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# Cytoplasmic variation between different tobacco cultivars revealed by mitochondrial-specific markers

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## Abstract

Tobacco (*Nicotiana tabacum* L.) is one of the most important commercial and industrial crops in the world. Genetic diversity studies provide estimates on the level of genetic variation among diverse materials that can be used in germplasm management, varietal protection, and tobacco improvement. Forty and two tobacco cultivars of three known ecotypes from different origins were genotyped by three mitochondrial-specific markers including two PARSs and one Atp1-sepecific primer pair. The most diversity in terms of Shannon index was observed within Burley ecotype. The largest genetic distance was observed between Virginia and Basma. A low Nei`s distance was obtained between 42 cultivars, indicating that a large proportion of valuable tobacco germplasm may already have been lost through the popularity of certain cultivars in commercial planting and the continuous artificial selection.. Cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) showed that the cultivars could be grouped into three classes, with no significant relationship between clustering groups and ecotypes.

**Key words:** Tobacco, Mitochondria, PARS, Marker, Diversity.

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## Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most important industrial plants in the world that has a main role in economical consumer and producer countries [5] and in economically goods is valuable and at least in 97 countries is cultivated for economical purposes [3]. Tobacco belongs to Solanaceae and is a grass yearlong, little grass perennial with 1-2 m height. There are many tobacco varieties and landraces and the number of new varieties in the world steadily increases.

Three ecotypes famous in the word are Burley, Basma and Virginia types. However, Virginia is the most common type to cultivate in the world. Tobacco is known as an economic crop [14].

Genetic diversity studies provide estimates on the level of genetic variation among diverse materials that can be used in plant breeding. Genetic diversity is useful for description of unique varieties and as a guide for selecting parents for crossing in plans of breeding plant. Morphological, karyotypical, and physiological characters have already been used to study the genetic diversity of flue-cured tobacco germplasm [4, 8, 18]. However, morphological characters usually vary with environments. The number of karyotypical characters is limited, and the study of genotypic diversity based on isozyme variation is restricted to a few polymorphic enzyme systems encoded by a small number of loci [8]. More information of genetic variation within commercially cultivated tobacco between cultivated forms of *N. tabacum* and its wild relatives should be revealed by molecular markers. Species-specific markers identified in studies are useful in identification of the true hybrids and monitoring introgression of useful genes from the wild relatives [17].

DNA-based molecular markers have acted as valuable tools and have found their own position in various scientific fields like characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding, and diagnostics. For example, Zhang et al (2006) used the Amplified fragment length polymorphism (AFLP) technique to clarify the genetic relationships between 51 distinct flue-cured tobacco accessions with desirable agronomic characteristics from the germplasm collections of the South China, Tobacco Breeding Research Center. Cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) showed that the cultivars could be grouped into American or Chinese types, with the Chinese types being further clustered into four subgroups and American ones into two subgroups. The findings of this study revealed that the nowadays commonly grown flue-cured tobacco germplasm has narrow genetic diversity among the cultivars, necessitating a sustained effort to preserve flue-cured tobacco germplasm resources. Further crosses should be made only with genetically distant varieties. Based on investigations of the structure and organization of mitochondrial and chloroplast genomes, Olmstead and Palmer (1991) demonstrated that a species similar to *N. sylvestris* donated the maternal genome of tobacco.

Recently, a molecular marker technique named Palindrome amplification by reverse sequence (PARS) has been developed to detect polymorphism within palindrome areas of genomic, mitochondrial and chloroplast DNA as well as viral, bacterial and fungal DNA [2]. PARSs are mostly co-dominant markers and densely distributed within genomes, even in virus sequences such as HIV. This technique was successful in producing markers in many mitochondrial genomes of plants, particularly for detecting polymorphism among different cultivars of tobacco [2].

In this study we report the first case of utilization of mt-DNA and PARS markers for screening mitochondrial genome of tobacco.

## Material and Methods

### Plant material

In this study, 42 cultivars of three tobacco ecotypes (including Burley, Basma and Viginia) were collected from Tobacco Research Institute, Tirtash, Northern Iran (table 1.). Leaf samples were stored in -80 °C untill DNA extraction.

### Genomic DNA Extraction

Young leaves were collected from 42 samples. Total genomic DNA was extracted by a modified CTAB method [2]. Leaves were ground in liquid nitrogen using mortar and pestle to a very fine powder. It was then transferred to pre-warmed extraction buffer and incubated at 65°C for 45 min and vortexed for 60s, then added an equal amount of chloroform: isoamyl alcohol (24:1), mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.7 volume of cold isopropanol to precipitate DNA. After centrifugation, the pellet was dried and dissolved in water. DNA was quantified on the agarose gel, diluted and used in PCR.

### Polymerase chain reaction (PCR) condition

Polymerase chain reaction (PCR) was performed in 15 µl volumes containing 0.75 µM/l of each primer, 7.5 µl Master Mix (CinnaGen, Iran) (including 200 µM/l dNTPs, 50 mM/l KCl, 10 mM/l Tris HCl, 1.5 mM/l MgCl2, and 1 unit of Taq DNA polymerase), 5µl H2O and 1µl DNA. The PCR profile was 94 oC for 5 min (denaturation), followed by 35 cycles of 94 $^{o}$C for 1 min, 50, 55 or 60 $^{o}$C (depend on the melting temperature of the primer pairs) for 1 min, 72 $^{o}$C for 2 min, and finally 72 $^{o}$C for 7 min in the final extension. The products from PCR reaction were resolved by electrophoresis in 2.5% agarose gel containing 0.5 µg/ml ethidum bromide.

### Primer synthesis

Cytoplasmic diversity was determined among cultivars using one mitochondrial-specific primer pair namely NtATP1mt01, and two PARS primers (NtFARSmt01 and NtFARSmt02) in PCR reaction. Tobacco mitochondrial sequence (GenBank accession number: NC006581) was used for designing primers. For distinguishing palindrome areas 2sqBLAST online program was used (www.ncbi.nlm.nih.gov\blast2seq) and suitable primers were designed with PRIMER 3.0 program (Table 2.). These primers had long lengths, thus they might amplify fragments of about 160 and 500 bp, respectively in PCR as expected from their location in mitochondrial genome of tobacco.

### Analyses

Data collected from banding pattern of markers were analyzed using Popgen32 software. Several criteria of population genetic, such as number of alleles ($N_o$), number of effective alleles ($N_e$), polymorphic loci, allele frequency, Shannon's information index (I) [12], genetic identity (GI) and genetic distance (GD). Similarity matrix was computed based on Nie's unbiased measures of genetic identity and genetic distance [9] and used to construct dendrogram by unweighted pair group methods with arithmetic average (UPGMA) method.

**Table 1. Plant material used in this study**

| ID | Cultivar name | Ecotype |
|---|---|---|
| 1 | Burley 21 | Burley |
| 2 | Cms Burley21 | Burley |
| 3 | Basma | Basma |
| 4 | Cms Basma | Basma |
| 5 | Coker347 | Virginia |
| 6 | Cms Coker347(RGH4xC347) | Virginia |
| 7 | Cms Coker347(NC100xC347) | Virginia |
| 8 | Orumia205 | Basma |
| 9 | Cms Orumia205 | Basma |
| 10 | Coker176 | Virginia |
| 11 | Cms Coker176 | Virginia |
| 12 | MN994 | Virginia |
| 13 | Cms MN994(NC100xMN994) | Virginia |
| 14 | Cms MN994(Ult128xMN994) | Virginia |
| 15 | Izmir | Basma |
| 16 | Cms Izmir | Basma |
| 17 | Tmv2 | Virginia |
| 18 | Cms Tmv2 | Virginia |
| 19 | Narocop 261 | Basma |
| 20 | Ourmia 209 | Basma |
| 21 | Basma 104.1 | Basma |
| 22 | Matianous | Basma |
| 23 | Imine | Basma |
| 24 | Kuklen 6 | Basma |
| 25 | So5 | Basma |
| 26 | D-566 | Basma |
| 27 | Polovidiv 58 | Basma |
| 28 | Orumia 347 | Basma |
| 29 | Kromov grad N.H.H 659 | Basma |
| 30 | Polovidiv 7 | Basma |
| 31 | Rila 544 | Basma |
| 32 | Kromov gral 544 | Basma |
| 33 | Haymanli | Basma |
| 34 | Basma S.31 | Basma |
| 35 | Zichna | Basma |
| 36 | Izmir | Basma |
| 37 | Erzigovin | Basma |
| 38 | Basma 178-2 | Basma |
| 39 | Basma 16.10 | Basma |
| 40 | K.P. 140 | Basma |
| 41 | P.Z. 17 | Basma |
| 42 | Melmik 261 | Basma |

**Table 2. Primer sequences and their locations in mitochondria genome of tobacco**

| *Primer symbol* | *Sequences* | *Location in NC006581* |
|---|---|---|
| NtPARSmt01 | 5´-CCGCCCTTATTAGTAGTAG-3´ | 123511- 123675 |
| NtPARSmt02 | 5´-GGAAGGCCGACGACTACAT-3´ | 200258- 200816 |
| NtATP1mt01 | F: 5´-GGACCACCTTAAGCAAATAG-3´<br>R: 5´-CTACCAGTCTCTCCTTTTTTTTATTCC-3´ | **261106-261235** |

## Results and Discussion

### Allele sizes and frequencies

This investigation was carried out to reveal genetic diversity within mitochondrial DNA of tobacco. In this study, enough cytoplasmic variation within marker loci was observed in mitochondrial genome. Allele sizes observed in three loci were within the range as the same as expected in the mitochondrial genome predicted by bioinformatics software. In these loci two alleles (A and B) for all of samples were observed and totally were produced 126 bands. Allele sizes were 160 and 165 bp for NtPARSmt02 primer, 500 and 507 bp for NtPARSmt03 primer, and 130 and 133bp for NtATP1mt01, respectively (Figure 1).

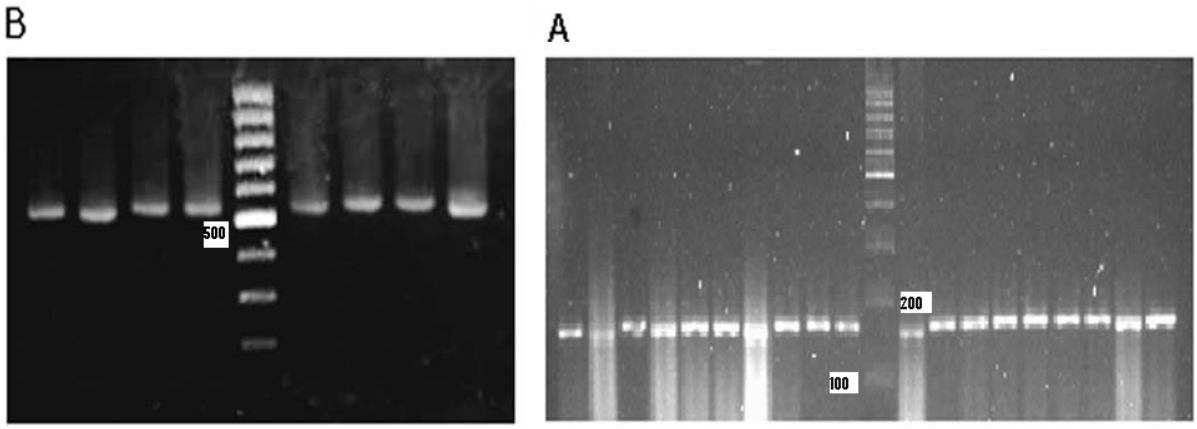


**Figure 1. A sample of banding patterns obtained with primers (A) NtPARSmt01 and (B) NtPARSmt02**

Result of analysis on marker loci showed that no heterozygosity exists in studied cultivars, confirming that open-pollination does not affect cytoplasm, because pollen does not carry cytoplasm and have maternal inheritance. Nei`s, Shannon and PIC indices for NtPARSmt02 were more than other ones, indicating being more diverse the locus, and hence is more effective the use of this marker for polymorphism detection and genetic diversity (Table 3).

**Table 3. Summary of genic variation statistics for all loci**

| Marker locus | Allele frequencies | | No. observed alleles | No. effective alleles | Nei`s distance | Shannon index |
|---|---|---|---|---|---|---|
| | A | B | | | | |
| NtPARSmt01 | 0.76 | 0.24 | 2 | 1.57 | 0.36 | 0.55 |
| NtPARSmt02 | 0.64 | 0.36 | 2 | 1.85 | 0.46 | 0.65 |
| NtATP1mt01 | 0.24 | 0.76 | 2 | 1.57 | 0.36 | 0.55 |
| Mean | - | - | 2 | 1.66 | 0.395 | 0.583 |

**Diversity analysis at ecotype level**

Analysis of basic statistics on three ecotypes of tobacco showed diversity level within ecotypes. The most and least genetic diversity was observed within Burley and Basma ecotypes, respectively (Table 4).

**Table 4. Shannon index (I) in different combinations of loci-ecotypes**

| | Basma | Virginia | Burley |
|---|---|---|---|
| Loci | I | I | I |
| NtPARSmt01 | 0.58 | 0.33 | 0.70 |
| NtPARSmt02 | 0.64 | 0.70 | -[a] |
| NtATP1mt01 | 0.33 | 0.48 | 0.7 |
| Mean | 0.52 | 0.57 | 0.70 |

a. No polymorphism was detected at this locus for Burley ecotype.

Genetic distance (DS) and genetic identity (GI) between ecotypes ranged between 0.14–0.2 and 0.82–0.87, respectively. Highest genetic distance among ecotypes was observed between Virginia and Basma (Table 5).

**Table 5. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal)**

| | Basma | Virginia | Burley |
|---|---|---|---|
| Basma | *** | 0.82 | 0.87 |
| Virginia | 0.20 | *** | 0.85 |
| Burley | 0.14 | 0.16 | *** |

Dendrogram obtained by UPGMA method is shown in figure 2. Scale above dendogram shows genetic distance. This dendogram shows two ecotypes Burley and Basma are more similar in mitochondrial genome.

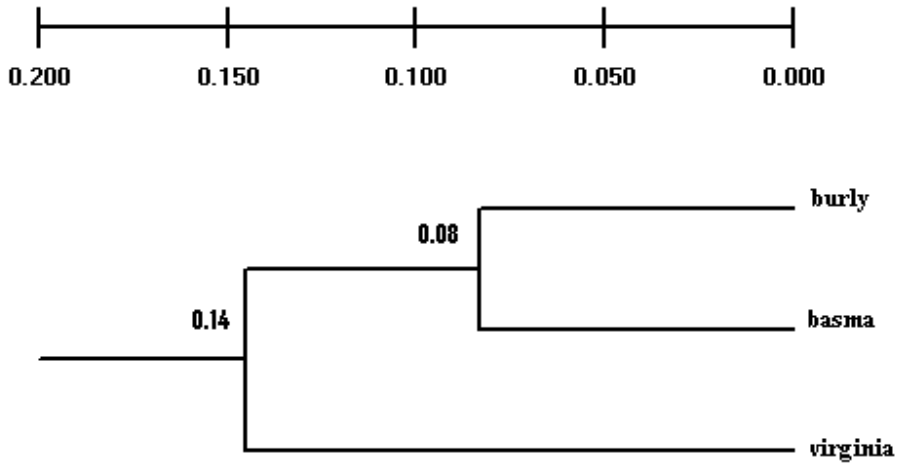


**Figure 2. Dendrogram produced between ecotypes by UPGMA method**

**Cluster analysis**

Forty and two genotypes were divided to three clusters (A, B and C), with the cluster A being further subdivided into two subgroups as shown in figure 3. As seen in the figure, there is no significant relationship between clustering groups and geographical ecotypes.

In this study we attempted to investigate mtDNA diversity within geographical ecotypes and among different cultivars of tobacco using mtDNA-specific markers. As shown in table 4, diversity in terms of Shannon's information index (I) within three ecotypes at all marker loci was slightly high, with maximum I=0.70 within Burley ecotype and with average of ~0.60 across all loci. The high diversity obtained for this ecotype can be associated to use of low number of cultivars in this ecotype for genotyping. Also, maximum genetic distance was obtained between Virginia and Basma (GD= 0.2) that is enough low. Wang and Zhou (1995) also obtained low GD between flue-cured tobacco cultivars and concluded that low level of genetic distance suggested the existence of limited genetic variation in flue-cured tobacco cultivars. The existence of low genetic diversity within cultivated flue-cured tobacco has been attributed to self-pollination [15].

A low Nei`s distance also was obtained between 42 cultivars in this study (0.395 in Table 3). A low DNA polymorphism level was also reported in several other self-pollinating plants such as wheat [6], pigeonpea [11], tomato [16], and coffee [13]. It is possible that a large proportion of valuable tobacco germplasm may already have been lost through the popularity of certain cultivars in commercial planting and the continuous artificial selection. Therefore, to avoid further degradation of germplasm resources, crosses should be made with genetically distant varieties or genotypes of diverse origin [17].

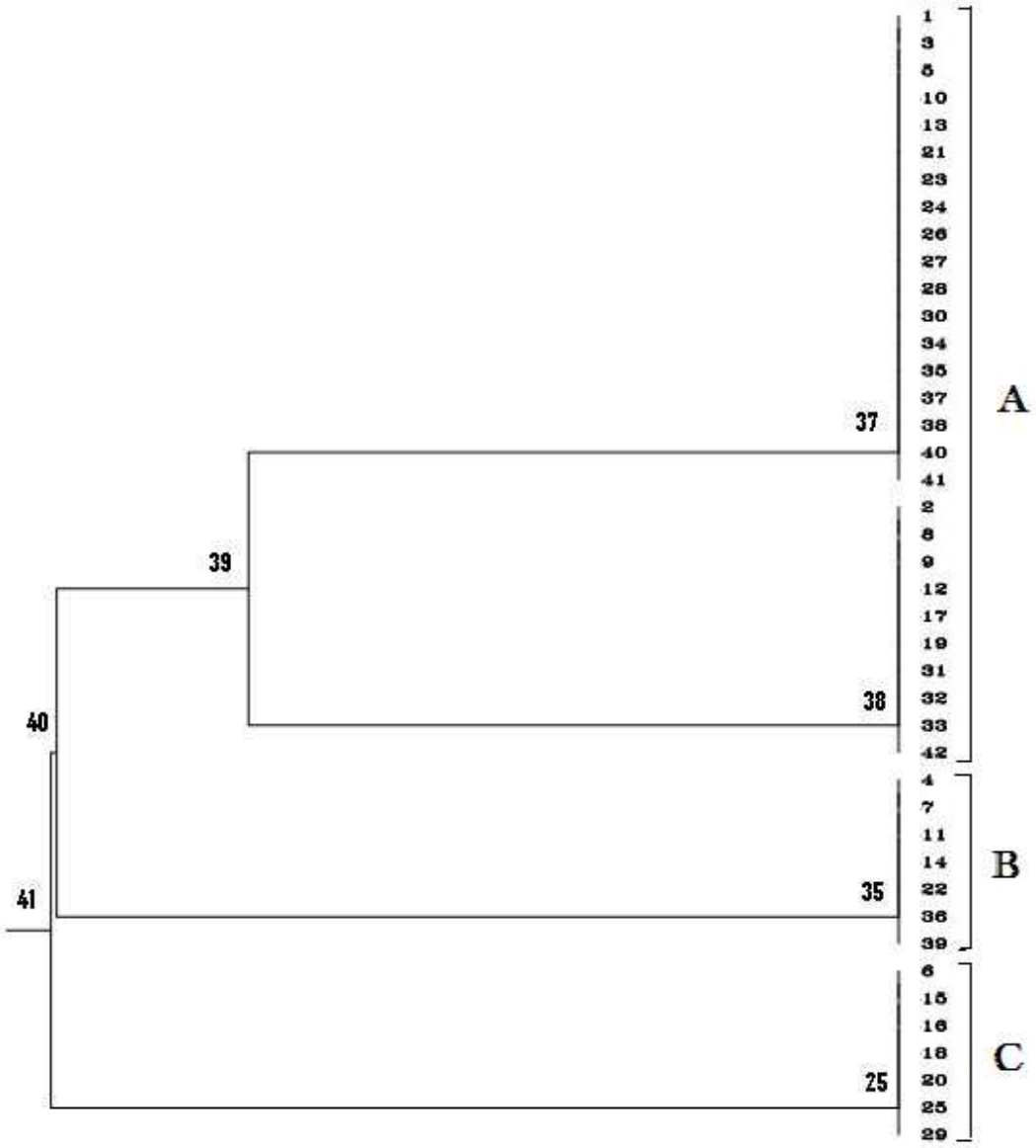


**Figure 3. Dendogram of 42 tobacco cultivars using UPGMA method**

## Conclusion

Mitochondrial-specific DNA markers developed in this study could detect polymorphism in mtDNA of tobacco cultivars. Although the variation in mtDNA within cultivars was relatively low, the mtDNA-specific markers could discriminate between geographical tobacco ecotypes.

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