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Assessing the genetic diversity of tobacco (*Nicotiana tabacum* L.) varieties

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ABSTRACT

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The genetic diversity of 40 tobacco (*Nicotiana tabacum* L.) cultivars was analyzed using 12 ISSR primers. The cultivars were evaluated for 14 traits using a simple lattice design (7×7) with two replications. Of 149 amplified bands, 142 (95.3%) were polymorphic. Maximum and minimum bands were generated by primers UBC825 (17 bands and 94.11% polymorphic) and UBC824 (7 bands and 100% polymorphic), respectively. Of the studied primers, UBC824 showed maximum Polymorphism Information Content (PIC) (0.43) and the highest genetic diversity, and thus could be used in future genetic diversity studies. In principal coordinate analysis using a similarity matrix, the first two coordinates, which are the main coordinates, explained 41.97% of the total variance. Cluster analysis was performed to determine the genetic relationship of tobacco cultivars using the un-weighted pair-group method with arithmetic average (UPGMA) based on morphological traits and ISSR markers separately. Plant genotypes were divided into nine main groups of flue-cured tobacco cultivars, and a cophenetic correlation coefficient was calculated (r = 0.9), indicating the usefulness of the UPGMA method for clustering plant genotypes. Cluster analysis based on morphological traits divided the studied genotypes into five groups using the UPGMA method. Results of canonical discriminate function analysis using the Fisher Linear method showed that the UPGMA method separated the genotypes with 100% accuracy. Both clusters were consistent with the geographical origins of the cultivars.

Keywords: cluster analysis, molecular marker, morphological traits, *Nicotiana tabacum*, polymorphism information content (PIC)

INTRODUCTION

Tobacco, a perennial plant of the *Nicotiana* genus of the Solanaceae family (Denduangboripant *et al.,* 2010a), is an economically important crop, widely cultivated all over the world (Yang *et al.,* 2007). *Nicotiana* originated in South America and comprises 76 diploid and polyploid species (Knapp *et al.,* 2004). Genetic diversity is important in crop breeding programs for selecting suitable parents to obtain heterotic hybrids (Ahmadikhah, 2007). Genetic identification can be performed by examining morphological or phenotypical characteristics but such characteristics are affected by environmental conditions (Chawla, 2002). However, DNA-based techniques allow scanning the genome directly without being environmentally affected (Doveri *et al.,* 2008). The most commonly used marker systems are restriction fragment length polymorphism (RFLP) (Soller and Beckmann, 1983), random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995), microsatellites or simple sequence repeats (SSRs) (Becker and Heun, 1994) and inter simple sequence repeats (ISSRs) (Zietkiewicz *et al.,* 1994). DNA markers, especially those based on microsatellites (such as ISSR), are useful for assessing a large number of accessions quickly and reliably (Gupta and Varshney, 2000). ISSR markers are simple and reproducible. They require small amounts of DNA and do not require information on DNA sequencing. ISSR markers have been used successfully for estimating the genetic diversity of important crops such as maize (Kantety *et al.,* 1995), wheat (Nagoaka and Ogihara, 1997), rice (Blair *et al.,* 1999), barley (Hou *et al.,* 2005), cotton (Liu and Wendel, 2001) and olive (Terzopoulos *et al.,* 2005). ISSR markers have also been used to study tobacco genetic diversity (Yang *et al.,* 2005) and to carry out genetic fingerprinting of *Nicotiana* spp. (Del Piano *et al.,* 2000). Chen *et al.* (2007) characterized the genetic diversity of 118 tobacco accessions, 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. Xiao and Yang (2007) studied the genetic diversity of 119 accessions, including various types of tobacco, using 21 ISSR markers. The objective of the present study was to study the genetic diversity of imported tobacco cultivars in Iran in relation to morphological traits and ISSR molecular markers and to generate information that could be a source of knowledge for tobacco genetic research and breeding in the future.

MATERIALS AND METHODS

Plant materials and field experiments

In this study, 40 tobacco cultivars (Table 1) were grown in the Tobacco Research Center of Guilan. In a simple lattice design (7×7) with two replications, the cultivars were evaluated for a total of 14 traits: plant height (cm), days to flowering, flowering duration, leaf shape index, leaf area coefficient, leaf width (cm), leaf length (cm), number of leaves, spade value, dry leaf per hectare (kg), fresh leaf per hectare (kg) and stem diameter (cm).

Data analysis (morphological)

Cluster analysis was performed with Genstat version 12 software using the un-weighted pair-group method with arithmetic average (UPGMA). To confirm the accuracy of cluster analysis, canonical discriminate function analysis was performed with Spss.16 software using the Fisher Linear method.

DNA extraction

Genomic DNA extraction from young leaves was carried out according to Doyle and Doyle (1987). The quality of extracted DNA was determined using 1% agarose gel electrophoresis, and 12 ISSR primers (CinnaGen Co.) (Table 2) were used to amplify regions of genomic DNA. The total volume of the PCR reaction mixture was 10 µL, which

Table 1. Names and origin of imported tobacco cultivars.

No.	Name	Origin	No.	Name	Origin
1	Pereg 234	Germany	21	Tiratash-17	Iran
2	Coker 258	USA	22	Amers Fortre	Belgium
3	Coker 347	Italy	23	Holandisher	USA
4	Coker 411	USA	24	Look Wood	USA
5	K.E 1	USA	25	RXT	Poland
6	K. 110	USA	26	GA. 955	Australia
7	MCNAIR944	USA	27	Coker 176	USA
8	TL 13	USA	28	NC.60	USA
9	Vinica	USA	29	IR aburboon	Iran
10	MC. 1	USA	30	P.B.D.6	France
11	MC.101	Japan	31	Bel	USA
12	Gewane Grone	Germany	32	Grixolla Soptenol	USA
13	Hicks Resistant	USA	33	Hick-RG	USA
14	Virginia115	Germany	34	R 30. N2	Iran
15	Virginia yold	Germany	35	C 319.R30	Iran
16	STNCB	Zimbabwe	36	C 319. C 411	Iran
17	TL33	South Africa	37	Erzegovinia	Erzegovin
18	NR 23	USA	38	K.S1.E.	USA
19	P49-4625	USA	39	Speight G. 28	USA
20	Tiratash-10	Iran	40	N.2	USA

Table 2. List of primers*, annealing temperature and sequences, number of bands, number of polymorphic bands and polymorphism information content (PIC).

No.	Primer name	Primer sequence	TM	Number of bands	Number of polymorphic bands	PIC
1	UBC811	5-(GA)8C-3	43.21	15	9	0.155
2	UBC812	5-(GA)8A-3	42.07	16	16	0.340
3	UBC813	5-(CT)8T-3	42.07	12	12	0.273
4	UBC814	5-(CT)8A-3	40.97	14	14	0.314
5	UBC815	5-(CT)8G-3	43.17	8	8	0.349
6	UBC816	5-(CA)8T-3	47.23	13	13	0.364
7	UBC817	5-(CA)8A_3	47.53	16	16	0.264
8	UBC823	5-(TC)8C_3	44.15	10	10	0.311
9	UBC824	5-(TC)8G-3	44.86	7	7	0.432
10	UBC825	5-(AC)8T-3	48.25	17	16	0.299
11	UBC826	5-(AC)8C_3	49.56	11	11	0.324
12	UBC873	5(GACA)4-3	43.34	10	10	0.391
Total				149	142	-
Mean				12.42	11.83	0.318

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

contained 30-40 ng of template DNA, 10 mM dNTP, 0.3 mM primer, 1X PCR Buffer and 1 U DNA Taq polymerase. Amplifications were performed using the Biometra thermo-cycler. Amplification cycles consisted of 35 cycles of 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 dissolved in a 1.5% agarose gel at 90 volts for 60 minutes. The bands were visualized under UV light by Gel DOC (Biometra; BioDocAnalyze, Bio-Rad).

Data analysis (moleculare)

The ISSR bands were scored as either 1 or 0 (presence or absence) (Fig. 1). Nei and Shannon's coefficients (Nei, 1973; Shannon, 1948) were employed to calculate accession similarities using Popgene 1.31 (Yeh and Yang, 1999). The polymorphism information content (PIC) value was calculated as $PIC= 1-\sum P_i^2$, where *pi* is the frequency of the *i*th allele (Smith *et al.,* 1997). Cluster analysis was performed to reveal the genetic relationship among genotypes. Genetic associations among varieties were evaluated using simple matching similarity coefficients for pair-wise comparisons based on the proportion of shared bands produced by the primers. Similarity matrices were generated using the SIMQUAL sub-program of NTSYS-PC software (Rohlf, 1998). Similarity coefficients were used for cluster analysis of varieties using the SAHN sub-program of NTSYS-PC software and dendrograms were obtained using the un-weighted pair-group method with arithmetic average (UPMA) sub-program of NTSYS-PC V.2.1. Principal coordinate analysis (PCoA) was performed using Genstat V.12 on a similarity matrix.

RESULTS AND DISCUSSION

Polymorphic revealed by ISSR markers

The ISSR primers used in this study produced a total number of 149 bands (an average of 12.42 bands per primer) ranging from 450 to 3000 bp; 142 of the 149 bands (95.3%) were polymorphic (Table 2).

The most important parameters of marker efficiency are PIC, heterozygosity and Shannon's information index. In this study, PIC varied from 0.15 to 0.43, with an average of 0.318 (Table 2). According to the importance of PIC in describing the polymorphic information content of each primer, primer UBC824 had the highest PIC (0.43). Also, of all the amplified primers used in this study, primer UBC824 showed the highest genetic diversity in tobacco cultivars.

Nei genetic diversity varied from 0.22 to 0.49, with an average of 0.44. Shannon's information index is calculated as an index of the genetic diversity of a population (Lewontin, 1974). The average of Shannon's information index was 0.63, indicating variation between genotypes. The highest and lowest Shannon's index belonged to UBC 815 (0.6918) UBC 816 (0.6918) and UBC 826 (0.5558) (Table 3).

Principal coordinate analysis

Principal coordinate analysis is used to explain genetic variation and show the variation pattern in a multidimensional pattern and a better interpretation of the relationship between individuals (Khayyam Nikoyiee *et al.,* 2009). The relative variance of each coordinate indicates the importance of the related coordinate of total variance and is expressed as a percentage. All the data obtained using 12 ISSR primers were used in principal coordinate analysis with simple matching coefficients of similarity.

The first 17 coordinates explained 90.63% of the

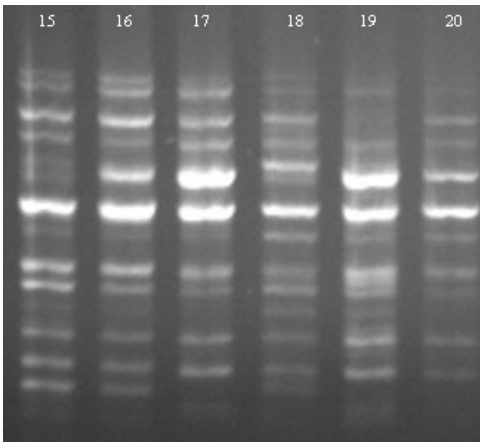

Fig. 1. ISSR-PCR amplification profiles of six accessions using UBC811 primer.

total variance. The first coordinate explained 30.55% of the total variance and the second, 11.42%. So the number of bands (142 polymorphic bands) was reduced to a smaller number and the primers were chosen accurately. In molecular data, the first two or three coordinates explained 10-20% of primer changes; this may not be appropriate for graphical views but indicates appropriate sampling of primers from the whole genome. The greater the number of coordinates, the greater the genome coverage (Siahsar *et al*., 2010). The first two coordinates explained 41.97% of the total variance, indicating that ISSR markers are scattered over different parts of the genome. A scatter plot of the genotypes was prepared based on the main coordinates. Tobacco genotypes were grouped into five different clusters according to their similar characteristics (Table 4, Fig. 2).

Table 3. Nei's index (H) and Shannon's information index (I) of the cultivars' genetic diversity.

No. of primer	Primer name	H	I
1	UBC811	0.3712	0.5560
2	UBC812	0.4676	0.6600
3	UBC813	0.4406	0.6301
4	UBC814	0.4445	0.6282
5	UBC815	0.4987	0.6918
6	UBC816	0.4987	0.6918
7	UBC817	0.4456	0.6348
8	UBC823	0.4450	0.6349
9	UBC824	0.4578	0.6483
10	UBC825	0.4333	0.6246
11	UBC826	0.3760	0.5558
12	UBC873	0.4303	0.6181
Mean		0.4424	0.6312

Table 4. Eigen values, variance and cumulative variance.

No. of coordinate	Eigen value	Variance (%)	Cumulative variance (%)
1	3.6813	30.55	30.55
2	1.3755	11.42	41.97
3	0.8474	7.03	49.00
4	0.4914	6.57	55.57
5	0.6989	5.80	61.37
6	0.5390	4.47	65.84
7	0.4683	3.89	69.73
8	0.3920	3.28	73.01
9	0.3702	3.08	76.09
10	0.3371	2.80	78.89
11	0.2964	2.46	81.35
12	0.2421	2.01	83.36
13	0.2210	1.83	85.19
14	0.1883	1.56	86.75
15	0.1690	1.40	88.15
16	0.1521	1.26	89.41
17	0.1466	1.22	90.63

Cluster analysis

Cluster analysis was performed to generate a dendrogram based on ISSR data (Fig. 3). Plant genotypes were divided into nine main groups. Values of similarity coefficients ranged from 0.34 to 0.98. Groups 1 to 9 consisted of 2, 1, 3, 1, 1, 25, 4, 1 and 2 genotypes, respectively. Accessions of different geographical origins were clustered separately; for example, GA955 from Australia was included in the fifth cluster and Virginia 115 from the USA in the second cluster. Accessions of the same tobacco type were clustered in the same group, for example, Coker 258, Coker 347 and Coker 176 from the USA. This clustering is consistent with the cultivars' geographical origin. Given that a higher cophenetic correlation coefficient (r = 0.9) indicates the usefulness of the method, it is clear that UPGMA clustering grouped the cultivars appropriately. A cophenetic correlation coefficient of greater than 0.8 has been recorded as appropriate in by other researchers (Mohammadi and Prasanna, 2003). Yang *et al.* (2005) used ISSR markers and cluster analysis with the UPGMA method to divide 24 flue-cured tobacco varieties into five major groups, the largest of which consisted of 12 varieties derived from Coker 319. They found that the 24 flue-cured tobacco cultivars (only 'Burley' and 'Virginia' cultivar groups) were closely related and had low genetic diversity. Xiao and Yang (2007) studied the genetic diversity of 119 accessions of various types

of tobacco using 21 ISSR primers. Cluster analysis using the UPGMA method divided the accessions into different subgroups or classes according to the types of tobacco. 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 subgroups and the American types into two subgroups. Results of the present study are in agreement with previous findings and also show that tobacco genotypes having the same geographical origin cluster together.

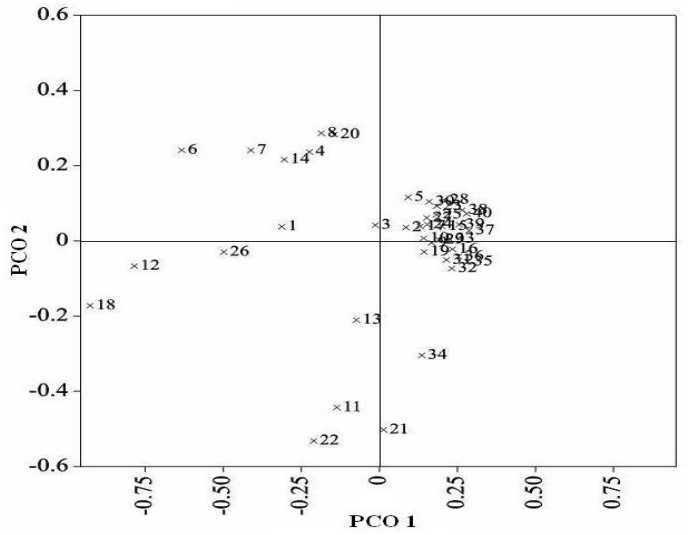

Fig. 2. PCO grouping of tobacco cultivars. Each digit is the ID of a tobacco cultivar according to Table 1.

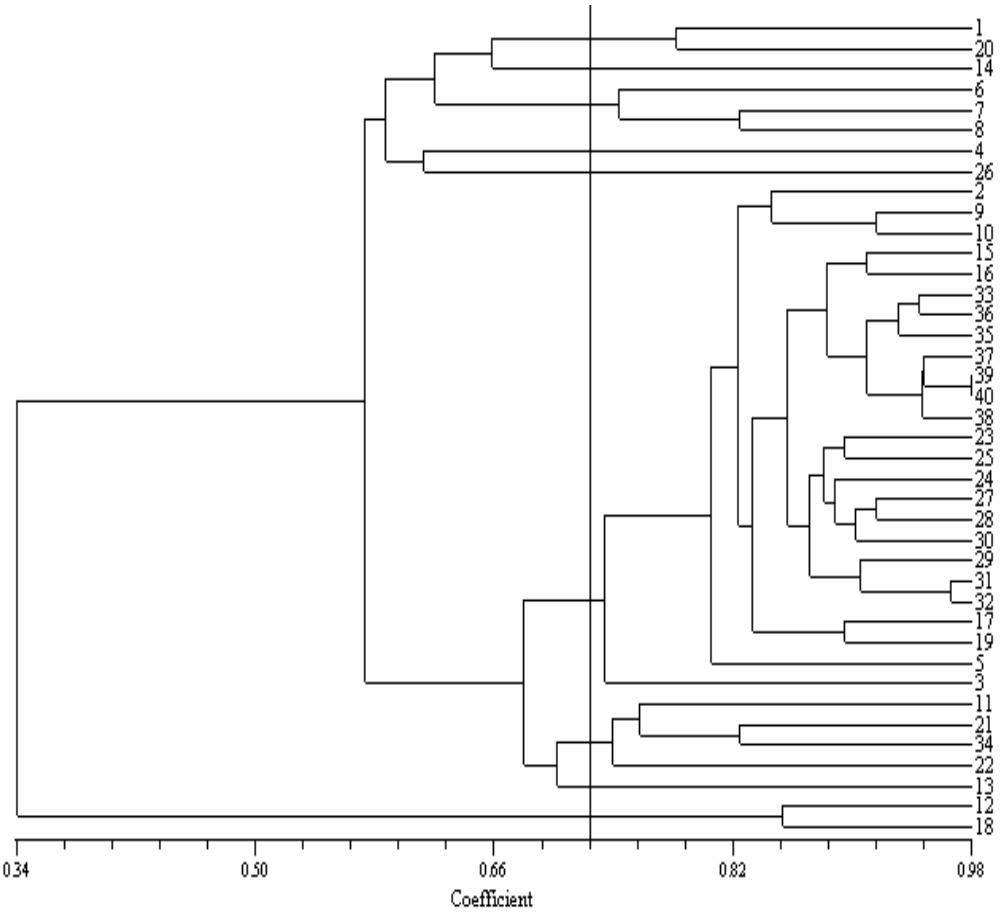

Fig. 3. Dendrogram generated by the UPGMA method based on intersimple sequence repeat (ISSR) amplification polymorphism analysis of 40 tobacco accessions. A cophenetic correlation coefficient was calculated (r = 0.9), indicating the usefulness of the UPGMA method in clustering plant genotypes.

Cluster analysis of morphological traits

Based on data on morphological traits measured in this study, cluster analysis was performed to generate a dendrogram using the UPGMA method. Plant genotypes were divided into five groups (Fig. 4). Groups 1 to 5 consisted of 6, 22, 1, 2 and 9 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 100% accuracy. Some of the genotypes that clustered together when molecular clustering was used again clustered in the same group using morphological clustering, but other genotypes were in different groups. This shows the consistency of clustering with the geographical origin of genotypes. Overall, morphological clustering was somewhat consistent with molecular clustering. Differences between these two clustering methods were caused by environmental factors and project conditions. However, molecular markers show genomic differences that are not influenced by environmental conditions and thus are more reliable.

CONCLUSION

Finally, according to the importance of PIC for describing the polymorphic information content of each primer, UBC824, with the highest PIC (0.43) and the greatest genetic diversity among the studied cultivars, was the most informative primer in this study, indicating its suitability for studying the genetic diversity of tobacco cultivars. Studying the genetic diversity of tobacco cultivars using ISSR markers showed that these markers are useful for identifying polymorphic loci. Therefore, the ISSR markers can be used to differentiate tobacco

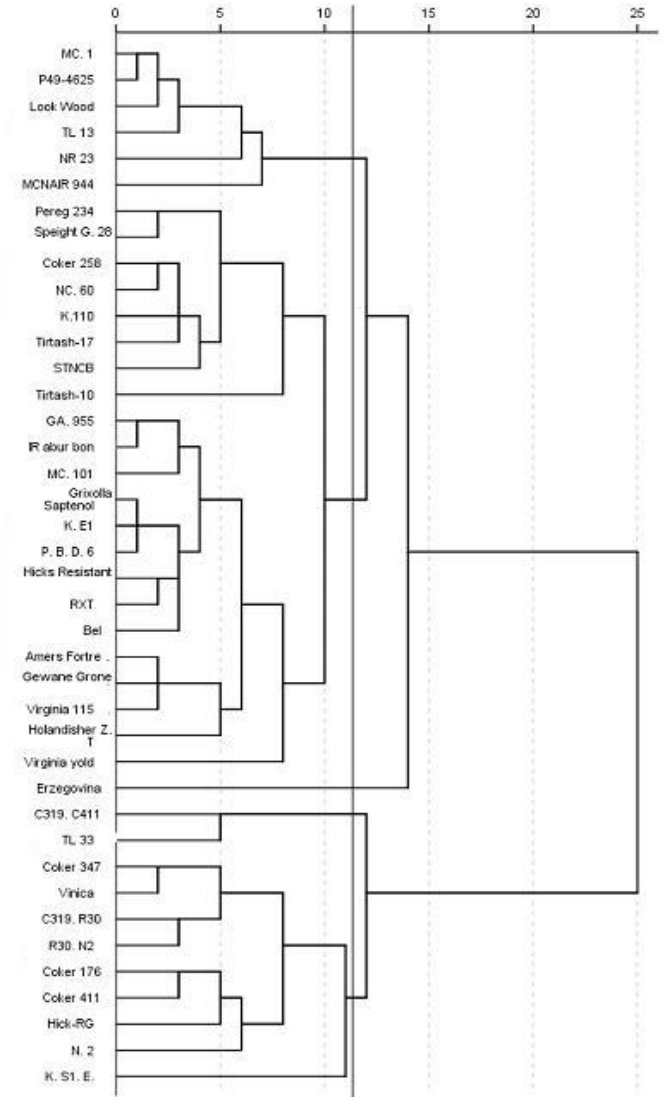

Fig. 4. Dendrogram generated by the UPGMA method based on morphological traits of 40 tobacco accessions. Results of canonical discriminate function analysis using the Fisher Linear method showed that the UPGMA method separated the varieties into five clusters with 100% accuracy.

cultivars (Denduangboripant *et al.,* 2010b). Knowledge of the genetic relationship between cultivars is useful for selecting superior and genetically divergent parents for hybridization to optimize the genetic variation of subsequent generations (Pradeep Reddy *et al.,* 2002). Results of this study could be an important source of knowledge for future tobacco research, such as genotyping of tobacco cultivars, germplasm improvement, and parental selection for breeding purposes.

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