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STUDY ON GENETIC DIVERSITY AMONG IRANIAN WATER PIPE'S TOBACCO (*NICOTIANA* SPP.) VARIETIES BY USING SIMPLE SEQUENCE REPEAT MARKERS

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Abstract

DARVISHZADEH, R., S. GHOLIZADEH, H. HATAMI MALEKI, B. ABDOLLAHI and I. BERNOUSI, 2013. Study on genetic diversity among Iranian water pipe's tobacco (*Nicotiana* spp.) varieties by using simple sequence repeat markers. *Bulg. J. Agric. Sci.,* 19: 557-562

Water pipe's tobacco is one of the most popular and important industrial plant cultivated in central parts of Iran. In this study, genetic variation among 13 Iranian water pipe's tobacco (*Nicotiana* spp.) genotypes was assessed by using simple sequence repeat (SSR) markers. 100 alleles were generated at 30 SSR loci. The mean number of alleles per locus (n_a) and the effective allele number (n_e) were 3.30 and 2.40, respectively. The expected heterozygosity ranged from 0.21 to 0.78 with average of 0.53. The Jaccard's similarity coefficients among studied water pipe's tobacco genotypes varied from 0.13 to 0.60 suggesting the presence of moderate genetic diversity among Iranian water pipe's tobacco genotypes. Based on un-weighted pair group method using arithmetic average (UPGMA) clustering algorithm the studied water pipe's tobacco genotypes were placed into 4 heterotic groups that could be applied in parent selection for water pipe's tobacco breeding programs. Our clustering could distinct two species of water pipe's tobacco including *N. rustica* and *N. tabacum* from each other successfully.

Key words: Genetic variability, heterotic groups, industrial plants, microsatellite markers, cluster analysis

Introduction

Genus *Nicotiana* belongs to family *Solanaceae* with more than 64 species (Goodspeed, 1954) and *Nicotiana tabaccum* is one of the most cultivated species among them (Narayan, 1987). Tobacco is consumed in many forms such as chewing tobacco, cigarettes, creamy snuffs, dipping tobaccos and water-pipe smoking. Water-pipe smoking originated nearly four centuries ago in ancient Persia and India and involves the passage of tobacco smoke through water before inhalation via a long pipe (Ghafouri et al., 2011). The shape, the size and the appearance of the water-pipe device as well as the type of tobacco, which are used through water pipe, are varying across regions (Maziak et al., 2004). Water-pipe's tobaccos as one of popular type of tobacco are cultivated in some regions of Iran including 'Bushehr', 'Khorasan', 'Khansar', 'Khomeyn', 'Golpayegan' and 'Lar'. To our knowledge, there is narrow study about water pipe's tobacco germplasm whereas it is necessary for its future breeding programmes.

Several traits such as agro-morphological (Wenping et al., 2009; Zeba and Isbat, 2011; Zhang, 1994), chemical and cytological traits (El-Morsy et al., 2009; Okumus and Gulumser, 2001) have already been used to study the genetic variation of several types of tobacco. Morphological and biochemical markers tend to be restricted to relatively few traits, display a low degree of polymorphism, are often environmentally variable in their manifestation and depend on the expression of several unlinked genes (Melchinger et al., 1991). Nowadays, with the emergence of molecular markers, this is possible to evaluate genetic divergence of plant germplasm in greater detail. In this sense, several studies were developed markers (Ren and Timko, 2001; Yang et al., 2007; Yao Zhang et al., 2008) to reveal the genetic diversity of *N. tabaccum*. Recently, with the advent of high-density SSR maps for tobacco it is feasible to estimate genetic variation with a large number of SSR markers that are well distributed across the tobacco genome (Bindler et al., 2007). SSRs as reproductively, co-dominant, wide genome coverage and multi allelic markers has

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been successfully employed to reveal the genetic variation of chewing tobacco genotypes (Siva Raju, 2011). In addition, Davalieva et al. (2010) could classify 10 Macedonian tobacco genotypes into 3 groups using 24 microsattelite markers. Regarding to a rich water pipe's tobacco germplasm from Iran and extensive cultivation of it, simple sequence repeat technique was used to assess the genetic variation of different local water pipe's tobacco varieties.

Materials and Methods

Plant material and DNA extraction

Thirteen local water pipe's tobaccos kindly provided by Urmia Tobacco Research Center were investigated in the present study (Table 1). The seeds of cultivars were cultivated in pots and grown in growth chamber at 25±2°C. Genomic DNA was extracted from the leaves of seedlings 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.

Polymerase chain reaction

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 (Table 2), 0.4 Unit of Taq DNA polymerase (CinnaGen, Tehran, Iran), 100µM of each dNTP (BioFluxbiotech, http://biofluxbiotech.com), 2µl 10X PCR buffer (CinnaGen, Tehran, Iran), 2mM $MgCl_2$ (CinnaGen, Tehran, Iran), ddH_2O and 25ng template DNA. Amplification was carried for 35 cycles consisting of a denaturation step at 94ºC for 1 min, annealing at 55ºC for 1 min and an extension step at 72ºC for 1.5 min. An initial denaturation step at 94ºC for 4 min and a final extension step of 10 min at 72ºC were also included. The reaction products were mixed with an equal volume of formamide dye (98% formamide, 10mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), resolved in 3% (w/v) agarose gel (0.5X TBE), stained with ethidium bromide (1.0µg ml^{-1}) and photographed under UV light (Gel Logic 212 PRO, USA).

Data analysis

The amplification products were scored for the presence (1) and absence (0) of bands across all studied genotypes to construct a binary data matrix. Mean number of allele per locus (n_a), effective allele number (n_e), allele frequency, observed and expected heterozygosities (H_o and H_e respectively) were estimated using the GenAlEx software version 6.41 (Peakall and Smouse, 2006). $n_a = \sum_{i=1n}^{n} n_{ai}/n$, where n_{ai} is the number of alleles at i^{th} locus. $n_e = \sum_{i=1}^{n} n_{ei}/n = \sum_{i=1}^{n} (1-\sum_{j=1}^{m_i} q^2_{ij})/n$, where n_{ei} is the effective allelic number at i^{th} locus, and q_j the frequency of the j^{th} allele. Allele frequency $= \frac{2N_{xx} + N_{xy}}{2N}$, calculated locus by locus (Hartl and Clark, 1997); 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), N = the number of samples. $H_o = \sum_{i=1}^{n} H_{oi}/n = \sum_{i=1}^{n} (1-\sum_{j=1}^{m_i} q^2_{ij})/n$ (Hartl and Clark, 1997), 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. $H_e = \sum_{i=1}^{n} H / n = \sum_{i=1}^{n} (1-\sum_{j=1}^{m_i} q^2_{ij})/n$ (Lynch and Milligan, 1994), 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. Different methods were used for calculating similarity among the studied genotypes and constructing dendrograms. The efficiency-of-clustering algorithms and their goodness-of-fit were determined based on co-phenetic correlation coefficients. Data analyses were performed using the NTSYS-pc version 2.11 software (Rohlf, 1998).

Results and Discussion

Out of 162 SSR primer pairs tested, 30 primer pairs were selected according to their polymorphism. One hundred alleles were generated at 30 SSR loci. The size of amplified PCR products ranged from 140 to 322 bp (Table 2). Number of allele per locus ranged from 2 to 6 (Table 3) with a mean number of 3.30 which is in agreement with the finding of Davalieva et al. (2010) reports in Macedonian tobacco genotypes. The effective allelic number (ne) was 2.40 on average and ranged from 1.28 to 4.64 (Table 3). This parameter takes into account both the number of alleles and their frequencies. It allows us to compare genotypes where the number and distributions of alleles differed drastically. The low n_e in this research could reflect the low heterozygosity (Nei, 1978).

Table 1
List of studied local water pipe's tobacco varieties

No.	Genotype	Species	No.	Genotype	Species
G1	Esfahani5	*N. tabaccum*	G 8	Jahrom14	*N. tabaccum*
G 2	Esfahani	*N. tabaccum*	G 9	Esfahani10	*N. tabaccum*
G 3	Shiraz23	*N. tabaccum*	G 10	Sarvestan31	*N. tabaccum*
G 4	Langhe	*N. tabaccum*	G 11	Esfahani4	*N. tabaccum*
G 5	Jahrom12	*N. tabaccum*	G 12	Esfahani2	*N. tabaccum*
G 6	Hakkan17	*N. tabaccum*	G 13	Borazjani	*N. rustica*
G 7	Baluchi	*N. tabaccum*			

Table 2
Names, sequences and linkage groups of the 30 simple sequence repeat (SSR) primer pairs applied to 13 Iranian water pipe's tobacco genotypes

Primer name	Range (bp)	Forward sequence (5'→-3')	Reverse sequence (5'→3')	Repeat motif	AT	LG
PT30021	224	CATTTGAACATGGTTGGCTG	CTCAACTCTCGTCGCTCTTG	TA	55	4
PT30132	216	CCTAACAGCATTTGCTACCCA	GATGGACAAGAGTGGCCTTT	TA	55	10
PT30202	225	TCGAAACCTCGAGGACAGTT	TATCCAAATCTCCAAAGCCC	GA	55	7
PT30159	197	GCATGCATATGAACATGGGA	TTTGACATCTCTACTCTTCCGTTT	TA	55	14b
PT30175	229	TTAGGCGGCGGTATTCTTAT	TATGCCTCAATCCCTTACGC	TA	55	14a
PT30285	177	CATCATGGCAAGTCACCATC	TGCTGGAAATTAGCGAGGTT	TA	55	18
PT30324	151	TGCTCTGCGTTAGAACAGGA	CGACGAGAGAAGATTAGTGAAAGA	TAA	55	12
PT20343	322	GGAACACCACCACCATAA	GGAGCTCAGGTTCCAATG	AC/AG/AT	55	4
PT30075	195	CGATCGGGTCGTTACACAAT	CCCATCAGGTTGTTGGGTTA	TA	55	11
PT30241	199	AAGTCTCGTGTGGTTGCTTT	AAAGGGCAATGTGTCTAGCTC	GA	55	15
PT30061	182	TCGTCCATTTCTTTCTCTCTCA	CATAAATAGTTGCTCATTCAATCG	TA	55	11
PT30144	266	TGATTTGTATTGACAGCGTGAAG	TTGTTTAGTTACCCTATTTGACTTGC	TA	55	16
PT30332	230	AAACCGAACCGAACTGATTT	TCAAATTTATGATTCTTGTAGCGAA	TA	55	16
PT30124	228	TCCTCCAACCAAACTCAAGC	TTTCTGTTCGCGTTTCAAAT	TA	55	4
PT30110	213	TTGTACGTTCCTCGCTGATG	GGCCGACAATAAAGTGGCT	TA	55	21
PT20275	184	GTTCTATTTGATCGCCCC	AACAGCACCAACAGCATT	CTT	55	5
PT30260	225	GGTAGGGTGGAACAAATTTATCA	AATATGGTCTATGCCCGCAA	TA	55	8a
PT30067	204	AAGCCTGGTCAGTTATCCCA	ATTCGCACCACTTAATCCCA	TA	55	2
PT30126	208	GTGATTCCAGCGGAAGACAT	TTCGAAATAAGTACCTAGAGTCGG	TA	55	10
PT30034	216	GACGAAACTGAGGATATTCCAAA	TGGAAACAAAGCCATTACCC	TAA	55	22
PT30008	192	CGTTGCTTAGTCTCGCACTG	GGTTGATCCGACACTATTACGA	TA	55	11
PT30165	224	ACCTCTGTGGCCGTAAGCTA	CCTCTACTTCAACAGGGTAAGAAA	TAA	55	19
PT30014	205	TGCCGTGTAAATTTCATTTGG	AGGATTCCTAACGTGTATTATGTTCT	TA	55	11
PT30272	140	GAACCTAACCTCGCTCCACA	AAATGGTAGCTGCGAGGAGA	GA	55	4
PT30171	218	CCCATGCATGCCTAATTTCT	CCCAGAAGCCCTTATACAACC	TA	55	24
PT30172	216	AAACAACGTCGAAGCATTTG	ACGCATGAAATTGTAAGGGC	GAA	55	4
PT30205	193	GGTCGATCCACAATTTAAACG	GCACTTGCTCCTTTGTACCC	TA	55	3b
PT20287	164	CGCCACAACAACTCACCTTA	TCATGCATGTTTCTCCTCCTT	AAG	55	3a
PT30250	177	GAACACACGTTCGTCATTGG	ATAAGTCCCTTTAATTTAATTGCG	TAG	55	10
PT30292	156	AAGACAGATTGGTGCGGAAC	AGCACTTGGACAGGCGAATA	TA	55	7

AT: annealing temperature. LG: linkage group.

Observed heterozygosity ranged from 0.00 to 0.88 with an average of 0.22 and 12 loci did not show any heterozygosity. The informativeness of each SSR locus was measured through expected heterozygosity. The expected heterozygosity ranged from 0.21 to 0.78 with an average of 0.53 (Table 3). SSR markers with high heterozygosity values such as 'PT30008' and ' PT30061' could be effectively used in water pipe's tobacco genetic diversity studies. Regarding to Table 3, there was a considerable difference between observed and expected heterozygosity for several SSR loci that implying the lack of Hardy-Weinberg equilibrium. This state could be consequence of selection, gene flow or genetic drift.

Different methods were used to construct the similarity matrices and dendrograms (Table 4). The co-phenetic correlation coefficients, as a measure of the correlation between the similarities represented on the dendrograms and the actual degree of similarity was calculated for each dendrogram (Table 4). Among the different methods, the highest value (r=0.76) was observed for the UPGMA method based on Jaccard's similarity coefficients (Table 4). Hence, Jaccard's

Table 3
Number of allele, effective allelic number, observed and expected heterozygosity and allele frequency of the 30 simple sequence repeat (SSR) loci studied on 13 Iranian water-pipe-tobacco varieties

Primer name	n_a	n_e	H_o	H_e	Allele Frequency					
					a_1	a_2	a_3	a_4	a_5	a_6
PT30021	3	2.50	0.25	0.60	0.41	0.45	0.12	-	-	-
PT30132	5	3.74	0.41	0.73	0.12	0.16	0.33	0.33	0.04	-
PT30202	4	2.78	0.81	0.64	0.04	0.45	0.13	0.36	-	-
PT30159	3	2.08	0.00	0.52	0.07	0.30	0.61	-	-	-
PT30175	3	1.69	0.07	0.41	0.03	0.23	0.73	-	-	-
PT30285	5	2.59	0.66	0.61	0.08	0.12	0.12	0.58	0.08	-
PT30324	3	2.00	0.33	0.50	0.16	0.66	0.16	-	-	-
PT20343	3	1.73	0.07	0.42	0.19	0.73	0.07	-	-	-
PT30075	5	3.13	0.83	0.68	0.29	0.12	0.45	0.04	0.08	-
PT30241	2	1.55	0.00	0.35	0.76	0.23	-	-	-	-
PT30061	5	4.27	0.30	0.76	0.19	0.30	0.26	0.15	0.07	-
PT30144	3	2.02	0.30	0.50	0.25	0.65	0.10	-	-	-
PT30332	3	2.17	0.00	0.54	0.60	0.30	0.10	-	-	-
PT30124	4	2.81	0.36	0.64	0.09	0.09	0.45	0.36	-	-
PT30110	4	2.91	0.09	0.65	0.18	0.22	0.09	0.50	-	-
PT20275	2	1.65	0.00	0.39	0.72	0.27	-	-	-	-
PT30260	2	2.00	0.00	0.50	0.50	0.50	-	-	-	-
PT30067	2	1.32	0.00	0.24	0.85	0.14	-	-	-	-
PT30126	4	2.50	0.07	0.60	0.07	0.57	0.19	0.15	-	-
PT30034	3	2.46	0.00	0.59	0.54	0.27	0.18	-	-	-
PT30008	6	4.64	0.33	0.78	0.08	0.08	0.12	0.29	0.12	0.29
PT30165	2	1.60	0.16	0.37	0.25	0.75	-	-	-	-
PT30014	4	3.44	0.55	0.71	0.38	0.22	0.27	0.11	-	-
PT30272	2	1.47	0.00	0.32	0.80	0.20	-	-	-	-
PT30171	2	1.97	0.00	0.49	0.44	0.55	-	-	-	-
PT30172	2	1.60	0.00	0.37	0.25	0.75	-	-	-	-
PT30205	3	2.16	0.88	0.53	0.38	0.05	0.55	-	-	-
PT20287	2	1.28	0.00	0.21	0.87	0.12	-	-	-	-
PT30250	3	2.46	0.00	0.59	0.27	0.54	0.18	-	-	-
PT30292	5	3.44	0.15	0.71	0.15	0.26	0.11	0.42	0.03	-
Mean	3.300	2.40	0.22	0.53						

n_a: observed number of alleles. n_e: effective allelic number; by definition it shows the number of equally frequent alleles. H_o: observed heterozygosity. H_e: expected heterozygosity.

similarity coefficient was used to depict the genetic diversity of studied genotypes. Considering to similarities results, the genetic similarity among the water pipe's tobacco genotypes varied from GS_j=0.13 (between Jahrom14 and Borazjan genotypes) to GS_j= 0.60 (between Jahrom14 and Jahrom12 genotypes) which indicate the moderate level of genetic variation among studied Iranian local water pipe's tobacco genotypes (data not shown). Ren and Timko (2001) was also reported low genetic variation within tobacco species. Similarly, Arsalan and Okumus (2006) using RAPD markers and Yang et al. (2007) by using inter-simple sequence repeat and inter-retrotransposon amplification polymorphism (IRAP) markers reported the low levels of genetic diversity among cultivars planted in eastern Anatolia of Turkey and Chinese flue-cured tobacco collection, respectively. Conversely, Davalieva et al. (2010) indicated a wide range of genetic diversity among the selected tobacco varieties by using SSR markers. In addition, Moon et al. (2009) by using SSR markers manifested that the most of the American *Nicotiana* germplasm collection are sufficiently distinct from each other.

Based on the dendrogram constructed using UPGMA clustering method (Figure 1), the studied water pipe's tobacco genotypes were classified into 4 groups. Group I was included genotypes Baluchi, Shiraz23, Esfahani, Esfahani4, Esfahani5 and Esfahani10. This was not unexpected because; Esfahani local water pipe's tobacco genotypes were sampled from Esfahan province of Iran. Likewise, these three regions (Baluchestan, Shiraz, Esfahan) located in the dry and hot climate of Iran. Genotypes comprising Langhe, Jahrom12, Hakkan17, Jahrom14 and Sarvestan31 were located in group II. All of group II genotypes were belonged to Shiraz province of Iran with exception of Langhe genotype. Two genotype including 'Borazjan' and 'Esfahani2' were located in group III and group IV respectively. In spite of other genotypes, genotype 'Borazjan' was possessed to *N. rustica* species. Therefore, cluster analysis based on SSR data could effectively distinguished two species of water-pipe tobacco from Iran.

Information on genetic diversity permits the classification of germplasm into heterotic groups, which is particularly important to hybrid breeding programs. Even though the genetic mechanisms that explain heterosis are not fully understood, it is well documented that crosses between unrelated, and consequently genetically distant parents show greater hybrid vigor than crosses between closely related parents (Stuber, 1994). It is also important in developing informative mapping populations for QTL identification.

Conclusions

Considering to SSR markers, there is moderate variation among local water pipe's tobacco varieties. Structure of

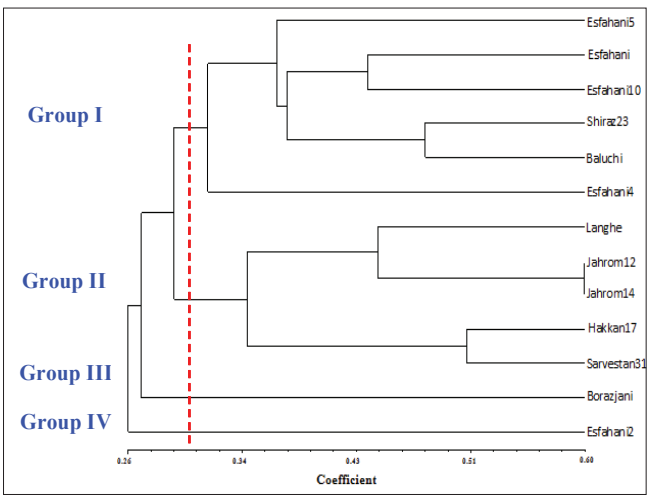

Fig. 1. Dendrogram showing Jaccard genetic similarity for 13 Iranian water pipe's tobacco varieties revealed by UPGMA clustering based on genetic fingerprints from 30 SSR loci

Table 4
Comparison of different methods for constructing similarity matrices and dendrogram in Iranian water pipe's tobacco varieties

Similarity matrices	Algorithm	Co-phenetic coefficient
Jaccard	UPGMA	0.76
Jaccard	Complete linkage	0.61
Jaccard	Single linkage	0.69
Dice	UPGMA	0.72
Dice	Complete linkage	0.56
Dice	Single linkage	0.65
Simple matching	UPGMA	0.73
Simple matching	Complete linkage	0.63
Simple matching	Single linkage	0.62

UPGMA – Unweighted Pair Group Method with Arithmetic Mean.

genetic variation of water pipe's tobaccos pursuit their geographical origins. Regarding reality of marker data, their information could be effectively used in water pipe's tobacco breeding program.

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