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Chloroplast Microsatellites Markers to Assess Genetic Diversity in Wild and Cultivated Grapevines of Iran

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Abstract: To assess the haplotype diversity and genetic relationship between them, A set of 69 Iranian cultivated accessions, six European cultivars and an accession of Vitis labrusca along with 63 wild grapevine individuals were studied using chloroplast microsatellite markers. Results showed that among analyzed cpssr loci only ccmp 3 and ccmp10 were polymorphic within cultivars and only ccmp3 was polymorphic in wild grape individuals. The size variants of both loci combine in a total of 4 different haplotypes. All the 4 haplotype are displayed in the cultivars while only 2 are presented in wild grapes. Sultani or keshmeshi Bidane cultivar has the haplotype III that there is not this haplotype among the wild grapes of studied regions. Concerning to existence of both haplotypes I and II in the number of Iranian cultivated and wild grapes, it is possible to consider that the wild grapes are ancestor of some of our native cultivars.

Key words: Wild grape, *Vitis vinifera*, chloroplast, microsatellite, haplotype

INTRODUCTION

Iran due to diversified climate is suitable for grapevine growth and cultivation. Iranian grape germplasm is a rich and complex germplasm consist of a large number of grapevine cultivars and wild populations (Tafazzoli, 1993, 1976). Unfortunately there is not any precise historical evidence about viticulture in Iran. However, McGovern (2003), suggested that human beings met wild grapes for the first time in the upland regions of eastern Turkey and in the north-western of Iran during the Palaeolithic era. The earliest evidences of grape cultivation dates back to the fourth millennium in the Middle East (Zohary and Hopf, 2000) and it has been proved that winemaking was already present in Iran during the second half of the sixth millennium B.C. (Mc Govern, 2003).

Wild grapevine prefers humid condition while the most part of domesticated accessions grow in dry habitats. Therefore, the mating system can be used as a efficient criteria to discriminate the two subspecies. Wild grapevine is dioecious while the domesticated relative is hermaphrodite (Grassi *et al*., 2003).

Because of non-recombinant, uniparental inheritance and haploid nature, cpDNA acts as a single heritable unit and due to the low genome mutation rate they are highly conserved (Ferris *et al*., 1998; Marchelli *et al*., 1998; Fineschi *et al*., 2002; Arroyo-Garcia *et al*., 2002). The availability of molecular markers to analysis genetic relationships provides new possibilities to define the genetic relatedness between wild and domesticated grapevines. In particular recently, Development of microsatellite or SSR (simple sequence repeats) markers based on chloroplast DNA provides powerful tool for analysis of phylogenetic relationships, cytoplasmic diversity, inheritance of plastids, determination of precise direction of cross between different genotype, monitoring gene flow and to study the history of wild grape domestication. (Arroyo-Garcia *et al*., 2002; Bowers *et al*., 1999; Provan *et al*., 1999). Universal angiosperm chloroplast microsatellite primers were developed by Weising and Gardner (1999).

In Iran, Wild grapes (*Vitis vinifera* ssp. *sylvestris*) as ancestor of cultivated today grapevines were found generally in humid regions of Alborz and Zagros

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mountains serial (Sabeti, 1976). Close to Zagros mountain serial there are many local grapevine cultivars that have been grown for a long time.

In this study, allelic variation at polymorphic cpSSR loci was used to characterise haplotypic diversity within and between Iranian *Vitis vinifera* cultivars and wild grapevine (*V. sylvestris*).

MATERIALS AND METHODS

Plant material and DNA extraction: A set of 69 Iranian cultivated accessions from Vitis germplasm collection of agricultural research centre of West Azerbayjan, Ourmieh-IRAN, 6 European cultivars (Cabernet sauvignon, Cabernet franch, Barbera, Muscat Alexandria, Pinot noir and Chardonnay) and an accession of Vitis labrusca along with 63 wild grapevine genotypes were studied using chloroplast microsatellite markers. Based on their morphological characteristics (especially dioecious mating system) the wild grapes were collected from different regions of West Azarbayjan and Kurdistan province, Iran All the sampled wild populations consisted of genotypes from typical wild grapevine habitats including wetlands and forests with a high degree of humidity along Zagros Mountains serial. Table 3, indicates names, identification code, number of individuals and geographical coordinates of populations belonging to each group. Every population was defined by a number of individuals ranging from 7 to 23 samples.

Cp-SSR analysis: Genomic DNA was extracted from young leaves of each sample using CTAB method as described by Labra *et al.* (2001). The 8 cp-microsatellite loci including ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7, ccmp9 and ccmp10 (Weising and Gardner, 1999; Grassi *et al.*, 2002) were used to amplified chloroplast DNA. The amplification was performed by using PCR-beads Ready-to-go KIT (Amersham- Bioscience, Italy) along with 10 ng of total DNA and 5 ng of forward and reverse primers. The forward primer was labelled with ^{33}P-ATP (Amersham, Italy). PCR amplification was carried out with the following thermal cycles: 3 min at 94°C; 35 cycles of denaturation (45 sec at 94°C), annealing (30 sec at 50°C) and extension (1 min at 72°C); then a final step for 7 min at 72°C. In the case of ccmp2, the annealing temperature was 51.5°C. A total of 5 μL of the PCR product was added to an equal volume of loading buffer (80% formamide, 1 mg mL^{-1} xylene cyanol FF, 1 mg mL^{-1} bromophenol blue, 10 mM EDTA, pH 8.0). A volume of 1.5 μL was loaded on a 6% denaturing polyacrylamide gel and electrophoresed in TBE buffer for 3 h at 80 W. The gel was fixed in 10% acetic acid and exposed to an X-ray film for 24 h. The resulting autoradiograms were scored by visual inspection.

Statistical analysis: Haplotype frequencies were measured as the percentage of individuals sharing the same haplotype in each group, both for cultivated and wild grapevines.

Gene Diversity (Nei, 1987), were estimated as $GD = 1-\Sigma p i^2$ where n is the number of alleles and p is the frequencies of the allele. Haplotype diversity was calculated in the same formula with n and p referring to haplotypes.

RESULTS

cpSSR alleles and haplotype definition: Among 8 analyzed cp-SSR loci, ccmp3 and ccmp10 loci showed polymorphism in the studied accessions. Two (106 and 107 bp) and three (114, 115 and 116 bp) different size variants were found at locus ccmp3 and ccmp10 respectively. Within the all cultivars analysed the 5 allelic variants identified at two chloroplast loci. At locus ccmp3 the most frequent fragment in the wild and cultivated samples were the 106 bp allele. Whereas, within the cultivated samples, 115 bp fragment was the most frequent at ccmp10 locus and only one sample (Pinot noir) had a 114 bp allele in this locus.

Both of the loci showed a uni-model distribution, with alleles differing by 1 bp from each other. This suggests that the observed variation confirms the stepwise mutation model (SMM), which can be explained by replication slippage at microsatellite loci (Freimer and Slatkin, 1996). Furthermore, the non-recombinant nature of the chloroplast genome means that unequal crossing-over cannot be the main causal mechanism for polymorphism.

Cultivated grapes haplotype diversity: Combinations of two loci alleles in the accessions resulted into 4 different haplotypes as showed in Table 1. The most of the cultivars had haplotype I (%39.47), that more than haplotype III (30.26%), II (28.94%) and IV (1.3%). From 17 native grapes in the regions of Sardasht, Piranshahr and Baneh, 15 cultivars have showed haplotype I this indicate that they have a probable common ancestors. The other two samples had haplotype II and III (Table 1). Haplotype diversity for all cultivated samples was 0.668 that very similar to the values obtained in other studies (Arroyo-Garcia *et al.*, 2002).

Heredity of plastids in angiosperms like grapes is maternally (Strefeler *et al.*, 1992). In this study Jig Jiga from Tabriz and Nazl grapes both had haplotypeIII.

Table 1: Haplotype defined basing on the ccmp3 and ccmp10 alleles sizes (bp) with list of cultivars

Haplotype	Cultivars
I (allele 107 + allele 115)	Agh Shani, Akuz Guzi, **Angotka**, Abi Balo, **Bol mazo**, **Bul mazu**, **Chava Ga**, Goi Maleki, **Gonka**, Hosaini, Inak Amjaii, **khoshnav**, **Klka Revi**, Lal Qermez, **Mam Braima**, **Rasha**, Sachakh, **Sayani**, **Sarghola**, Shirazi, **Seyah Mamoli**, **Taiefi**, **Zardka**. Cabernet Sauvignon, Cabernet Franch, Barbera, Ormia 63, Yaghoti and Saghal solian2
II (allele 106 + allele 115)	Agh Melhi, At Ouzum, Dizmari, Fakhri, Qara Ouzum, Qara Shira, Qara Melhi, Lal Bidaneh, Lal Seyah, MaieMo, Makaii, Mosli, Rezghi, Rishbaba Sefid, **Sorav**, Saghal solian, Muscat, Jig jiga, **Seyah Sardasht**, Labrusca, Ormia 65 and Ormia 65
III (allele 106 + allele 116)	Alhaghi, Askari, Dastarchin, Galin Barmaghi, Saghal solian 1 Qara Gandoma, Qara Shani, Qzl Ouzum, Gazandaii, **Kazhav**, Keshmesh Sefid, Bidane Qermez, Khalili, Kalati, Tabarza Qermez, Rejin, Rishbaba Qermez, Sahebi, Bidane sefid, Tabarze sefid, Jig jiga tabriz, NAZ1 and Chardonnay
IV (allele 106 + allele 114)	Pinot noir

Grapevines cultivars from baneh, sardasht and piranshahr regions are in bold

Considering that Naz1 is a hybrid between Jig Jiga (as a mother parent) and Rupestris species (Table 1).

According to results of chloroplast markers, three homonymous were detected between Jig Jiga from Tabriz and Jig Jiga from Urmia, Rishbaba Sefid and Rishbaba Qermez and three samples in name Saghal solian. These cultivars showed a completely different haplotype from each other. Labrusca grape collecting from the north forest of Iran and aromatic Muscat grape both had the same haplotype (Table 1). Sultani or Keshmeshi Bidane cultivar revealed haplotypeIII that this haplotype did not observe among wild grape accessions.

The Rasha is an important grape cultivar that is predominantly cultivated in the regions of North West and west of Iran. It is very similar to wild grapes in some fruit characteristics such as colour and shape but unlike of wild grapes, it is hermaphrodite. We observed the haplotypeI in Rasha and numbers of wild grapes (Table 1 and 3).

The sample of European cultivars used in this study showed haplotype diversity. Among European cultivars Cabernet Sauvignon, Cabernet Franch and Barbera revealed haplotype I that exist in both Iranian cultivars and wild grapes and chardonnay had haplotype III which were common in Iranian cultivars only. Haplotype IV was only observed in Pinot Noir from Europe. This may suggest distinct origin of domestication for this cultivated grapevine (Table 1).

Wild grapes haplotype diversity: In this study 63 wild grapes from three different regions of northwest and west of Iran were analysed by Cpssr markers (Table 2). Among all the used Cpssr loci only ccmp3 was polymorphic and revealed two different variant in wild grape genotypes that in combination with monomorphic ccmp10 allele size (115 bp) two haplotype was detected in the wild grape accessions. The frequency of haplotypes I and II in wild accessions was 49.3 and 50.7%, respectively.

The wild genotypes from Piranshahr region showed high frequency of haplotypeI unlike the accessions from Sardasht and Baneh with high frequency of haplotypeII (Table 3). Because of high number and wide distribution of wild populations of Sardasht region, they were divided into three subgroups; S-Grjal. without haplotypeI and S-Shalmash ans S-Qasmarash with the both haplotypes. Haplotype diversity for all wild samples was 0.5.

Table 2: List of wild grape populations, code and number of samples analysed in the population (N) and their geographic coordinate

Population name	Code	N°	Coordinates
Sardasht-Qasm rash	SGR	23	36°13'; 45°22'
Sardasht-shalmash	SSH	10	36°5'; 45°30'
Sardasht-Grjal	SG	7	36°25'; 45°22'
Piranshahr- Prdanan	PRP	14	36°14'; 45°5'
Banah- Tazhan	BT	9	35°59'; 45°53'

Table 3: Wild grape samples and their haplotype frequency

Population	Haplotype I	Haplotype II	Total individual
Baneh	0.03	0.11	9
Piranshahr	0.2	0.015	14
S-Shalmash	0.11	0.047	10
S-Grjal	0	0.11	7
S-Qasmarash	0.14	0.22	23
total	0.493	0.507	63

Table 4: Allele size, frequency and gene diversity values for two cpSSR loci in cultivated samples

Loci	Allelic size (Frequency)	Gene Diversity (GD)
cpSSR3	106 (0.6)	0.48
	107 (0.39)	
CpSSR10	114 (0.01)	0.44
	115 (0.68)	
	116 (0.3)	

Genetic diversity: To assess chloroplast genetic diversity within cultivars, the allelic variation was studied at the two cpSSR polymorphic loci. Gene diversity levels for these loci varied from 0.44 to 0.48 (Table 4) which is within the range of diversity found for cpSSR loci in other species (Arroyo-Garcia *et al*., 2002).

DISCUSSION

Due to the maternal inheritance of chloroplast genome in angiosperm (Dumolin *et al*., 1995), cp-microsatellite variants accumulate in a uniparental lineage providing information's about the level and distribution of genetic diversity at the regional and individual level. Data deriving from organelle genomes may better define events governing and leading population and

evolutionary processes (Provan *et al*., 2001). However, in the present study, the few loci were polymorphic across the genotypes, but these few cp-microsatellite markers were suitable to assess genetic variability in *Vitis vinifera* L. and the occurrence of common haplotypes in both subspecies (wild and cultivated), allowed the evaluation of their relationships.

Iranian accessions showed Haplotypes I, II and III that lower than the haplotype diversity that reported by Grassi *et al.* (2002) in Italian populations. Basing on archaeological and historical studies (Zohary and Spiegel-Roy, 1975; Zohary and Hopf, 2000), the primary centre of grape domestication was located in Near East regions and all grapevine cultivars should have derived from this area. The low rate of haplotype diversity in Iranian germplasm may be due to: 1) genetic erosion of several autochthonous cultivars growing in Iran, 2) the existence of secondary domestication centres out of these regions that domestication could have been taken place and 3) less number of polymorphic markers in the present study.

The presence of common haplotypes in wild and domesticated samples combined with historical information (Olmo, 1976; Forni, 1990) seems to support the existence of primary domestication events in the near and Middle East regions but some varieties could derive from secondary domestication events or local breeding program from wild and cultivated accessions occurring in the Mediterranean basin (Grassi *et al*., 2003). CpSSR markers have been developed in the last years and proved to be particularly useful in the study of cultivars origin (Palmé and Vendramin, 2002; Snoussi *et al*., 2004). Haplotype analyses would help to determine the local or imported nature of a given variety.

Concerning to existence of both haplotypes I and II in the number of cultivated and wild grapes in these areas, it is possible to consider that the wild grapes are ancestor of some of our native cultivars on the other hand cultivars with haplotype I and II are derived from these wild one. Since a number of Iranian cultivars (Table 1) had haplotypeIII which did not find in the wild grapes, we could hypothesise that they may be domesticated elsewhere and imported from other neighbour regions. To understand the source of haplotypeIII in Iranian cultivated grapes, it is suggested to study wild grapevine populations from other part of country especially from west and north forests.

Absence of haplotype I in the subgroup S-Grjal from Sardasht wild grapes population could be result of decline in size of this population due to human impact and spread of pests and diseases. Shalamsh and Qasmarash wild populations have haplotypes I and II that must be considering for in situ biodiversity conservation.

In conclusion we underline the usefulness of chloroplast genome markers to study the relationships between wild and cultivated grapes from different regions of west and North West of Iran. To earn the more information about domestication events and genetic relatedness, application of other marker systems such as nuclear SSRs with co-dominant heritage and wide genome coverage will be suitable.

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