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Full Length Research Paper

Study of genetic relationships between wild and domesticated grapevine in the north of Morocco

El Oualkadi A[1,2]*, Ater M[2], Laucou V[1], Boursiquot J.M[1], Lacombe T[1], Peros J.P[1] and This P[1]

[1]UMR 1097, DIAPC, INRA, Equipe "Diversité, Génétique et Génomique Vigne", 2 Place Viala, 34060 Montpellier, France.
[2]Diversité et Conservation des Systèmes Biologiques, Faculté des Sciences de Tétouan, B.P. 2062, M'hannech II Tétouan, Maroc.

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In Morocco, knowledge about the wild grapevines has been absent. Until now, no accurate inventory was available and the characterization of this wild grapevine group was lacking. In the present work, prospecting conducted in the North of Morocco (Rif) permitted us to inventory 18 sites with wild grapevines (10 to 50 km away from each other). A total of 168 individuals have been found. This material was analyzed with the 20 nuclear, 3 chloroplast SSR markers and 2 genes involved in the anthocyanin metabolic pathway. We compared the diversity of this material with 48 individuals from cultivated grapevines prospected in the same region, 50 cultivated grapevines from Tunisia and 30 from Algeria along with 128 wild grapevines from France. The diversity observed in the Moroccan wild group was slightly higher compared to other groups. Studies of the genetic structure were then carried out. Within the wild grapevines, the French and Moroccan samples were well differentiated. The wild group (of French or Moroccan origin) was also differentiated from the cultivated, thus revealing the wild status of this indigenous material. The analyses of the sequences showed the same SNP in the different group but the haplotypes reconstruction revealed the presence of specific haplotypes in the Moroccan material when compared with the cultivars group used in this study. This work, confirms the existence and the interest in the conservation of the wild grapevine in Morocco. We suppose that these wild vines contain interesting genes which facilitate the adaptation of grape to its environment.

Key words: *Vitis vinifera*, gene flow, genetic diversity, Morocco, plant conservation genetics.

INTRODUCTION

Many modern crops have been selected from their wild relatives and landraces (example, the ancient or primitive cultivars of crop plants) to meet our demands for food. To ensure sustainable and developmental crop consumption, it is essential to investigate the variations in the wild relatives and landraces that may be of possible value in crop breeding programs, example, confirming disease resistance and cold hardiness. In addition, it is also important to preserve natural plant populations and the habitats of wild progenitors (that is, *in situ* conservation of plant genetic resources) for future use (Matsuo, 1998). Domesticated plants often have the potential to spontaneously hybridize with those wild relatives that are growing in close proximity (Ellstrand et al., 1999). Such hybridization leads to gene flow: 'the incorporation of genes into the gene pool of one population from one or more populations' (Futuyma, 1998). The reduction of diversity attributale to domestication and breeding appears to be weak on a genome-wide scale, a few notable changes in morphology have emerged since grape domestication, including perfect flowers, larger berry sizes, higher sugar content, and a wide range of berry colors (Olmo, 1995). Myles et al. (2011) find that haplotype diversity in western viniferae is slightly reduced comapred with eastern vinifera suggesting that the grape experienced a modest reduction in genetic diversity as it was brought to western

*Corresponding author. E-mail: mater20@gmail.com. Fax: 212.0524.44.63.80.

Europe. Also, because gene flow tends to genetically homogenize populations (Slatkin, 1987) and because crops are typically genetically depauperate compared to their wild relatives (Ladizinsky, 1985), overwhelming gene flow from crops is expected to deplete genetic diversity in wild populations (Ellstrand et al., 1999).

In *Vitis vinifera,* the wild grapevines represent a unique and invaluable genetic resource for cultivated grapevines (Negrul, 1938). Nowadays, wild grapevines exist in small populations in diverse natural ecosystems throughout Europe, the Mediterranean region of Northern Africa, the Middle East, and Western Asia (Ocete et al., 2002). Recent studies of the residual wild grapevine sites in Europe warn that wild grapevines may be near extinction (Arnold, 1998). Introduction of diseases and pests (Phylloxera), climatic accidents and anthropic pressure represent the principal causes of this decline (Boursiquot, 2000; Arnold et al., 2005). The intensive cultivation of the grapevine in large areas, using only a few varieties and clones, has drastically decreased genetic variability in cultivated vines. The preservation of wild populations of V. *vinifera* L. subsp. *Silvestris* is thus essential for the maintenance of genetic variability and to limit genetic erosion.

Wild grapevines have been analyzed against cultivated grapevines with the nuclear microsatellite DNA level to map genes, as well as to catalogue biodiversity for evolutionary and conservation. This study was carried out in Europe (Perret, 1996; Cuisset, 1998; Arnold et al., 1998; Ocete et al., 1999; Holub and Prochazka, 2000; Lacombe et al., 2002a; Labra et al., 2002; Rossetto et al., 2002; Grassi et al., 2003a; Forneck et al., 2003; Kozjak et al., 2003; Grassi et al., 2003b; Aradhya et al., 2003; This et al., 2004; Arroyo et al., 2006; DiVecchi et al., 2008) and in Tunisia by Zoghlami et al. (2003a). Evaluation of the genetic diversity, differentiation and relationship among wild grape specimens from different areas will contribute to a better understanding of the process of grapevine domestication.

In North Africa, the Phoenicians cultivated the vine more than 4 thousand years ago. The crop has thus existed in the Maghreb for a very long time. Through the various historical times until today the North African natives have used this crop as a food complement (Chetouh, 1991). The gathering of the wild grape always constituted an appreciable complementary resource for the mountain inhabitants of North Africa. In Morocco, the inhabitants of the Atlas also consumed this fruit.

The destruction of the natural habitats and the phylloxera crisis around 1860 caused the disappearance of this taxon and currently the exact status of this taxon in Maghreb is still unknown.

In Morocco, the presence of the wild grapevine in the Rif area (North of Morocco) was noted in several Arab historical books and on Roman coins with grape representation confirming the important economic value of the vine in this area (Nachat, 2006). In the valleys of this mountainous area, lambrusques or wild grapevines can still be found. Until now, no accurate inventory was however available and the characterization of this wild grapevine in this area has been studied.

In this work, the prospecting of wild grapevines was performed in wild conditions in the Rif area. This material was then analyzed using 20 nuclear, 3 chloroplast SSR markers and 2 genes involved in the anthocyanin metabolic pathway in order to analyze the extent of a structural and genetic diversity as a prerequisite for adequate conservation of this material in Morocco. A previous study used the same markers to characterize the diversity existing in collection from Morocco (El Oualkadi et al., 2009). The prospected material was then compared to cultivated material prospected in the same region and to samples from France (Lacombe et al., in prep) in order to confirm the material status and to confirm whether it contains specific genes.

METHODS

Plant material

The samples described in this study were located in the Rif region of Morocco. In total, 168 samples from wild grapevines were collected (Figure 1; Table 1). In the same region, 83 specimens were collected from cultivated vines. The 128 wild grapevines in this study were compared with 83 cultivated grapevines collected in the same region or originating from the SODEA collection (El Oualkadi et al., 2009). We also compared these two samples with 50 cultivated grapevines from Tunisia, 30 from Algeria and 128 wild grapevines from France who were randomly selected among a population of 175 wild grapevines.

Molecular analyses

DNA was extracted from young dried leaves, collected in the spring; and freeze dried after harvesting. Extraction was performed with a Qiagen DNeasy (Qiagen IN, Valencia CA, USA) Plant Mini Kit with minor modifications: addition of 1% w/v of PVP-40 to the AP1 solution, addition of 180 µl AP2 and centrifugation for 10 min at 6000 rpm.

Microsatellite analyses were performed on 20 microsatellite markers (nSSRs) well distributed across the 19 grape chromosomes (Doligez et al., 2006) as previously described (Lacombe et al., 2007), 2 of the VMC series (VMC1b11, VMC4f3; Vitis Microsatellite Consortium, Adam- Blondon et al., 2004), 9 of the VVI series (VVIb01, VVIn16, VVIn54, VVIn73, VVIp31, VVIp60, VVIv37, VVIv67, VVIq52; Merdinoglu et al., 2005), 8 of the VVMD series (VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD28, VVMD27, VVMD32; Bowers et al., 1996, 1999), and VVS2 (Thomas and Scott, 1993b; Thomas et al., 1994). We also used the three chloroplast loci (ccmp3, ccmp5 and ccmp10; Powell et al., 1995) found to be polymorphic in *V. vinifera* samples (Arroyo-carcia et al., 2002). The amplification method was performed, electrophoresis and analysis as previously described by Di Vecchi Staraz et al. (2007a) was also done.

The genes that were sequenced were located on two separate chromosomes. The genes are involved in the anthocyanin metabolic pathway: the dihydroflavonol 4-reductase (DFR, gi 499017), (LG located 18) present in one copy in the genome of V. *vinifera* L. and the leucoanthocyanidin dioxygenase (L-DOX gi

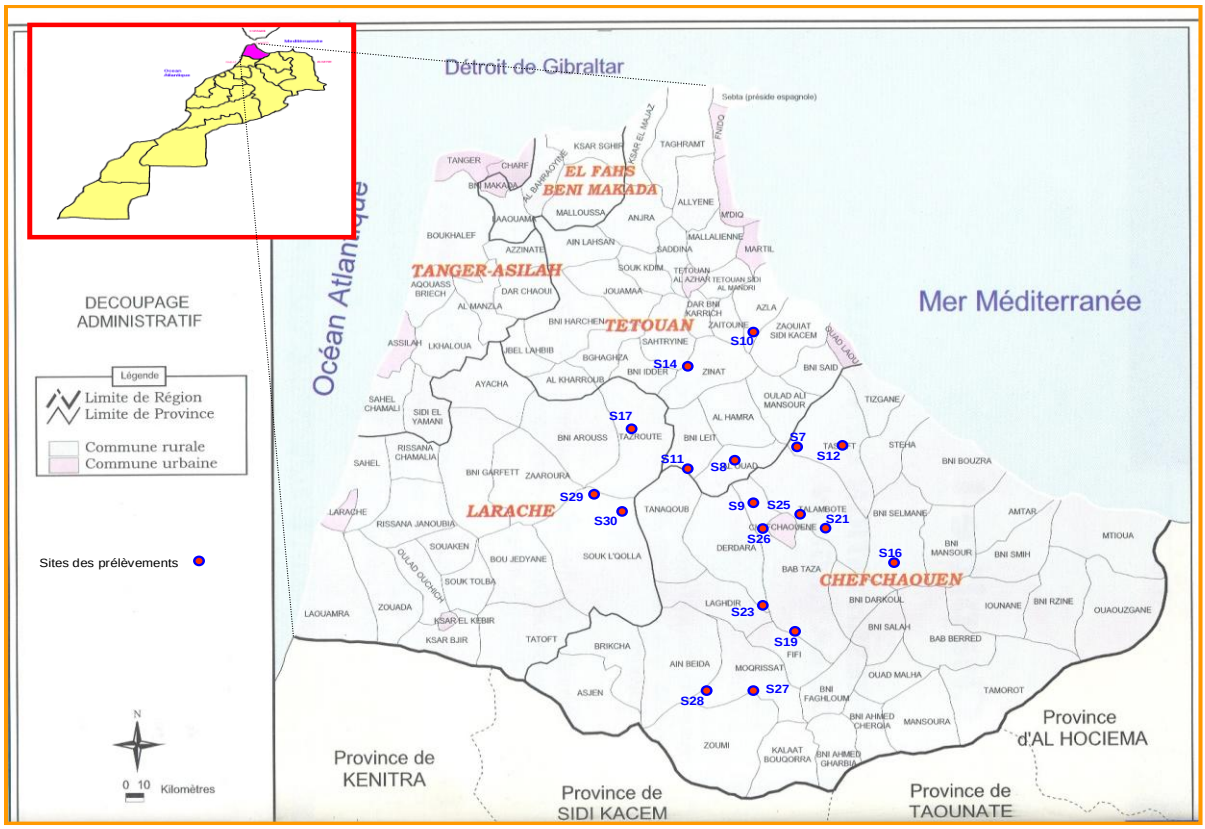

Figure 1. Sample location of wild grapevine in north Morocco.

Table 1. Sampling locations and sizes for wild populations prospected.

							Chlorotypes			
Population	**Acronym**	**Location**	**Latitude, Longitude**	**Altitude (m)**	**N^t**	**N^p**	**A**	**B**	**C**	**D**
1 Oued amsa	S7	Amsa	35°30' N, 5°14'E	110	8	8	8	-	-	-
2 Zerka	S8	Zaytouna	35°31'N, 5°20'E	120	9	3	2	1	-	-
3 Béni hassan	S9	Al Hamra	35°18'N, 5°21'E	569	15	15	14	-	-	1
4 Oued smir	S10	Tétouan	35°41'N, 5°22'E	09	24	18	17	1	-	-
5 Béni idder	S11	Moulay abdeslam	35°24'N, 5°30'E	421	15	12	10	-	2	-
6 Oued tamrabet	S12	Amsa	35°32'N, 5°11'E	100	9	5	4	-	-	1
7 Khmis anjra	S14	Khmis anjra	35°39'N, 5°30'E	100	9	4	2	-	2	-
8 Oued kannar	S16	Stehat	35°14'N, 5°005'E	111	8	2	1	-	-	1
9 Béni harchan	S17	Beni harchan	35°29'N, 5°47'E	610	10	9	9	-	-	-
10 Khezana	S19	Bab taza	35°02'N, 5°10'E	750	9	6	6	-	-	-
11 Akchour	S21	Chaouen	35°14'N, 5°10'E	439	9	9	9	-	-	-
12 Dardara	S23	Dardara	35°05'N, 5°16'E	403	12	9	9	-	-	-
13 Talamboute	S25	Al Ouad	35°17'N, 5°14'E	150	6	4	2	-	1	1
14 Sidi Bousaada	S26	Dardara	35°01'N, 5°29'E	350	2	2	-	-	2	-
15 Route de Moukrissate	S27	Ain baida	35°01'N, 5°24'E	190	2	2	-	-	1	1
16 Route de Ouazane	S28	Dardara	35°01'N, 5°28'E	177	3	3	3	-	-	-
17 Ayacha	S29	Ayacha	35°20'N, 5°40'E	130	10	10	7	3	-	-
18 Tardane	S30	Béni Aross	35°20'N, 5°35'E	360	8	7	6	-	1	-
Total					168	128	16	3	6	5

N^p: number of putative individuals.N^t: number of prospected individuals.

499021), (LG located 8) were present in at least three copies in the genome of *V. vinifera L*. based on the NCBI database. PCR amplification, sequencing, and SNP detection were performed by Le Cunff et al. (2008), using the following primers for DFR and LDOX respectively (primer forward: GCTGACAGATTTGGGGTTTGA; primer reverse: TTGGGCCATTCCGTTTTATTA) and (primer forward: TTGAGCCCAATCATATTAGTTCC; primer reverse: GTGGCATGACCATTCTCCTC).

Data analyses

Among the 83 specimens collected, SSR data had already been obtained for the 39 cultivars of the collection (EL Oualkadi et al., 2009).The 128 wild grapevine prospected in France (Lacombe et al., in prep; DiVecchi et al., in prep) were included in this study.

Parentage analyses were performed using FaMoz software (Gerber et al*.,* 2003) as previously described (Lacombe et al*.,* 2007). The 10,000 simulated pairs performed by FaMoz for parentage analysis identified a Log of the odds ratios (LOD) score threshold of 8 to assess a potential parent pair with 20 nSSRs. Based on this threshold, only pairs with LOD scores higher than 8 were considered to be valid in the current data set. We authorized a discrepancy of maximum 3 loci to cover possible data errors (Ewen et al*.,* 2000), null alleles (Dakin and Avise, 2004), and clonal mutations as previously described by Di Vecchi Staraz et al*.* (2007a).

Standard measures of genetic variation, including number of alleles per locus, allele frequencies, Nei unbiased gene diversity (GD; Nei 1987), observed heterozygosity (Ho), and their standard deviation were calculated using the macro MS® Excel Microsatellite Toolkit elaborated by Park (2001). A comparison of the identity of profiles was also performed using this macro. Using GenALex 6 (Peakall et al., 2006) we calculated the genetic parameters of each wild population: A, mean number of alleles; P, percentage of polymorphic loci; Ho, Fis, mean fixation index over polymorphic loci for each population estimated according to Weir and Cockerham (1984). Pairwise genetic distances (Nei et al., 1972, 1978) and geographical distances (km) among the 18 groups were also calculated using GenALex.

The isolation of a population by distances for the eighteen populations was computed through the Mantel procedure using GenAlex, using 1000 random permutations of the data. The Genic differentiation for each population pair was calculated using Genepop 4.0 (Rousset, 2008), using 1000 iterations and 1000 iterations per batch.

SSR data were also analyzed using Principal Component Analysis (PCA) using the R v.2.8.1 software with Package FactoMineR (Husson et al., 2008).

Allelic and haplotypic frequencies within each sample were directly estimated. Haplotype diversity (HD) was calculated with the formula:

$HD= (1-\sum p_i^2)$ (Weir, 1996)

Where p_i is the frequency of haplotype i.

A clustering was built using DARwin v.5.0.156 software (Perrier et al., 2003) and applying the weighted neighbour-joining method to the dissimilarities matrix.

The structure of the sample was also analyzed using Structure v2.2 software (Falush et al., 2007) setting 500 000 burnings and 500 000 repetitions after burnings with 15 run.

The DNA sequences were analysed using the Staden Package (Bonfield et al., 1995). Heterozygous SNPs were identified as double pics on the chromatograms and coded according to international codes (nucleotide codes of the International Union of Biochemistry). Haplotype reconstruction was made by the Gevalt software (Davidovich et al., 2007).

RESULTS

Characterization of prospection material

A total of 168 wild specimens were collected. This number does not take into account all observed specimens, but solely the specimens for which we could take readings and collect data and leaves. These individuals were considered as wild or “lambrusques” on the basis of their leaf morphology and sex when observed.

The greatest number of individuals was observed in Oued smir with 24 individuals including 18 possible lambrusques. Then in Béni Hassan and Béni Idder with 15 individuals. The populations (10 to 50 km away from each other), per a mean number of individuals by population equal to 9 (ranging from 2 to 18) (Table 1).

The analysis of these 168 samples using the nSSR markers enabled the identification of redundant material and of individual showing clear parentage relationships with cultivars (Table 2) such as for example S7-4, S7-6 and S7-7 of the station Oued amsa; S12-6 and S12-9 of the station Oued tamrabet, S19-7 and S19-8 of the Khezana station, and S25-5 and S27-1 of the Talamboute stations and road of Moukrissate respectively. The majority of the individuals have parentage relationships with the cultivar Taferielt which is frequent in the area of study. None of these individuals were analysed further. We also identified parentage relationships between individuals of distant stations (for example Oued Kannar (S16) and Oued amsa (S7); Béni Hassan (S9) and Dardara (S23); Oued Tamrabet (S12), Béni Hassan (S9) and Oued amsa (S7); Béni Hassan (S9) and Akchour (S21); Oued amsa (S7) and Béni Hassan (S9)) (Table 2).

Finally we kept 128 supposedly “true” wild grapevines. The continuation of the analyses thus concerns only 128 individuals thought to be “lambrusques” (Table 1).

Similarly, 83 cultivars were observed in the Rif in an abandoned vineyard. Most of them were identified based on their morphology to cultivars of SODEA (EL Oualkadi et al., 2009) except 38 individuals which were analyzed by molecular markers. Only 9 of these cultivars did not correspond to cultivars from SODEA.

Diversity of the wild grapevine

Analyses nSSR

Genetic parameters for each of the eighteen populations are given in Table 3. For the eighteen populations of wild grapevine, the average number of alleles per locus (A) ranged from 1.65 in S16 to 5.7 in S10. The mean percentage of polymorphic loci, was 96% and the genetic

Table 2. List of wild individuals for which parents with relationships direct of relative parent/child were identified using the Famoz software.

Individual	Parent 1 (Lod score)	Parent 2 (Lod score)	Parent 3 (Lod score)
S27-1	Bezoult el aouda (17.29)		
S12-9	Trebbiano (11.76)		
S19-8	Taferielt (9.5)		
S19-6	Campinas IAC (1.31)		
S16-5	S7-4 (10.95)		
S7-6	Belz el Ansa (2.63)		
S12-6	Taferielt (12.64)	Blanc de Rhafsaï (9.98)	
S9-12	S9-3 (10.02)	S23-11 (9.78)	S9-1 (8.39)
S29-3	S23-11 (6.44)		
S19-7	Taferielt (9.25)		
S12-1	S9-13 (9.25)	S7-4 (9.02)	
S9-13	S21-6 (20.4)	S9-16 (19.49)	S7-3 (18.79)
S17-10	S9-16 (3.19)		
S7-7	Taferielt (8.39)		
S7-4	Taferielt (14.99)		
S25-5	Airen (15.18)		

Table 3. Genetic diversity values of 18 *Vitis vinifera Silvestris* populations based on twenty microsatellite loci.

Population	N	A	P	He	Ho	F
S7	8	4	100	0.63	0.69	-0.12
S8	3	3.2	100	0.63	0.8	-0.27
S9	15	5.55	100	0.69	0.69	-0.01
S10	18	5.7	100	0.68	0.68	-0.01
S11	12	5.4	100	0.65	0.7	-0.07
S12	5	3.85	100	0.66	0.79	-0.2
S14	4	3.05	100	0.52	0.6	-0.14
S16	2	1.65	65	0.33	0.65	-
S17	9	5.05	100	0.68	0.7	-0.04
S19	6	2.85	90	0.5	0.69	-
S21	9	4.7	100	0.61	0.66	-0.06
S23	9	5.45	100	0.67	0.68	-0.03
S25	4	2.9	90	0.49	0.58	-
S26	2	2.25	90	0.45	0.73	-
S27	2	1.9	90	0.45	0.9	-
S28	3	3.15	100	0.55	0.53	0.01
S29	10	4.65	100	0.64	0.68	-0.08
S30	7	4.9	100	0.66	0.72	-0.09
Population mean		3.9	95.83	0.58	0.69	-

A, mean number of alleles; P, percentage of polymorphic loci (0.99 criterion); Ho, observed heterozygosity; He, expected heterozygozity; F, mean fixation index over polymorphic loci.

diversity (He) was 0.58. The highest values of He were detected in Béni hassan (S9), in Oued smir (S10) and in Béni harchan (S17) with values of diversity of 0.69 and 0.68, respectively. While a low level of diversity, He= 0.33, was found in Oued kannar (S16). The Oued kannar (S16) population possessed the lowest level of polymorphism (P= 65%), while thirteen populations (S28, S14, S21, S29, S23, S10, S7, S9, S11, S17, S30, S21 and S8) had the greatest level of polymorphism (P = 100%) and high values of expected heterozygosity

Table 4. Genetic differentiation of the Moroccan populations of wild grapevine.

	S7	S8	S9	S10	S11	S12	S14	S16	S17	S19	S21	S23	S25	S26	S27	S28	S29	S30
S7																		
S8	Nt																	
S9	0.056***	Nt																
S10	0.044NS	Nt	0.029***															
S11	0.058*	Nt	0.050***	0.068***														
S12	0.057NS	Nt	0.071***	0.067***	0.072***													
S14	0.101*	Nt	0.085***	0.069**	0.110***	0.149***												
S16	Nt	Nt	Nt	Nt	Nt	Nt	Nt											
S17	0.060NS	Nt	0.043***	0.045***	0.068***	0.070NS	0.097***	Nt										
S19	0.100*	Nt	0.084 NS	0.095***	0.102***	0.127NS	0.150***	Nt	0.092NS									
S21	0.050NS	Nt	0.052***	0.053***	0.067***	0.096***	0.088***	Nt	0.056***	0.111***								
S23	0.057NS	Nt	0.037***	0.034***	0.065***	0.070**	0.098***	Nt	0.050***	0.091NS	0.061***							
S25	0.101NS	Nt	0.098***	0.097***	0.100***	0.113NS	0.132***	Nt	0.107*	0.164NS	0.086**	0.093NS						
S26	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt					
S27	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt				
S28	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt			
S29	0.058NS	Nt	0.045***	0.039***	0.072***	0.081***	0.083***	Nt	0.037*	0.112***	0.063***	0.048***	0.113***	Nt	Nt	Nt		
S30	0.042***	Nt	0.046***	0.052***	0.056***	0.072***	0.091***	Nt	0.056***	0.096NS	0.056***	0.060***	0.117***	Nt	Nt	Nt	0.051***	

Nt, not tested because a lower number of individuals. The values of FST. The values of significance χ^2 calculated with probabilities p=0.05 (*), p=0.01 (**), and p=0.001 (***, highly significant), (NS: not significant).

(He = 0.52 to 0.69). Observed heterozygosity (Ho) ranged from 0.53 (S28) to 0.9 (S27). A significant excess of heterozygotes existed in 12 populations, as indicates by negative Fis values (P<0.01) (Table 3).

Analyses per individuals using nSSR data was down using the Darwin software. The clustering did not distinguish any differentiation with the individuals according to their geographical localization, only some populations like S10, S11 and S9 were well differentiated (Data not shown). The genetic differentiation among populations was also analyzed using the Fst statistic (Table 4). Small populations, less than 4 individuals, were removed from analysis. Genetic differentiation was significantly higher between the population S11 and the all other population. It was also higher between the population S9 and all others except population S19. However the genetic differentiation was not significant for the population S7 and the all other population, similarly for the population S17 and S19 were genetic differentiation was not significant in comparison with the other populations.

No significant pattern of isolation by distance was observed, differentiation measured as $F_{st}/(1-F_{st})$ was not correlated with the logarithm of the geographical distance between populations (Figure 2). The mantel tests show no correlation between Nei genetic distance and geographical distance (P>0.001, R^2=0.013) suggesting that populations may not be different according to geographical distances.

Analyses SSRcp

The distribution of the four cpDNA haplotypes was not homogenous (Table 1). The Haplotype A was present in 53.33%, Haplotype C presents 20%, Haplotype D 16.67% and Haplotype B only 10%. Haplotypes A was almost ubiquitous; it is

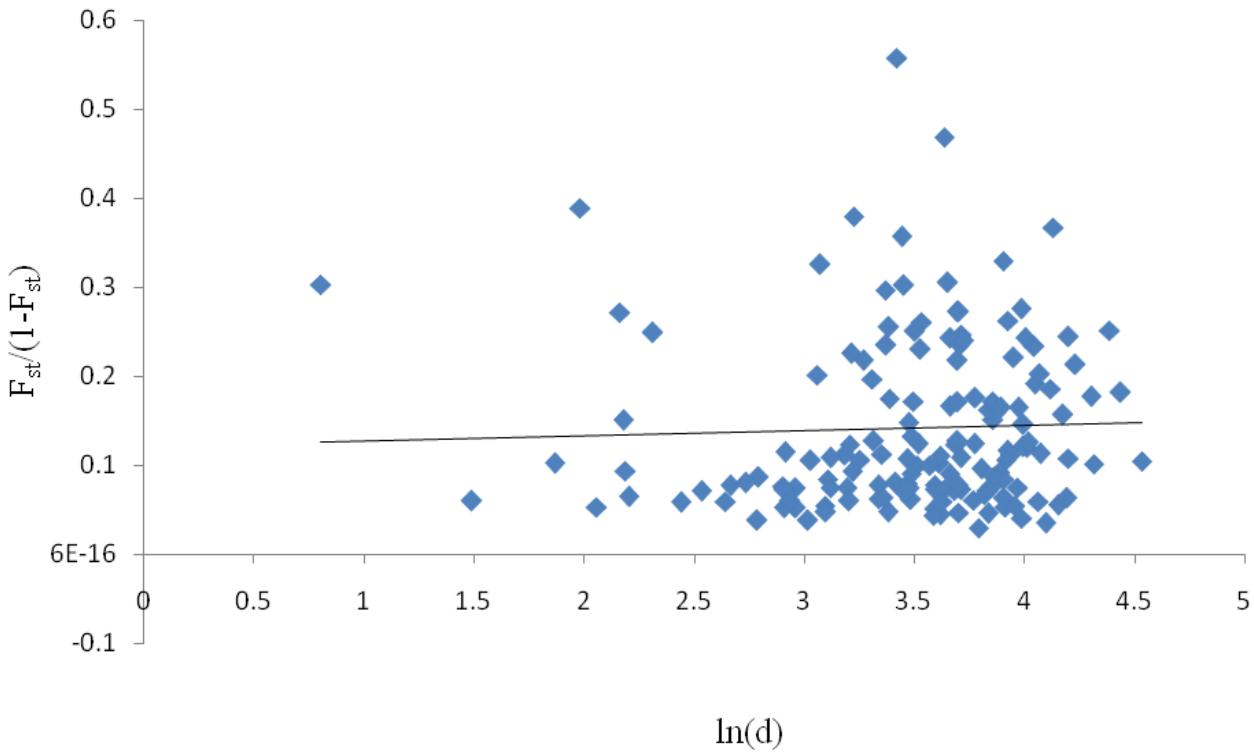

Figure 2. Plot of pairewise $F_{st}/(1-F_{st})$ ratios against the logarithm of distances (in km) between populations from Morocco.

present in all 18 populations. The other haplotypes were less present in the populations. We also calculated the haplotypes diversity in each one. The population S14, S27 and S25 have the highest haplotype diversity (HD = 0.56). The population S12, S16 and S29 have a similar haplotype diversity (HD = 0.44) while the haplotype diversity was null for the population S7, S17, S19, S21, S23, S26 and S28.

The diversity of wild vines was compared with that of a similar number of French wild vines, the 48 cultivars from Morocco (39 from SODEA plus the 9 new samples from the prospections), 30 cultivars from Algeria and 50 from Tunisia (Table 5).

Comparison of the wild and cultivated grapevine

The number of alleles for SSR markers generated in the wild grapevine from Morocco was 201 alleles with a mean equal to 10.05 while in the French wild grapevine it was 192. The number of alleles in cultivated grapevines from Maghreb was 208 with a mean equal to 10.4 alleles per locus. The mean number of the observed heterogeneity was 0.74 but was lower in the wild grapevines from Morocco (mean 0.71) and France (mean 0.67). The Nei gene diversity was calculated for each group. It was 0.74 for the cultivated grapevine from Maghreb, 0.73 for the wild grapevine from Morocco and 0.69 for the French wild grapevine (Table 5). The samples from the 128 specimens collected were compared to a sample of cultivated grapevines originating from Maghreb. PCA was carried out on this data. The projection of the individuals in the plan of axes 1 (15.48%) and 2 (7.73%), is presented in Figure 3 according to the type of the individuals and their geographical origin. We observe a very clear differentiation between the three groups (Lambrusque France, Lambrusque Morocco and Cultivars Maghreb). Some cultivated individuals find themselves however in the group of the Moroccan wild grapevines (Kremed talaka, Hybride blanc and Aneb daradara) while in the French group clustered individuals (Plant d'Ouchtata and Tabarka 3) from Tunisia. Ampelographic analyses had previously suspected the cultivars Plant d'Ouchtata 1 and Tabarka 3 as cultivated accessions (Boursiquot and Lacombe, communication personnelle) but molecular data prove their strong classification within the wild grapevines classification. Conversely, some of the identified collected grapevines were categorized as *sativa* were indeed found among the group of the cultivated varieties, whereas others remain in the group of the wild grapevines. Concerning the geographical origin of the individuals, their situations were also very different. Concerning the wild grapevines, the distinction between Moroccan and French individuals were very clear. With regards to the relations between wild and cultivated

Table 5. Genetic diversity indices of the grapevine populations analyzed in this study (wild populations and cultivars) based on 20 nSSR markers.

	N_{ind}	$N_{alleles}$	A	Ho	He	GD	nSNP LDOX	nSNP DFR
Lambrusque Morocco	128	201	10.1	0.71	0.74	0.73	6	10
Lambrusque France	128	192	9.6	0.67	0.7	0.69	-	-
Cultivars Morocco	48	158	7.9	0.72	0.70	0.71	6	8
Cultivars Alegria	30	132	6.6	0.73	0.69	0.7	6	9
Cultivars Tunisia	50	176	8.8	0.76	0.76	0.76	6	10

N_{ind}, number of individuals; $N_{alleles}$, number of alleles; A, mean number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; GD,gene diversity.

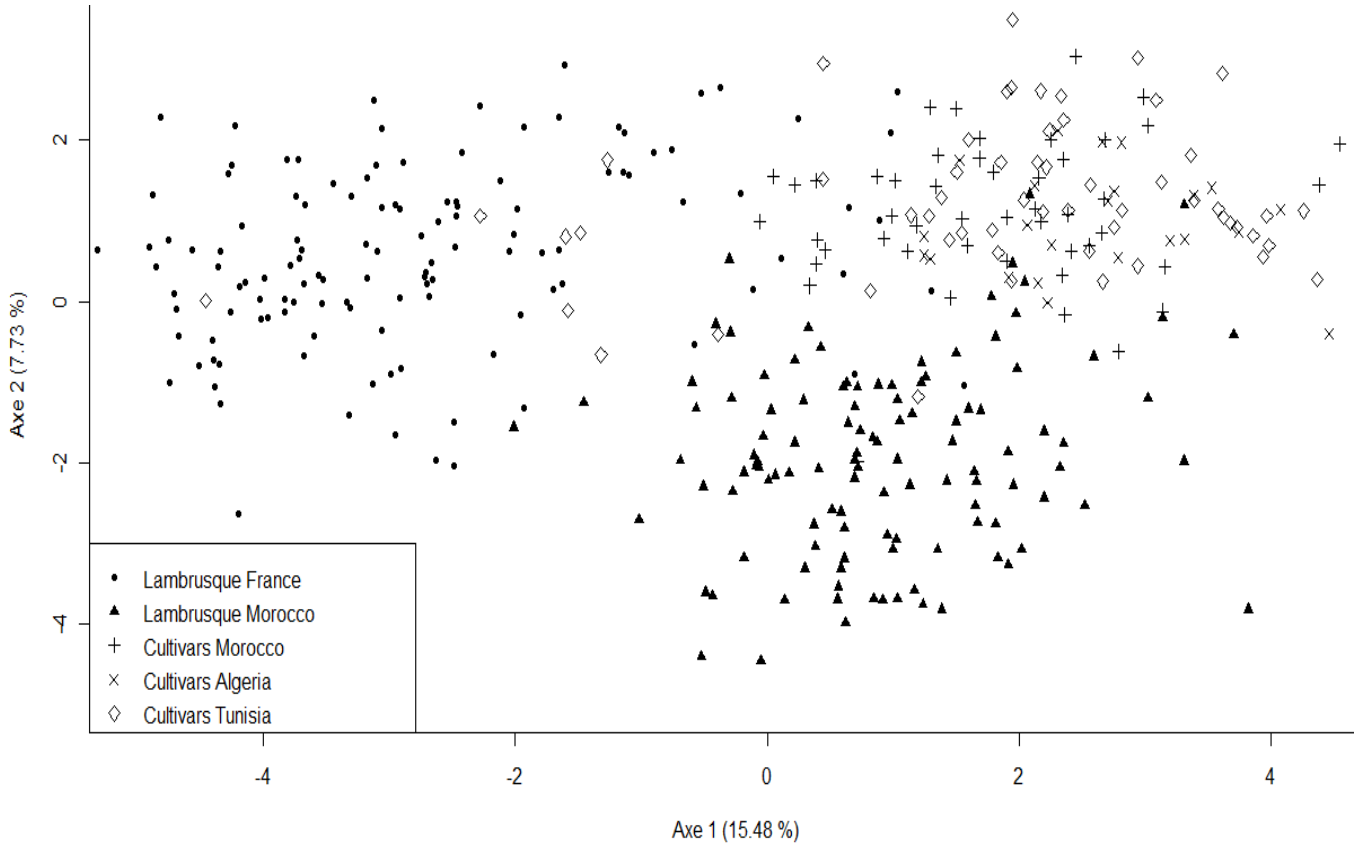

Figure 3. Results of the PCA analysis carried out on the data of the 20 locus microsatellites. Projection of the 348 individuals on the plan Axes 1 and 2 of the PCA. The codes correspond to the geographic origin of the individuals.

species, the data clearly showed that the cultivated varieties originating in Morocco were found nearer to the Moroccan wild grapevines than to the varieties originating from Tunisia or Algeria.

The same result was obtained using the Structure Software. Three groups were shown one grouping all of the cultivars, one showing wild grapevines from Morocco, and the last showing wild grapevine from France Figure 4.

The differentiation was also confirmed by the calculation of the parameter of genetic differentiation between populations. All pairwise comparisons between the five grapevine gene pools yielded highly significant differentiation values. F_{st} pairwise values between cultivated grapevine populations were low ranged from 0.018 (CUL DZA- CUL MAR) to 0.020 (CUL DZA- CUL TUN) but significantly different from zero. However results showed higher F_{st} pairwise value when the wild population was compared to other populations of cultivated grapevines. A high pairwise value was also observed between the wild grapevines from Morocco and wild grapevines from France (F_{st} = 0.083). The sizes of

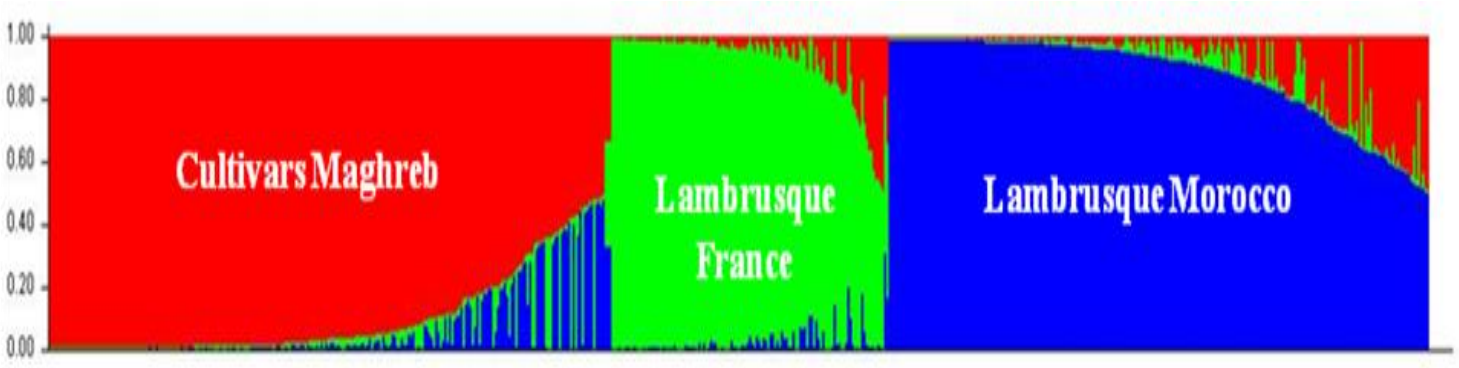

Figure 4. Membership of 348 cultivars in 5 genetic populations identified by the software structure (each colour corresponds to a different population).

the aligned fragment were 267 and 302 bp for DFR and LDOX respectively. The number of SNP calculated for the two fragments of LDOX and DFR genes for the lambrusques from Morocco, and the cultivated grapevine from Tunisia, Algeria and Morocco, were similar (Table 5). The number of the polymorphic bases for the two genes DFR and LDOX revealed in the lambrusques from Morocco and cultivated grapevines from the Maghreb was 10 and 6 respectively, nine and five SNP was detected in the Intron for DFR (104 bd size) and LDOX (168 bd size) respectively, however only one SNP for the two gene fragment was detected in Exon (103 bd size in DFR and 147 bd in LDOX).

The distribution of the haplotype was not homogenous (Table 6). For the gene DFR, 17 haplotypes were observed in Tunisian group, 14 in Algeria group and only 11 in Moroccan group, while in the Lambrusque group 13 haplotypes were observed. In the LDOX gene, the distribution of the haplotype was more homogenate, 6 haplotype was observed in Moroccan group, 5 in Tunisian group and only 4 in the Algerian group, the Moroccan Lambrusque group represent the most number of the haplotype (9 haplotypes) for the gene LDOX. The lambrusque group represent the specifics haplotypes compared with the other groups (Table 6).

For the chloroplastique materials study the whole grapevine was analyzed. The 7 allelic variants identified at three chloroplast loci combined only into 4 different haplotypes (Table 7). Overall frequencies distinguished Haplotype A in more than 60% of the grapevine analyzed. The most common chlorotypes was A in *silvestris* and the C in the cultivated grapevine. Among wild grapevine the four haplotypes were present in the Morocco but in the French population only two haplotypes (A and D) were present. In the cultivated grapevine from Maghreb the four haploytpe were observed. The comparison with this samples revealed that the Haplotypes A and C were the most frequent in the sativa group but the Haplotype C was the most frequent in the *Silvestris* samples (Table 7). Haplotype diversity values (HD) was higher in cultivated grapevines from the Maghreb (HD= 0.72) and was lower in the wild grapevine from Morocco (HD=0.32) and France (HD=0.31).

DISCUSSION

Genetic diversity revealed by SSR markers

The study of diversity from 18 populations of wild grapevine collected in the North of Morocco, show a mean number of allele for all population equal to 3.9. The lowest number of allele that were observed in the populations S27 and S16 can be explained by the lower number of individuals collected prospected in those populations. The microsatellites data indicate that most wild grapevine populations from Morocco have a significant excess of heterozygotes. Significantly, a negative Fis value within populations indicates low levels of inbreeding in *V. vinifera subsp Silvestris*.

The populations for the wild grapevine from northern Morocco were differentiated based on nuclear SSR polymorphisms. But were not differentiated according to geographical distances probably because the populations are not distant enough from each other (with the modes of dissemination per seed (birds) and cutting through the courses of water, or by the gene flow and exchange of pollen). The detection of an IBD pattern greatly depends on the spatial scale studied (Rousset, 1997; Castric and Bernatchez, 2003). The capacity to detect isolation by distance also depends on the range of distance values investigated, and on the variance of estimators that is probably lower in short distances (Rousset, 1997). According to Kitamoto et al. (2005), in the study of spatial genetic structure for populations of Primula with SSRs markers, the emergence of isolation by distance would probably be impeded by some pollen flow and high mutation rate. The three Moroccan areas in which we sampled the most number of wild grapevines are Oued

Table 6. The number and frequency of the haplotype detected by the two genes (LDOX and DFR).

Genes	Haplotypes	Cultivars Tunisia		Cultivars Algeria		Cultivars Morocco		Lambrusques Morocco	
		Nb	Frequency	Nb	Frequency	Nb	Frequency	Nb	Frequency
LDOX	CCGCTA	25	53.19	14	46.67	6	35.29	94	98.94
	GTGTCG					15	88.23		
	CCACTA	33	70.21	23	76.67	11	64.70	34	35.78
	GTGTCG	13	27.65	15	50	11	64.70	9	9.47
	GCGTCG	21	44.68	10	33.33	3	17.64	28	29.47
	CCGCTG							18	18.94
	GCGCTA					2	11.76	1	1.05
	CCGCCA	2	4.25						
	CCACTG							3	3.15
	GCGCTG							2	2.10
	GCATCG							1	1.05
DFR	GAGCTACCGT	1	2.22	5	16.67			64	67.36
	GAGCAACGGT	21	46.67	19	63.33	7	41.17	21	22.10
	GAACAACGGT	5	11.11	6	20	2	11.76	44	46.31
	GAGCTACGGT	13	28.89	7	23.33	1	5.88	19	20.00
	GAACTACCGT	5	11.11	1	3.33			23	24.21
	GAACTACGGT	7	15.56	5	16.67	8	47.05	2	2.10
	GAGTTGCCCT	4	8.89	5	16.67	6	35.29	2	2.10
	GAGTTACCCC	10	22.22	1	3.33			4	4.21
	GGACTACCGT	7	15.56					6	6.31
	GAATTACCGT			4	13.33				
	GAATTACCCC	5	11.11	4	13.33				
	GAGTTACCCT					2	11.76		
	GAACAACGGC					2	11.76		
	GAATTGCCCT	5	11.11	1	3.33	2	11.76	2	2.10
	GAATTACCCT	1	2.22	2	6.67	1	5.88		
	GAGTTACCGT			1	3.33	1	5.88		
	GAACTGCCCT					1	5.88		
	GAATTAGCGT	2	4.44	1	3.33				
	TAACAACGGT							2	2.10
	GAATTACGGT	1	2.22						
	GAGCTGCCCT	1	2.22						
	GAACTACCCC	1	2.22						
	TAATTGCCCT	1	2.22						
	GAGTTAGCGT							1	1.05
	GAGCAACGGC							1	1.05

smir (with 24 individuals), Béni Hassan and Béni Idder (with 15 individuals). The analysis of these 168 samples reveals individuals who are identical, and those which result from parentage relationships, such as S7-4, S7-6 and S7-7 of the station Oued amsa; S12-6 and S12-9 of the station Oued tamrabet, S19-7 and S19-8 of the Khezana station, and S25-5 and S27-1 of the Talamboute and road of Moukrissate stations respectively. The wild grapevines who meet in these zones seem to be almost exclusively rootstocks or interspecific hybrids returning to their original state or.

Some of the cultivated and wild Moroccan grapevines of the same area are closer in proximity. This result can be explained by gene flow and migrant inter groups. This could suggest that they were derived from a local domestication event. Similar results were obtained in the study of the olive tree that proposes multilocal domestication (Bersnard and Bervillé, 2000). However, the high level of differentiation between cultivated and wild populations was recorded, indicating that the cultivated population do not come directly from wild populations or could mostly correspond to imported

Table 7. Chloroplasts haplotypes and frequencies in the *V. vinifera* samples analyzed.

Loci				Frequency				
Haplotype	**Ccmp3**	**Ccmp5**	**Ccmp10**	**Silvestris Morocco**	**Silvestris French**	**Sativa Morocco**	**Sativa Algeria**	**Sativa Tunisia**
A	106	105	114	82.93	81.31	31.92	33.33	33.33
B	106	105	115	4.06	-	14.9	-	10.41
C	106	105	116	8.13	-	40.42	40.74	25
D	107	104	115	4.88	18.69	12.76	25.93	31.25
N	-	-	-	4	2	4	3	4
HD	-	-	-	0.32	0.31	0.71	0.67	0.74

N = number of haplotypes; HD = haplotype diversity, HD ($1-\sum pi^2$).

materials introduced during historical times or derived from crossing between them.

Specificity of the wild samples

The analyses of 128 individual wild grapevines from Morocco with SSR markers, show higher diversity present in these specimens compared with the cultivated grapevines from the Maghreb. In the other study, using different markers and number of wild grapevines in a different region, the number of alleles was also lower. Perret et al. (2000) revealed 7 alleles detected by 10 SSR markers, in 35 wild grapevine; Aradhya et al. (2003) detected 94 alleles in 22 wild grapevine with 8 SSR markers; Zoghlami et al. (2003b) observed 104 alleles in 58 wild grapevines with 10 SSR markers; Lacombe et al. (2003) detected 123 alleles in 154 wild grapevine with 13 SSR markers; however DiVecchi et al. (2007) revealed 277 alleles in 512 individuals of wild grapevine with 20 SSR locus. Generally, the high level of gene diversity detected in wild grapevine populations can be correlated with the mating system of these dioecious plants.

The comparison of the two varieties of wild grapevine analyzed in this study showed that the wild grapevine from Morocco revealed higher number of alleles to that obtained in the wild grapevine from France. We suggest that this result can be due to the fact that during the time we have observed important loss diversity to the wild grapevine in the Europe favorite by unfavorable climatic conditions compared in the North Africa, how the loss diversity of wild grapevine was not important due to favorable conditions climatic. It was also thought that this loss of diversity can be due to the anthropic action which was more observed in France than in Morocco, its due mainly in Morocco the wild grapevine was primarily in places where the access was generally more difficult and far from the activity human.

The values of GD (genetic distance) are quite similar in wild and cultivated grapes (except in the Tunisian group where these values are higher. This analysis is however influenced by the important difference in number of individuals for the 2 groups, especially in the cultivated group and by the geographical areas, the cultivars of the Maghreb vs wild grapevines from France. In fact values are commonly observed at the perennial species (Tavaud, 2002; Burczyk et al., 2004; Coart et al., 2006), but also at annual species like the wheat (Roussel et al., 2005). The genetic diversity of wild groups was usually higher than the cultivars of grapevine. The wild groups was generally more genetically diverse than the cultivars (Pernes, 1984; Pitrat and Foury, 2003), since at the origin of this last, such as for example at apple cultivars (Coart et al., 2006) or corn (Vigouroux et al., 2005). During domestication, a restricted portion of the wild progenitor's gene pool was used to create a new cultivated "species." The reduction in the size of a population through domestication should cause a reduction in genetic diversity (Gepts, 2004; Ladizinsky, 1985; Doebley, 1989), and thus all genes in any domesticated plant necessarily have a history that includes a recent demographic event, the bottleneck associated with domestication. The bottleneck reduces diversity in neutral genes, but selection decreases diversity beyond that caused by the bottleneck alone (Ross-Ibarra et al., 2007). Genes important for domestication were also subjected to conscious or unconscious directional selection, experiencing a reduction in variation over and above that associated with any demographic events. The level of diversity remaining at a given locus in a domesticated is thus expected to be inversely proportional to the locus adaptive importance during domestication. Thus, the major genes contributing to agronomically important traits may lack variation entirely (Ross-Ibarra et al., 2007). This phenomenon may explain the difference of diversity within wild and cultivated grapevine.

Genotypic analysis of Moroccan wild grapevine and cultivated grapevines from the Maghreb at twenty nuclear microsatellite loci showed that sets of samples maintain the same levels of genetic variation. This diversity was higher than those reported by other authors in different grapevine cultivar sets and using different SSR markers and number of samples analyzed (Bower et al., 1996; Sefc et al., 1998; Sánchez-Escribano et al., 1999; Ibañez,

2000a; Martín et al., 2002; Ibañez et al., 2003; Snoussi et al., 2004). This result was confirmed by the study of the allelic diversity from the two genes implicated in the anthocyanin metabolic pathway: the same number of SNP was obtained in each group analyzed. The distribution of the haplotypes for the two genes (DFR and LDOX) seemed to differ among the groups compared. The Moroccan lambrusque group revealed the presence of specific haplotype for the two genes (DFR and LDOX) compared with the cultivars group. This result confirms that this material was interesting and can contain interesting genes implicated in the adaptation of the grape to its environment.

The cultivated groups appeared differentiated but were found to be significantly different from zero in all pairwise comparisons while F_{st} values between cultivated and wild accessions presented high levels. Low F_{st} values in the case of cultivated grapevines populations it's probably due to the exchange of material between regions.

The chloroplast microsatellite polymorphisms confirmed the same results obtained in the nuclear microsatellites. The chloroplasts markers showed presence of the four chlorotype in the wild and cultivated populations from Morocco, Arroyo et al. (2006) obtained 8 chlorotypes in whole of 688 samples from different countries, but in North Africa only 4 Chlorotypes A, B, C and D were observed. These results parallel our finding. In the 128 wild grapevines from France we analyzed only newly identified Chlorotypes A and D but Chlorotypes B and C existed in the global sample of 175 individuals (data not shown). The Haplotype A was the most present in wild populations, but in the cultivated populations Haplotype C occured most frequently, this result was did not correlate with those obtained by Arroyo et al. (2006) in North Africa.

The structural analysis of the wild-cultivated also highlights three groups well differentiated; the Moroccan wild grapevines are considerably different from the French wild grapevines, whereas cultivated vines of the Maghreb were clustered in the intermediate group. Our study, confirmed the results (Cuisset, 1998; This et al., 2001; Lacombe et al., 2003b; DiVecchi, 2007a; DiVecchi et al., in prep) concerning the clear differentiation of the subsp *Silvestris* compared to the subsp *vinifera sativa*. In Tunisia, in the study of the relationship between the cultivated and the wild grapevine (Snoussi et al., 2004), showed differentiation with the domesticated and wild grapevine and suggested the possibility of local domestication. In contrast, other studies on diversity, which included wild and cultivars, showed less differentiation between wild and cultivated grapevines, using nuclear (Aradhya et al., 2003; Grassi et al., 2003a) and chloroplast (Arroyo-Garcia et al., 2006; Grassi et al., 2006; Imazio et al., 2006) SSR markers. Myles et al. (2011) findings showed that haplotype diversity in Western vinifera is slightly reduced compared with eastern vinifera, suggesting that the grape experienced a modest reduction in genetic diversity as il was brought to Western Europe.

The extent of grape diversity in Morocco – conservation aspects

Our study allowed a first precise prospection and analysis of the wild grapevine in the North of Morocco.

Approximately 168 individuals of putative wild grapevine were observed in the Rif area of Morocco. This number does not necessarily reflect the reality of the presence of the wild grapevine on the Moroccan territory. Indeed, of many stations we observed in which we met a majority of rootstocks or interspecific hybrids and, to a lesser extent, varieties of *V. vinifera* ssp. *sativa* that we were able to recognize. These sites correspond for the majority probably to old abandoned vineyards. In the same way, we observed a greater number of individuals probably wild during the prospections, without being able to reach them. It exists at the present time in Morocco of the zones in which we could identify wild grapevine.

We also identified parentage relationships between individuals of distant stations. These can be explained by the flux gene or exchanges of pollen between the various seedlings, but this cannot be the case in this study, because the populations are distant at least 10 km, Di Vecchi et al. (2007) verified that the pollen cannot be dispersed at long distances. It can also be a question of a limitation related to the markers used and additional markers will make it possible to confirm these direct relationships. It can finally be a question of very old individuals belonging to the same ancestral genetic population. Generally an important consanguinity it is shown for many populations for the crossing are often carried out between close and connected individuals.

With hardly more than 168 individuals prospected on the northern part of Morocco, the wild grapevine is thus a threatened species. Moreover the lambrusques were often observed either isolated or with a very small number by population. The median number per station was 9 on the unit of the individuals. The populations distant from 10 to 50 km, with number of individuals by population equal to 9 for the whole of the individuals and 7 for the possible lambrusques. They are not strictly populations in the genetic term. In the populations with small number, mortality of individual generates a risk of extinction of the population and/or reduction of their genetic diversity and adaptability (Huenneke, 1991; Ellstrand and Elam, 1993; Ellstrand et al., 1999; Couvet, 2002; Burczyk et al., 2004). This risk was observed in some of these samples as previously observed in France (DiVecchi et al., 2008).

Conclusion

This works could show the existence of the wild grapevine in the north of Morocco, it acts on the

populations with little effect and which is capable with the hydride of cultivated grapevine; this enables us to think that this material deserves being preserved. This material was distinguished to the other wild grapevine from France indicating that material was an interesting specific genetic material. Even the same wild material is in party resulting from old crossings with compartment cultivated; it is a very original material. Taking into account the prospection and localization of a considerable part of the wild grapevines in high-risk zones (hedges with the turn of the pieces or along roads) and of the ignorance of the presence of wild grapevines per manager of natural spaces, we think that this subspecies is relatively threatened. It thus seems important to us to set up a policy of conservation of the lambrusques Moroccan. They could be initially the conservation *ex situ* on under sample selected lambrusques, representing the genetic diversity of this compartment, according to the principles of the realization of core-collections. We also suggest the conservation *in-situ* or the cryoconservation of this material. This material contains specific genes and can be utilized in the program of genetic improvement by crossings with cultivated for improvement of the quality and the production of the local grapevine.

In perspective, other prospections must be made in the other areas of Morocco, in order to know the differentiation of the populations that enter the various zones, for better vision of the history of the grapevine in the Maghreb and also in the entire Mediterranean circumference in comparison with other wild grapevines of the same area.

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