DOI: http://dx.doi.org/10.22092/cbj.2012.100453

Assessing the genetic diversity of 89 flue-cured tobacco varieties using morphological traits and inter-simple sequence repeat markers

H. Samizadeh Lahiji[a]*, M. Mohsenzadeh Golfazani[b], K. Edrisi Maryan[c], M. Shoaeid Deylami[d] and A. Aalami[e]

[a, b,c,e] School of Agricultural Science, University of Guilan, Rasht, Iran.
[d] Guilan Tobacco Research Center, Guilan, Rasht, Iran.
*Corresponding author's E-mail: hsamizadeh@yahoo.com

Received: January 2013
Accepted: September 2013

ABSTRACT

Samizadeh Lahiji, H., Mohsenzadeh Golfazani, M., Edrisi Maryan, K., Shoaeid Deylami, M., and Aalami, A. 2013. Assessing the genetic diversity of 89 flue-cured tobacco varieties using morphological traits and inter-simple sequence repeat markers. **Crop Breeding Journal 3(2): 79-85.**

The genetic diversity of 89 flue-cured tobacco varieties was examined using 12 ISSR primers. These cultivars were evaluated at the Guilan Tobacco Research Center, Rasht, Iran, using a 7×7 simple lattice design with two replications, and 12 morphological traits. The total number of PCR amplified products was 143 bands ranging from 450 to 3000 bp, of which 108 bands (74.28%) were polymorphic. Primers UBC 811 and UBC 814 with 16 bands and UBC 825 with 6 bands generated the highest and lowest number of bands, respectively. Of all the primers, UBC817, UBC824 and UBC873 showed the maximum amount (0.47) of polymorphism information content (PIC) and the greatest diversity. To determine the genetic relationship among tobacco cultivars, cluster analysis was performed based on either morphological traits or ISSR markers using the un-weighted pair-group method with arithmetic average (UPGMA). Tobacco genotypes were divided into five main groups. Principal coordinate analysis (PCoA) on a similarity matrix of genotypes showed that the first 12 coordinates explained 60.16% of the total variance, whereas the first two coordinates explained only 28.96% of total variance. Cluster analysis of morphological traits divided tobacco genotypes into five groups. Based on canonical discriminate function analysis using the Fisher linear method, the UPGMA method separated the genotypes with 78.5% accuracy. UBC817, UBC824 and UBC873 were the most informative primers and thus could be used to assess the diversity of tobacco cultivars. In addition, UBC813, UBC823 and UBC826 would be appropriate ISSR primers because of the reasonable amount of PIC, Nei and Shannon's information index.

Keywords: bootstrap analysis, cluster analysis, genetic relationship, molecular marker, Shannon's information index

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is the most important non-food crop in the world (Anonymous, 2011). Evaluating the genetic diversity of tobacco germplasm is useful in breeding programs and for variety identification (Davalieva *et al.*, 2010). It can also be used to select suitable parents (Yang *et al.*, 2007) and develop new cultivars for improving crop productivity (Shaha *et al.*, 2009).

Historically, morphological, cytogenetic, pedigree and chemical analyses have been used to study plant diversity (Volis *et al.*, 2001). Evaluating genetic diversity based on morphological traits is time-consuming and requires extensive field trials and evaluation (Astarini *et al.*, 2004). In recent years, many molecular techniques have been used to identify crop cultivars (Pivoriene and Pasakinskiene, 2008) and have largely overcome problems associated with phenotype-based classification (Awasthi *et al.*, 2004). DNA markers have proved to be valuable tools in crop breeding, especially in studies on genetic diversity and gene mapping (Pradeep–Reddy *et al.,* 2002).

Several molecular techniques are available for detecting genetic differences within and among cultivars (Williams *et al.*, 1990). Different molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), microsatellites or simple sequence repeats (SSRs) (Becker and Heun, 1994) and inter-simple sequence repeats (ISSRs) (Zietkiewicz *et al.*, 1994) have been used to assess genetic variation at the DNA level. These molecular methods are different in principle, as well as in application, type, amount of polymorphism detected and time requirements (Naghavi *et al.*, 2004).

One of the most efficient molecular marker

methods—in terms of the ability to produce polymorphic markers within a comparatively short time and with a limited budget—is ISSR profiling for total genomic DNA (Pivoriene and Pasakinskiene, 2008). This method has several advantages over other techniques: first, it is able to discriminate between closely related genotypes (Hodkinson *et al.*, 2002) and, second, it can detect polymorphisms without any previous knowledge of the crop's DNA sequence (Kumar *et al.*, 2006). The ISSR marker technique involves polymerase chain reaction (PCR) amplification of DNA using a single primer composed of a microsatellite sequence such as GACA (Zietkiewicz *et al.*, 1994).

ISSRs have proven to be a rapid, simple and inexpensive approach for assessing genetic diversity (Sarla *et al.*, 2003), identifying closely related cultivars (Martins-Lopes *et al.*, 2007), and studying evolutionary processes such as reproductive systems (Liston *et al.*, 2003) and gene flow (Wolfe *et al.*, 1998). The genetic diversity of important crops such as wheat (Nagaoka and Ogihara, 1997), rice (Blair *et al.*, 1999) and maize (Kantety *et al.*, 1995) has been estimated by ISSR markers. Del Piano *et al.* (2000), Yang *et al.* (2005) and Xiao and Yang (2007) have studied tobacco genetic diversity using ISSR markers.

The present study reports on the use of ISSR markers for assessing the genetic diversity and relationships among 89 imported flue-cured tobacco cultivars and comparing the results with the morphological diversity of these cultivars for future breeding purposes in Iran.

MATERIALS AND METHODS

Plant materials and field experiments

Eighty-nine imported and domestic flue-cured tobacco varieties (Table 1) were grown and evaluated at the Guilan Tobacco Research Center, Rasht, Iran using a 7×7 simple lattice design with two replications. Twelve morphological traits were evaluated during the growing season or after harvest. Days to flowering, flowering duration, leaf shape index, leaf area coefficient, plant height, leaf width, leaf length, number of leaves, stem diameter, SPAD value, fresh leaf yield ha^{-1} and dry leaf yield ha^{-1} were measured on five samples from each plot.

Morphological analysis

Cluster analysis for morphological traits was performed with Genstat Version 12 software using a simple matching similarity coefficient and the un-weighted pair-group method with arithmetic average (UPGMA). Canonical discriminate function analysis was performed with SPSS.16 software using Fisher's linear method in order to confirm the accuracy of cluster analysis.

DNA extraction

DNA was extracted from young leaves based on the method described by Doyle and Doyle (1987). Extracted DNA was qualified using 1% agarose gel electrophoresis. Twelve ISSR primers were used to amplify regions of genomic DNA (Table 2). Total volume of the PCR reaction mixture was 10 µL, containing 30-40 ng of template DNA, 10 mM dNTP, 0.3 mM primer, 1X PCR buffer and 1 U DNA Taq polymerase.

DNA amplification was carried out using a thermocycle (Biometra) as follows: initial denaturation at 94°C for 4 minutes; 35 cycles of denaturation at 94°C for 40 seconds, annealing at 40°C/49°C (depending on the primers used) (Table 2) for 40 seconds, extension at 72°C for 2 minutes, and final extension at 72 °C for 5 minutes. The PCR products were separated on 1.5% agarose gel in 10X TAE buffer and at a constant voltage of 90 for 60 minutes. The bands were visualized under UV light by a Gel DOC (Biometra; BioDocAnalyze, Bio-Rad).

Molecular analysis

ISSR bands were scored as present (1) or absent (0) for each primer. A data matrix was generated for each reaction according to PCR banding patterns. There are several parameters for evaluating the efficiency of a primer. Some important parameters of marker efficiency are PIC, Nei's index and Shannon's information index. Nei and Shannon's coefficients (Table 2; Nei, 1973; Shannon, 1948) were calculated using Popgene 1.31 (Yeh and Yang, 1999). The PIC value was calculated as $PIC= 1-\sum P_i^2$, where P_i is the frequency of the allele (Smith *et al.*, 1997). Similarity coefficients and cluster analysis with UPGMA were performed by NTSYS-PC software (Rohlf, 1998). Reliability of the cluster was estimated by bootstrap analysis (100 samples).

Principal coordinate analysis was performed using GenStat V.12 on a similarity matrix. PIC analysis was performed using Excel software. To confirm the results of cluster analysis, canonical discriminate function analysis using the Fisher linear method was performed by SPSS software (Ver.16). Two similarity matrices based on molecular and morphological clusters were constructed and compared using the Mantel test (Mantel, 1967; Mantel and Valand, 1970) and Genstat Version 12.

RESULTS AND DISCUSSIONS

A total of 143 bands were generated by 12 ISSR

Table 1. Identification number, name, and country of origin of the 89 tobacco varieties.

ID	Cultivar name	Source	ID	Cultivar name	Source
1	'Coker-254'	USA	46	'TL1112'	USA
2	'Coker-298'	USA	47	'Ex.4.PR-1'	USA
3	'Bel-61-10'	USA	48	'GoldenGift'	Britain
4	'ChemicalMutant'	Australia	49	'C258.MC944'	Iran
5	'Bel-71-500'	USA	50	'Pereg 234'	Germany
6	'Bel-71-501'	USA	51	'Coker 258'	USA
7	'Bel-61-9'	USA	52	'Coker 347'	Italy
8	'Virgin'	Germany	53	'Coker 411'	USA
9	'R-9'	Iran	54	'K.E 1'	USA
10	'R-30'	Iran	55	'K. 110'	USA
11	'Fixed-A1'	USA	56	'MCNAIR944'	USA
12	'Honggarten-Blatt'	Germany	57	'TL 13'	USA
13	'Delhi'	Canada	58	'Vinica'	USA
14	'VirginiaAmerican'	USA	59	'MC. 1'	USA
15	'VirginRP37'	USA	60	'MC.101'	Japan
16	'Hicks55'	USA	61	'GewaneGrone'	Germany
17	'PreviStammV6'	USA	62	'HicksResistant'	USA
18	'HicksBroadLeaf'	USA	63	'Virginia HR'	Germany
19	'VirginiaH.R.'	USA	64	'Virginia yold'	Germany
20	'VirginiaRee40'	USA	65	'STNCB'	Zimbabwe
21	'NortCarolina88'	USA	66	'TL 33'	South Africa
22	'PrevStammv3'	USA	67	'NR 23'	USA
23	'VirginiaBright88'	USA	68	'P49-4625'	USA
24	'VirginiaRee488'	USA	69	'Tiratash-10'	Iran
25	'PeeDee'	Germany	70	'Tiratash-17'	Iran
26	'NOD8'	Africa	71	'AMERSFORTER'	Belgium
27	'NC.95XCHMUTANTNO2	Iran	72	'Holandisher'	USA
28	'Soth-Carolina'	USA	73	'Look Wood'	USA
29	'VirginiaRP.37'	USA	74	'RXT'	Poland
30	'Tirtash-4'	Iran	75	'GA. 955'	Australia
31	'Tirtash-33'	Iran	76	'Coker176'	USA
32	'PeregR.2-228'	Germany	77	'NC.60'	USA
33	'PeregR.2-234'	Germany	78	'IRaburboon'	Iran
34	'BadisherGeudert'	Germany	79	'P.B.D.6'	France
35	'ComstockSpanish'	USA	80	'Bel'	USA
36	'Manilla-Geel'	USA	81	'GrixollaSoptenol'	USA
37	'MontcalmBrum'	Switzerland	82	'Hick-RG'	USA
38	'Alida'	USA	83	'R 30. N2'	Iran
39	'Pfatzer'	USA	84	'C 319.R30'	Iran
40	'AllPurpose'	USA	85	'C 319. C 411'	Iran
41	'Pennbel69'	USA	86	'Erzegovinia'	Erzegovin
42	'Parfum-ditalie'	Canada	87	'K.S1.E.'	USA
43	'RosecanNela'	Canada	88	'Speight G.28'	USA
44	'BERGERAC-C'	France	89	'N.2'	USA
45	'TRUMPF'	Germany			

Table 2. List of primers,* primer sequence, annealing temperature (TM), number of bands, number of polymorphic bands, polymorphism (%), PIC, Nei's index (H) and Shannon's information index (I).

Primer no.	Primer name	Primer sequence	TM	Bands (no.)	Polymorphic bands (no.)	Polymorphism (%)	PIC	H	I
1	UBC 811	5-(GA)8C-3	43.21	16	14	87.5	0.38	0.47	0.66
2	UBC 812	5-(GA)8A-3	42.07	13	10	76.9	0.43	0.46	0.66
3	UBC 813	5-(CT)8T-3	42.02	13	12	92.3	0.44	0.46	0.65
4	UBC 814	5-(CT)8A-3	40.97	16	12	75.0	0.45	0.42	0.6
5	UBC 815	5-(CT)8G-3	73.17	11	8	72.7	0.45	0.35	0.53
6	UBC 816	5-(CA)8T-3	47.23	12	9	75.0	0.43	0.46	0.65
7	UBC 817	5-(CA)8A_3	47.53	14	10	71.4	0.47	0.44	0.63
8	UBC 823	5-(TC)8C_3	44.15	14	11	78.5	0.46	0.45	0.64
9	UBC 824	5-(TC)8G-3	44.86	8	5	62.5	0.47	0.38	0.56
10	UBC 825	5-(AC)8T-3	48.25	6	4	66.6	0.44	0.42	0.61
11	UBC 826	5-(AC)8C_3	49.56	13	8	61.5	0.46	0.45	0.64
12	UBC 873	5(GACA)4-3	43.34	7	5	71.5	0.47	0.38	0.57
Total				143	108				
Mean				11.92	9	74.28	0.44	0.43	0.63

*Source: Yang *et al.* (2007).

primers ranging from 450 to 3000 bp, of which 108 bands (74.28%) were polymorphic. Primers UBC811 and UBC814 with 16 bands and UBC825 with 6 bands had the highest and lowest number of bands, respectively. The average numbers of amplified and polymorphic bands per primer were 11.92 and 9, respectively (Fig. 1 and Table 2).

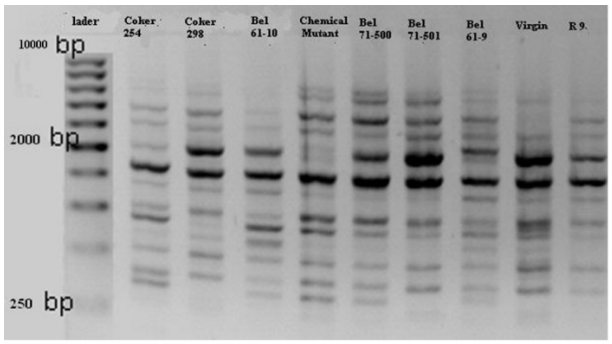

Fig. 1. ISSR amplification profile of 10 tobacco varieties by primer UBC811.

PIC varied from 0.38 to 0.47, with an average of 0.44 (Table 2). Regarding the importance of PIC for primer efficiency, it is interesting that UBC817, UBC824 and UBC873 had the highest PIC (0.47). Four primers—UBC826 (0.46), UBC823 (0.46), UBC814 (0.45) and UBC 815 (0.45)—had the highest PIC after UBC817, UBC824 and UBC873.

The correlations between Nei genetic diversity, Shannon's information index and PIC were also estimated (Table 3). There was a significant positive correlation (r = 0.995**) between Nei genetic diversity and Shannon's information index, but no correlation between PIC and these two diversity indices (Nei genetic diversity and Shannon's information index). There was a negative and significant correlation (r = -0.512*) between Nei genetic diversity and PIC, as well as between Shannon's information index and PIC (r = -0.512*). These correlations indicate there is a direct relation between Nei genetic diversity and Shannon's information index, but no relation at all between PIC and the other two diversity indices (Nei genetic diversity and Shannon's information index).

Table 3. Correlation between Nei, Shannon's information index and PIC.

	PIC	H	I
PIC	**1**	**-0.521***	**-0.521***
H		**1**	**0.995****
I			**1**

PIC = Polymorphic information content.
H = Nei genetic diversity.
I = Shannon's information index.

Denduangboripant *et al.* (2010) used 20 ISSR primers to study genetic relationships between introduced and local tobacco cultivars grown in Thailand. The 20 ISSR primers that were preliminarily screened on a total of 11 tobacco samples generated 128 PCR bands whose sizes ranged from 280 to 1,600 bp. Between 1 and 11 polymorphic bands were generated, with an average of 5 bands per primer. Five primers (UBC807, UBC809, UBC813, UBC823, and UBC836) were found to be highly polymorphic and generated reproducible bands. As shown in this study, UBC813 and UBC823 primers had the highest reasonable amount of PIC (Table 2), as well as high Nei and Shannon's information indices (Table 4), indicating the validity of these two primers for identifying genetic diversity among tobacco cultivars.

Table 4. Eigenvalues, variance and agglomerative variance.

Coordinates	Eigenvalues	Variance (%)	Agglomerative variance (%)
1	**8.85**	**22.44**	**22.95**
2	**2.57**	**6.52**	**28.96**
3	**1.76**	**4.46**	**33.42**
4	**1.63**	**4.14**	**37.56**
5	**1.53**	**3.87**	**41.43**
6	**1.38**	**3.50**	**44.93**
7	**1.18**	**3.01**	**47.94**
8	**1.56**	**2.93**	**50.87**
9	**1.04**	**2.63**	**53.50**
10	**0.96**	**2.43**	**55.93**
11	**0.87**	**2.19**	**58.12**
12	**0.80**	**2.04**	**60.16**

Principal coordinate analysis:

Principal coordinate analysis (PCoA) was performed using simple matching coefficients of similarity. The first 12 coordinates explained 60.16% of the total variance. The first two coordinates explained 28.96% of the total variance. The first coordinate explained 22.45% and the second one explained 6.52% of the total variance. A scatter plot of genotypes was constructed based on the two main coordinates. Tobacco genotypes were grouped into five different clusters according to their similar characteristics in the PCoA biplot (Fig. 2 and Table 4).

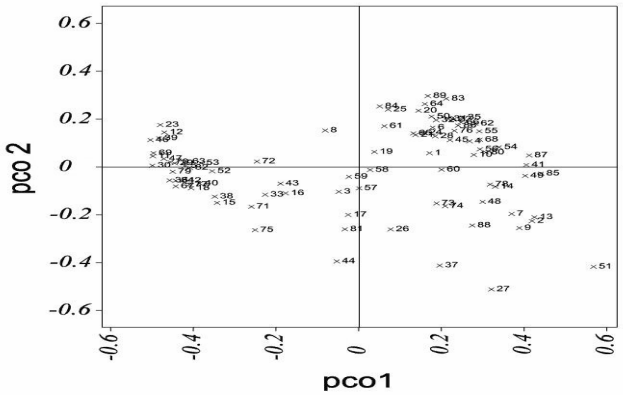

Fig. 2. PCO grouping of tobacco cultivars. Each digit is the ID of tobacco variety (Please see Table 1).

Cluster analysis (molecular)

Cluster analysis was performed to develop a UPGMA dendrogram (Fig. 3). Tobacco cultivars were divided into five main groups based on the value of the similarity coefficient (0.33-0.97). Groups 1 to 5 consisted of 28, 19, 7, 6 and 29 genotypes, respectively (Table 5). Tobacco cultivars of the same geographical origin were clustered

together. For example, 'Coker-254' and 'Coker-298' from USA were clustered in the first group, and 'Tirtash-4' and 'Tirtash-33' from Iran in the third group. This indicates the consistency of the molecular profile and the influence of the cultivars' geographical origin.

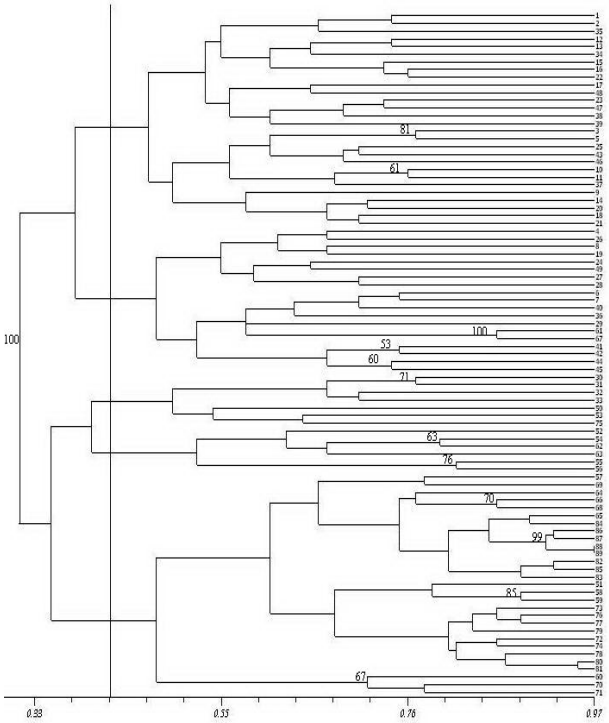

Fig. 3. Dendrogram generated by the simple matching coefficient based on ISSR amplification polymorphism analysis of 89 tobacco varieties. Bootstrap values of less than 50% are not shown.

The cophenetic correlation coefficient (r = 0.81) was calculated to evaluate the usefulness of the UPGMA method in clustering plant genotypes. A high cophenetic correlation coefficient (> 0.8) has been recorded as an appropriate coefficient (Mohammadi and Prasanna, 2003). Therefore, our results indicated that UPGMA clustering grouped the cultivars appropriately.

Yang *et al.* (2005) studied 24 flue-cured tobacco varieties by ISSR markers and cluster analysis using the UPGMA method, which separated the varieties into five main groups. In their study, the largest group consisted of 12 varieties derived from 'Coker-319'. These authors suggested that 24 flue-cured tobacco cultivars ('Burley' and 'Virginia' types) were closely related and had low genetic diversity. Zhang *et al.* (2006) studied the genetic diversity of 51 flue-cured tobacco cultivars using AFLP markers. Cluster analyses using the UPGMA method showed that the cultivars could be grouped into American or Chinese types, with the Chinese types being further clustered into four sub-groups and the American types into two sub-groups. Molecular analysis of variance (AMOVA) showed that 55.76% of genetic variation came from cultivars having different origins and 44.24% from cultivars having the same origin.

Chen *et al.* (2007) characterized the genetic diversity of 118 tobacco cultivars including flue-cured tobacco, sun-/air-cured tobacco, burley tobacco, oriental tobacco and wild tobacco using inter-simple sequence repeat (ISSR) and inter-retrotransposon amplification polymorphism (IRAP) markers. Although low levels of genetic diversity within and among cultivated tobacco types were found, cultivars from the same tobacco types were clustered into the same group. Xiao and Yang (2007) studied the genetic diversity of 119 cultivars of various types of tobacco using 21 ISSR primers. In their study, cluster analysis using the UPGMA method divided the tobacco cultivars into different sub-groups or classes according to type.

Raju *et al.* (2009) reported that 24 tobacco varieties were grouped into 5 clusters using 18 RAPD primers and the UPGMA method for cluster analysis. They also reported that their results were in agreement with their expectations.

Table 5. Name of tobacco varieties clustered together using ISSR markers.

Varieties	Cluster
'Coker-254', 'Coker-298', 'ComstockSpanish', 'Honggarten-Blatt', 'Delhi', 'Badisher Geudert', 'VirginRP37', 'Hicks55', 'PrevStammv3', 'PreviStammV6', 'GoldenGift', 'VirginiaBright88', 'Ex.4.PR-1', 'Alida', 'Pfatzer', 'Bel-61-10', 'Bel-71-500', 'PeeDee', 'RosecanNela', 'TL1112', 'R-30', 'Fixed-A1', 'MontcalmBrum', 'R-9', 'VirginiaAmerican', 'VirginiaRee40', 'HicksBroadLeaf', 'NortCarolina88'	**1**
'ChemicalMutant', 'NOD8', 'Virgin', 'VirginiaH.R.', 'VirginiaRee488', 'C258.MC944', 'NC.95XCHMUTA, 'Soth-Carolina', 'Bel-71-501', 'Bel-61-9', 'AllPurpose', 'Manilla-Geel', 'VirginiaRP.37', 'GewaneGrone', 'NR 23', 'Pennbel69', 'Parfum-ditalie', 'BERGERAC-C', 'TRUMPF'	**2**
'Tirtash-4', 'Tirtash-33', 'PeregR.2-228', 'PeregR.2-234', 'Pereg 234', 'Coker 411', 'GA. 955'	**3**
'Coker 347', 'K.E 1', 'HicksResistant', 'Virginia HR', 'K. 110', 'MCNAIR944'	**4**
'TL 13', 'Tiratash-10', 'Virginia yold', 'TL33', 'P49-4625', 'STNCB', 'C 319.R30', 'Erzegovinia', 'K.S1.E.', 'Speight G.28', 'N.2', 'Hick-RG', 'C 319. C 411', 'R 30. N2', 'Coker 258', 'Vinica', 'MC. 1', 'Look Wood', 'Coker176', 'NC.60', 'P.B.D.6', 'Holandisher', 'RXT', 'IRaburboon', 'Bel', 'GrixollaSoptenol', 'MC.101', 'Tiratash-17', 'AmersFortre'	**5**

Denduangboripant *et al.* (2010) reported that the PCR patterns of two of three major groups of imported tobacco cultivars of 'Burley' and 'Virginia' types were similar to each other, which suggested that they were closely related genetically. As is clear from clustering results obtained in this

study, most tobacco genotypes of the same geographical origin were clustered together; these results are in agreement with previous findings.

Cluster analysis of morphological traits

Cluster analysis was performed to generate a dendrogram using the UPGMA method based on data recorded for morphological traits. Tobacco cultivars were clustered into five groups (Fig. 4). Groups 1 to 5 consisted of 10, 8, 43, 22 and 6 genotypes, respectively. Results of canonical discriminate function analysis using the Fisher linear method showed that the UPGMA method separated the genotypes into five clusters with 78.5% accuracy. Although some genotypes were located in different groups when compared to clustering using molecular information, some genotypes formed similar groups. This shows the consistency of clustering based on morphological traits and geographical origin of genotypes.

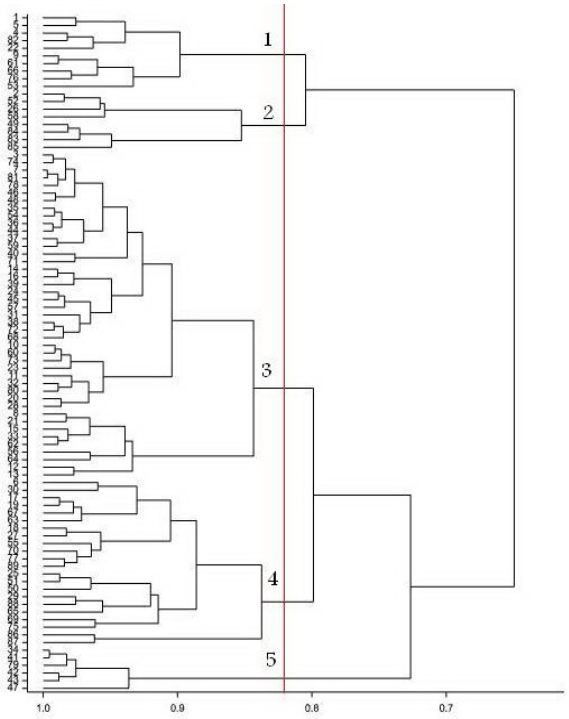

Fig. 4. Dendrogram generated by the UPGMA method based on morphological traits of 89 tobacco varieties.

Although morphological clustering was consistent with molecular clustering to some extent, two similarity matrices were evaluated using Mantel's test to compare molecular and morphological clusters. Results showed that there was no significant correlation between morphological traits and molecular similarity matrices ($r = 0.05$**)**. A likely reason for this may be the nature of molecular markers, because the amplification may have occurred in heterochromatic regions. Under these conditions, the resulting bands are not appropriate indicators of genes whose expression could develop into quantitative traits.

CONCLUSIONS

Based on the importance of the PIC value that describes the polymorphic information content of each primer, UBC817, UBC824 and UBC873 were the most informative primers in the present study and thus could be used to assess the diversity of tobacco cultivars. UBC813, UBC823 and UBC826 could also be used as appropriate ISSR primers with reasonable PIC, Nei's and Shannon's information index, which indicates the high efficiency of these primers in differentiating tobacco genotypes. The findings of this study may be useful in genotyping, germplasm enhancement, and parental selection for breeding purposes in tobacco breeding programs.

ACKNOWLEDGEMENTS

We would like acknowledge and thank the Guilan Tobacco Research Center, Rasht, Iran, for the financial support.

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