hundred genotypes of tobacco (*Nicotiana tabacum* L.) with different growth-type and origins were investigated under filed conditions (Tabela 1).

Table 1- Name and origin of tobacco genotypes

Genotype	Origin	Genotype	Origin
C.H.T.269-12e	Iran	Pobeda 1	Russian
Ts 8	-	Pl 7	Bulgaria
F.K.40-1	Iran	L 17	Bulgaria
Samsun 959	Turkey	Melnik 261	Bulgaria
Samsun katenizi	Turkey	Triumph	Yugoslavia
Try-Kula	-	B.S.31	Greece
ss-289-2	-	SPT 403	Iran
B 12-2	Iran	SPT 405	Iran
B 16-10	Iran	SPT 406	Iran
B 104-1	Iran	SPT 408	Iran
B 181-1	Iran	SPT 409	Iran
K.B		SPT 410	Iran
G.D.165	Bulgaria	SPT 412	Iran
Pobeda 2	Russian	SPT 413	Iran
Kromograid	Bulgaria	SPT 420	Iran
Kramograd N.H.H. 565	Bulgaria	SPT 430	Iran
Immni 3000	-	SPT 432	Iran
kharmanli 163	Turkey	SPT 433	Iran
Izmir	Turkey	SPT 434	Iran
Ploudive 58	Bulgaria	SPT 436	Iran
T.K.23	-	SPT 439	Iran
OR-205	Iran	SPT 441	Iran
OR-379	Iran	P.D.324	Iran
Trabozan	Turkey	P.D.325	Iran
Line 20	Iran	P.D.328	Iran
Jahrom 14	Iran	P.D.329	Iran
K.P.Ha	-	P.D.336	Iran
Neverkop	Bulgaria	P.D.345	Iran

Continuação Tabela 1

Mutant 3	Iran	P.D.364	Iran
L 16	Iran	P.D.371	Iran
C.H.T.209.12e	Iran	P.D.381	Iran
Xanthi	Iran	Mutant 4	Iran
C.H.T.283-8	Iran	C.H.T.209.12e×F.K.40-1	Iran
C.H.T.266-6	Iran	T-B-22	Iran
C.H.T.273-3b	Iran	Ohdarsuma	Yugoslavia

The 'SPT' and 'Jahrom' lines known as 'Chopogh' and water pipe tobacco respectively were selected from our local landraces. The 'PD' lines are recombinant inbred lines coming from the cross between Basma S. 31 and Dubec 566. Other genotypes used in this study are inbred lines from different countries introduced from the CORESTA (Cooperation Center for Scientific Research Relative to Tobacco, Paris, France) collection or pure lines kindly provided by Iranian Tirtash Tobacco Research Centre (ITTRC).

Tobacco seeds were sown at a rate of approximately 5 g m^{-2} in bed. After sowing the seeds, a fine layer of well fermented and sieved sheep manure was spread on top of beds. Then tobacco seedlings were transplanted to plots when plant averaged about 12 cm in height. The experiment was conducted in a simple square lattice design (10×10) with two replications. Each plot was comprised of three lines of 5m, with a spacing of 65 × 20 cm. The plants were not topped as is common with most other tobacco types (such as: Virginia and Burley). The agro-morphological traits were plant height (PH), stem girth (SG), leaf number (LN), leaf length (LL), leaf width (LW) and day to 50% flowering (DF) that were recorded on 5 random plants in total competition per plot (KARA; ESENDAL, 1995). Dry leaf yield and fresh leaf yield were evaluated using total plants of plots with exception of border effects (KARA; ESENDAL, 1995).

DNA extraction and polymerase chain reaction

Considering to availability of leaf sample just for 70 genotypes out of 100 genotypes, total DNA was extracted from these 70 genotypes leaves following the method described by Doyle and Doyle (1987). Concentration of DNA samples was determined spectrophotometrically at 260 nm (BioPhotometer 6131; Eppendorf, Hamburg, Germany). The quality of the DNA was checked by running 1µl DNA in 0.8% (w/v) gels in 0.5X TBE buffer (45mM Tris base, 45mM boric acid, 1mM EDTA pH 8.0). DNA samples that gave a smear in the gel were rejected.

13 SSR primer pairs out of 278 from the tobacco SSR database (BLINDER *et al*., 2007) were used for DNA fingerprinting (Tabela2). The choice of SSR markers was based on clarity of produced bands. Polymerase chain reaction (PCR) was performed in a 20µl volume using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany). The reaction mixture contained 2.5mM of each primer (Tabela 2), 0.4 Unit of Taq DNA polymerase (Cinna Gen Inc., Tehran, Iran), 100µM of each dNTP (BioFluxbiotech, http://biofluxbiotech.com), 2µl 10X PCR buffer, 2mM $MgCl_2$ (CinnaGen, Tehran, Iran), ddH_2O and 25ng template DNA. Amplification was

Table 2 - Name, sequence, linkage group and position of 13 SSR primers applied to 70 oriental tobacco genotypes

Name	Sequence of primer F (5′→3′)	Sequence of primer R (5′→3′)	Linkag group	Position (CM)a
PT30021	CATTTGAACATGGTTGGCTG	CTCAACTCTCGTCGCTCTTG	4	22.7
PT30027	CCGAGAGTTGCATTTGAATTT	AGGGTTCTACGCAAGAGATTG	13	98.6
PT30014	TGCCGTGTAAATTTCATTTGG	AGGATTCCTAACGTGTATTATGTTCT	11	76.7
PT30241	AAGTCTCGTGTGGTTGCTTT	AAAGGGCAATGTGTCTAGCTC	15	0
PT30250	GAACACACGTTCGTCATTGG	ATAAGTCCCTTTAATTTAATTGCG	10	90.7
PT30202	TCGAAACCTCGAGGACAGTT	TATCCAAATCTCCAAAGCCC	7	0
PT30285	CATCATGGCAAGTCACCATC	TGCTGGAAATTAGCGAGGTT	18	55.3
PT20343	GGAACACCACCACCATAA	GGAGCTCAGGTTCCAATG	4	0
PT30172	AAACAACGTCGAAGCATTTG	ACGCATGAAATTGTAAGGGC	4	36
PT30165	ACCTCTGTGGCCGTAAGCTA	CCTCTACTTCAACAGGGTAAGAAA	19	0
PT30008	CGTTGCTTAGTCTCGCACTG	GGTTGATCCGACACTATTACGA	11	39.9
PT30126	GTGATTCCAGCGGAAGACAT	TTCGAAATAAGTACCTAGAGTCGG	10	0
PT30034	GACGAAACTGAGGATATTCCAAA	TGGAAACAAAGCCATTACCC	22	0.1

[a]Genetic distance from the upper telomere estimated according to framework genetic linkage map of tobacco (BINDLER *et al.*, 2007)

carried for 35 cycles consisting of a denaturation step at 94 ºC for 1 min, annealing at 55 ºC for 1 min and an extension step at 72 ºC for 1.5 min. An initial denaturation step at 94 ºC for 4 min and a final extension step of 10 min at 72 ºC were also included. The reaction products were mixed with an equal volume of formamide dyes (98% formamide, 10Mm EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and resolved in a 3% (w/v) agarose gel in 0.5X TBE buffer, stained with 1.0 μg ml^{-1} ethidium bromide and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA).

Data analysis

Analysis of variance (ANOVA) followed by descriptive statistics were calculated for total genotypes based on agro-morphological traits using general linear model in the SAS 9.13 software (SAS Institute, Cary, NC). In order to comparing the classification results of agro-morphological data with marker data, 70 genotypes that had also marker data, were undertaken to clustering. Classification of genotypes using agro-morphological data was performed by means of un-weighted pair-group method using arithmetic average (UPGMA) algorithm based on squared Euclidean distances. Prior to squared Euclidean distance calculation, the data were standardized to have a mean of zero and a variance of one. Data processing was performed using SPSS 15.00 statistical software (SPSS/PC-15, SPSS Inc., Chicago, IL, USA; http://www.spss.com). The pseudo F statistic and the pseudo T^2 statistic (JOBSON, 1992) were examined to establish the optimum numbers of morphological clusters by using SAS 9.13 software (data not shown).

About marker data, the amplification products were scored for the presence (1) and absence (0) of bands across the 70 genotypes to construct a binary data matrix. Several indices such as mean number of allele per locus (n_a), effective allele number (A_e), allele frequency, gene flow (N_m), observed heterozygosity (H_o) and expected heterozigosity (H_e) were estimated using GenAlEx 6.41 software (PEAKALL; SMOUSE, 2006) according to the following equations:

$$n_a = \sum_{i=l}^{n} n_{ai} / n \quad (1)$$

where n_a is the number of alleles at i^{th} locus and n is the number of loci;

$$A_{e=} \frac{1}{\sum_{i=l}^{n} p_i^2} \quad (2)$$

where A_e is the effective allelic number at a locus, and P_i is the frequency of the i^{th} allele in a locus (HARTL; CLARK, 1997);

$$\text{Allele frequency} = \frac{2N_{XX} + N_{XY}}{2N} \quad (3)$$

which was calculated locus by locus, where N_{XX} is the number of homozygotes for allele $X(XX)$, N_{XY} is the number of heterozygotes containing the allele $X(Y$ can be any other allele), and N = the number of samples (HARTL; CLARK, 1997);

$$Nm = [(1/F_{ST})-1]/4 \quad (4)$$

where F_{ST} represents the degree of population genetic differentiation (FRANKHAM *et al*. 2004);

$$H_o = \sum_{i=l}^{n} H_{oi} / n = \sum_{i=l}^{n} 1 - \sum_{j=1}^{mi} q^2_{ij}) / n \quad (5)$$

where H_{oi} represents the observed heterozygosity of the i^{th} locus, and q_{ij} is the frequency of the j^{th} allele at i^{th} locus (HARTL; CLARK, 1997);

$$H_e = \sum_{i=1}^{n} H_i / n = \sum_{i=1}^{n} (1 - \sum_{j=1}^{mi} q^2_{ij}) / n \quad (6)$$

where H_i is the expected heterozygosity of the i^{th} locus**,** and q_{ij} is the frequency of the j^{th} allele at i^{th} locus (LYNCH; MILLIGAN, 1994).

It was assumed that the gene frequency within a population was under Hardy-Weinberg equilibrium. Genetic similarity among individuals was calculated using Jaccard's similarity coefficient (JACCARD, 1908). Dendrograms were constructed by the un-weighted pair group method using arithmetic average (UPGMA) algorithm. The efficiency-of-clustering algorithms and their goodness-of-fit were determined based on co-phenetic correlation coefficients by using NTSYS-pc version 2.11 software (ROHLF, 1998). The significance of co-phenetic correlation was tested using the mantel matrix correspondence test (MANTEL, 1967). All analysis was performed using the NTSYS-pc 2.11 software package (ROHLF, 1998).

RESULTS AND DISCUSSION

Agro-morphological traits

According to univariate statistical analysis (Tabela 3), there is wide ranges of genetic variation between oriental-type tobacco genotypes for all studied agro-morphological traits which show the possibility of selection among genotypes for improving tobacco.

Leaf number varied from 8.7 to 52 and leaf length ranged from 19.1 to 52.5 cm. Traits including leaf width, fresh leaf yield and dry leaf yield ranged from 10.7 to 33.3 cm, 1.6 to 26.4 kg and 0.4 to 5 kg, respectively. Stem height and stem girth fluctuated from 70 to 198.7 cm and 4.7 to 10.3, respectively. Days to 50% flowering ranged from 23 to 134 days.

Table 3 - Variation observed among the tobacco genotypes for the traits under study

Trait	F value	Mean	SD	Range
Leaf number	25.4**	29.06	8.34	8.7-52.0
Leaf length	5.9**	32.09	6.03	19.1-52.5
Leaf width	4.5**	16.70	3.67	10.7-33.3
Fresh leaf yield	4.5**	9.06	3.85	1.6-26.4
Dry leaf yield	6.6**	1.91	0.73	0.4-5.0
Stem height	8.5**	137.03	29.7	70.0-198.7
Stem girth	9.5**	6.37	0.94	4.7-10.3
Day to %50 flowering	50.6**	58.13	16.41	23-134

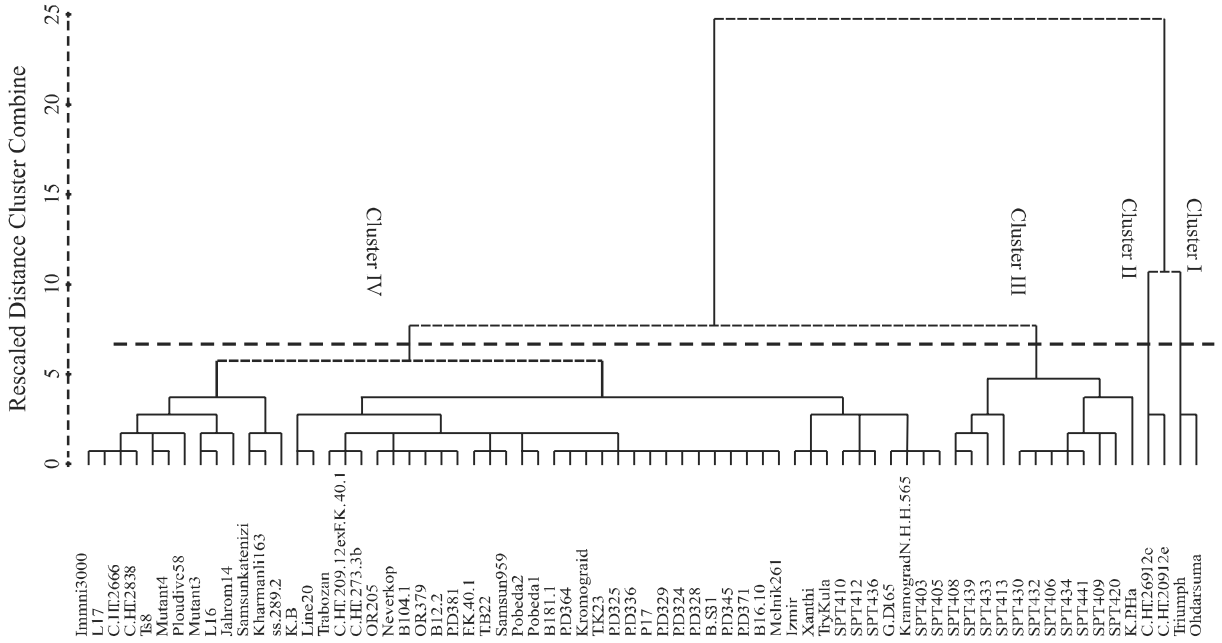
** Significant at a level of 1%

Maximum (29.7) and minimum (0.73) standard deviation corresponded to stem height and dry leaf yield, respectively. Relatively, large variation was detected for studied traits (Tabela 3). Utility of univariate statistical techniques in identification of tobacco genetic diversity has been reported by Wenping *et al*., (2009) and Zeba and Isbat (2011). Evaluation of fifteen diverse tobacco genotypes based on agro-morphological traits depicted that there is statistically significant differences between tobacco genotypes based on all studied morphological traits such as plant height, leaf number, leaf length, leaf width, stem girth and day to 50% flowering (ZEBA; ISBAT, 2011). Genetic variation for traits comprising leaf appearance, percent of dry matter, leaf area index, leaf number and leaf length was also reported in F_2 population of Burley tobaccos (HONARNEJAD; SHOAIE-DEYLAMI, 2004).

There were some reports implying genetic variation of tobacco based on qualitative traits such as nicotine content (TSO *et al*., 1983), sodium, potassium and chlorine concentration in leaf (DARVISHZADEH *et al*., 2011; TSO *et al*., 1983) as well as susceptibility to disease such as stem rot (ELLIOT *et al*., 2007) and powdery mildew (DARVISHZADEH *et al*., 2010).

Classification of genotypes based on agro-morphological traits using UPGMA clustering algorithm separated them into four main groups (Figura1). Cluster I included two tobacco genotypes originated from Yugoslav that had distinguishable morphological performance in field conditions. Genotypes C.H.T.209.12.e and C.H.T.269.12.e that belong to Mazandran province of Iran established cluster II. Breeding lines known as SPT that

Figure 1 - UPGMA clustering of oriental tobacco genotypes based on squared Euclidean distances by using agro-morphological traits



derived from Iran`s northwest landraces by single seed descent method were located in cluster III. Other studied genotypes that had several sympatric origin grouped into cluster IV. Wenping *et al*. (2009) and Zeba and Isbat (2011) used multivariate statistical analysis such as cluster analysis and principle component analysis to grouping tobacco genotypes and identifying important agro-morphological traits. Identification of groups of genotypes with large distances could be effective in recognition of parental lines that might produce hybrid vigor in breeding programs. Several reports (ALEKSOSKI, 2010; KARA; ESENDAL, 1995) showed that there were heterosis for several agro-morphological traits in tobacco such as stem height, leaf number and dry leaf yield.

In this research, clustering of oriental-type tobacco based on morphological traits was in agreement with their geographical distribution and growth characteristics. Therefore, there is acceptable genetic diversity within oriental-tobacco genotypes that is accommodated by Darvishzadeh *et al*. (2011) based on chlorine concentration in leaves.

SSR markers

Similar to the agro-morphological traits, high molecular genetic variability was also observed among the genotypes studied which are in agreement with the finding of Ren and Timko (2001), Yang *et al*. (2007) and Davalieva *et al*. (2010) by means of AFLP, ISSR and SSR markers, respectively. The number of alleles detected for each SSR locus varied from 2 to 3 alleles per locus and a total of 35 alleles were detected over all loci (Tabela 4). The mean number of allele per locus was 2.7, which was parallel with Davalieva *et al*. (2010) reports in Macedonian tobacco with average of 3 alleles per locus. There was not any rare allele (an allele that will be detected once in the 70 genotypes) in studied tobacco genotypes.

The effective number of alleles varied from 1.50 to 2.96 (Tabela 4). It was inferred from low differences among observed and effective number of alleles (Tabela 4), that there is low standard deviation between allele frequencies in each SSR loci. The observed homozygosity values ranged from 0.48 in locus PT30008 to 1.00 in PT30021, PT30241, PT30250, PT30202, PT30172, PT30165, PT30126 and PT30034 loci, with an average of 0.88 across all loci and the observed heterozygosity values ranged from 0.00 in loci PT30126, PT30034, PT30172, PT30165, PT30241, PT30250, PT30202, PT30021 to 0.52 in locus PT30008 with an average of 0.11 across all loci (Tabela 4). The expected homozygosity values ranged from 0.33 in locus PT30014 to 0.67 in locus PT30165, with an average of 0.43 across all loci (Tabela 4). The expected heterozygoty values also ranged from 0.33 in locus PT30165 to 0.67 in locus PT30014, with an average of 0.55 across all loci (Tabela 4).

The genetic similarity based on Jaccard similarity coefficient varied from a maximum of 0.92 (between Pobeda1 and C.H.T.209.12e genotypes) to a minimum of 0.00 (between C.H.T.269-12e and SPT 405 genotypes) with average of 0.32. So, there is a wide range of genetic variation among all oriental-type genotypes.

Table 4 - A summaries of genetic parameters across single sequence repeat loci in oriental tobacco germplasm

SSR loci	n_a	A_e	Obs_{Hom}	Obs_{Het}	Exp_{Hom}	Exp_{Het}	Frequency of alleles			HW	Probability
							A	B	C		
PT30021	3	2.57	1.00	0.00	0.39	0.61	0.34	0.49	0.16	1.00	0.00
PT30027	3	2.84	0.57	0.43	0.35	0.65	0.41	0.36	0.22	0.83	0.00
PT30014	3	2.96	0.92	0.08	0.33	0.67	0.33	0.38	0.28	0.88	0.00
PT30241	2	1.90	1.00	0.00	0.52	0.48	0.61	0.38	-	1.00	0.00
PT30250	2	2.00	1.00	0.00	0.50	0.50	0.52	0.47	-	1.00	0.00
PT30202	2	1.73	1.00	0.00	0.58	0.42	0.30	0.69	-	1.00	0.00
PT30285	3	2.63	0.61	0.39	0.38	0.64	0.21	0.50	0.27	0.29	0.09
PT20343	3	2.33	0.97	0.03	0.43	0.57	0.31	0.56	0.12	0.94	0.00
PT30172	3	1.95	1.00	0.00	0.51	0.49	0.15	0.67	0.16	1.00	0.00
PT30165	2	1.50	1.00	0.00	0.67	0.33	0.20	0.79	-	1.00	0.00
PT30008	3	2.94	0.48	0.52	0.34	0.66	0.40	0.28	0.31	0.41	0.02
PT30126	3	2.39	1.00	0.00	0.42	0.58	0.15	0.55	0.28	1.00	0.00
PT30034	3	2.79	1.00	0.00	0.36	0.65	0.30	0.45	0.23	1.00	0.00

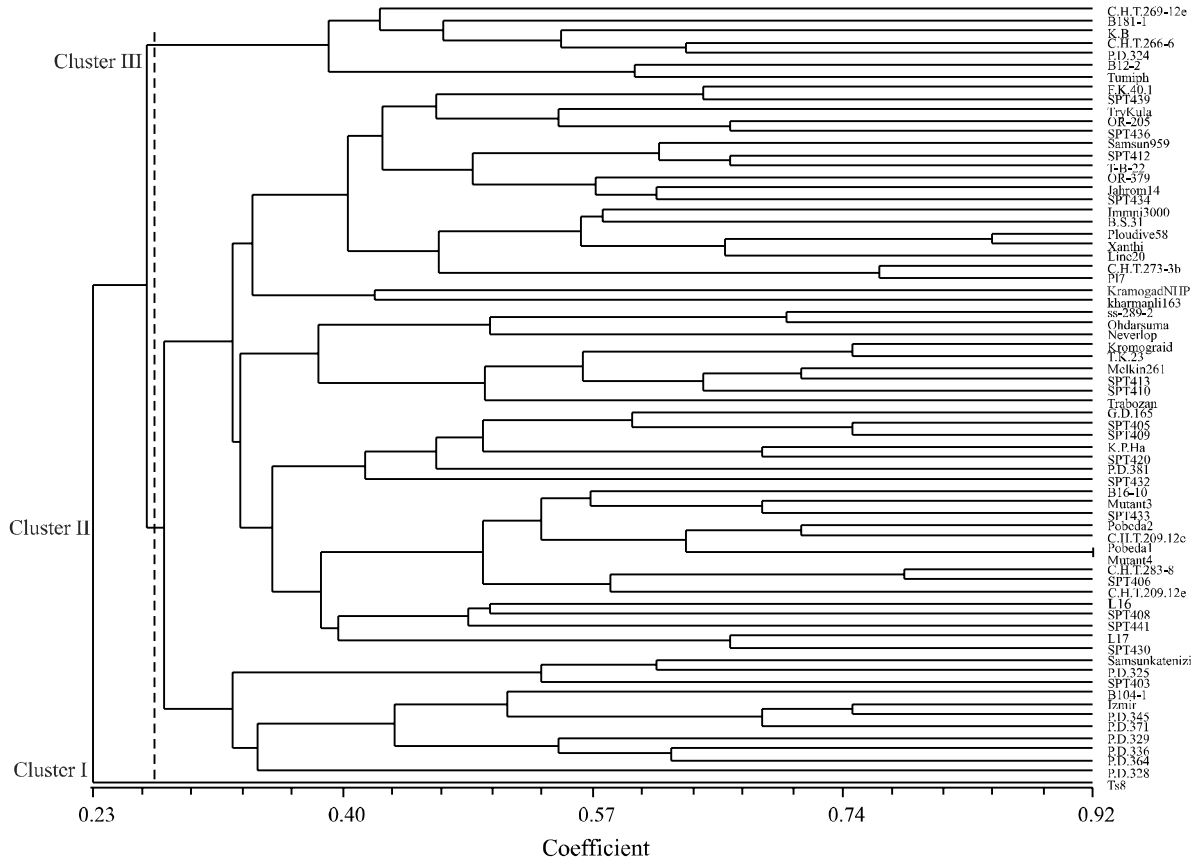
n_a = observed number of alleles; A_e = effective number of alleles; Obs_{Het} = observed heterozygosity; Exp_{Het} = expected heterozygosity

These diverse values of similarity between genotypes could validate that this collection is a valuable tobacco germplasm that already have not been exposed to degradation. The lowest value of similarity (0.0) was belong to two genotypes including C.H.T.269-12e and SPT 405 which were originated from two difference regions of Iran with very differed geographical conditions. SPT series genotypes are dwarf type with low flowering period that are distinguished dramatically them from others such as C.H.T.269-12e. Genotype C.H.T.209.12e belonged to Iran's north province (Mazandaran) and had highest value of similarity with genotype Pobeda1 originated from Russian country near to north province of Iran.

Classification of genotypes based on SSR data by using UPGMA clustering method separated them into three main clusters (Figura 2). The first, second and third clusters comprised 1.4%, 88.6% and 10% of genotypes, respectively. In this study, molecular marker data based clustering of oriental tobacco genotypes did not pursuit agro-morphological based grouping. This is similar to finding of Yao Zhang *et al*. (2008). Since, 77% of the total genomic DNA in cultivated tobacco is composed of repetitive sequences (NARAYAN, 1987), therefore, there is low amount of non-repetitive DNA in the genome of tobacco that responsible for any morphological and quality traits variation. Therefore, recommended to use functional markers such as EST-SSR as barley (SALEM *et al*., 2010) to achieving precise evaluation of tobacco germplasm. In contrast to agro-morphological traits, there were not completely concurrences among geographical distribution of oriental-type genotypes and established clusters based on SSR marker data. Yao Zhang *et al*. (2008) also indicated that dendrogram constructed by using RAPD and AFLP markers in flue-cured tobaccos could not indicate any clear pattern of their geographical origins.

Considering both agro-morphological and SSR marker classifications, some genotypes trend to be located in the same group that is not unexpected because all of studied genotypes considered as oriental or semi oriental tobacco genotypes.

Figure 2 - UPGMA clustering of oriental tobacco genotypes based on Jaccard's similarity coefficient by using SSR data



CONCLUSIONS

There were several studies in the genus *Nicotiana* that used growth characteristics and cytogenetic attributes to describing genetic diversity but there is little information about using agro-morphological traits accompanying with molecular markers data to reveal genetic variation within *N. tabaccum*. Considering to agro-morphological traits and SSR markers, there is significant variation within oriental-type tobacco germplasm. Structure of genetic diversity of oriental-type tobacco genotypes does not pursuit their geographical origins. There is not completely accommodation between classification based on agro-morphological traits and SSR markers. Regarding to reality of markers data, their information could be effectively used in tobacco heterosis breeding program.

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