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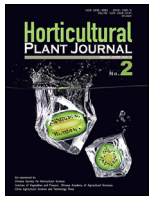

Genetic Diversity and Structure of Tunisian Local Pear Germplasm as Revealed by SSR Markers

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A B S T R A C T

Growing pear has a long tradition in Tunisia, and numerous local cultivars possessing an excellent adaptability and resilience potential to climatic variation are present. This large adaptability is associated with an important genetic diversity, which is threatened to erosion. Appropriate measures have to be taken in order to properly evaluate and conserve this local material. Microsatellite markers were used to assess the level of genetic diversity among Tunisian pear germplasm, and compare it with some European varieties and wild pear species. 61 pear accessions representing eight groups (six groups from Tunisia, one from Northern Europe and another group composed of wild pear) have been genotyped using SSR markers derived from apple and pear. The pear accessions showed a significant polymorphism and 95 polymorphic alleles were found. The number of alleles per locus varied from 5 for CH04e03 locus to 14 for CH01d09 locus with an average of 9.4 alleles per locus. Moreover, the mean gene diversity (H_e) per locus ranged from 0.192 to 0.752. Genetic distance values and cluster analyses revealed high genetic similarities among the Tunisian groups. Factorial correspondence analysis (FCA) categorized the accessions into three independent groups where Tunisian local accessions agglomerated together distantly from European and wild pear accessions. Additionally, UPGMA dendrogram grouped accessions into two clusters, confirmed thereafter by the Bayesian model-based Structure analysis. The results showed 16 putative triploid accessions found in the local germplasm. This study provides valuable information to develop strategies of local pear conservation and use.

Keywords: *Pyrus communis*; molecular marker; genetic diversity; local accession

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1. Introduction

Pear (*Pyrus* spp.) is one of the oldest-known fruit trees, grown in temperate regions of 87 countries in 2017 (http://www.fao.org/faostat/fr). Pear trees are usually diploid with a basic chromosome number $x = 17$ (Rodger and Campbell, 2002) and an estimated genome size of 600 Mb (Chagné et al., 2014). However, occasional triploid or tetraploid species and cultivars were also found in the genus (Janick and Paull, 2008; Puskás et al., 2016). *Pyrus* was believed to be native to mountainous regions of western and southwestern China (Rubtsov, 1944; Zheng et al., 2014). Based on their geographical distribution and domestication area, *Pyrus* species are traditionally divided into two groups: occidental pears and oriental pears (Rubtsov, 1944; Teng et al., 2002).

Pyrus communis L. was the main cultivated pear species in Europe, North Africa, North America, Australia and other temperate regions of the Southern hemisphere (Yamamoto and Chevreau, 2009). A wild species, *P. syriaca* Boiss occurred in the Eastern Mediterranean region (Mir Ali et al., 2007) including the mountainous regions in Northern Tunisia (Carraut, 1986). Due to its intense bloom and good compatibility, *P. syriaca* was sometimes used as a pollinizer for *P. communis* in commercial orchards (Zisovich et al., 2010). The European pear has been cultivated for a long time under different edaphoclimatic conditions in Tunisia. In the Northern and Central-Western area, pear cultivation was based on commercial European and American varieties. These varieties are characterized by moderate to high chilling requirements. By contrast, various autochthonous low chilling cultivars were grown in a warmer coastal area in Central-Eastern Tunisia (Mars et al., 1994; Brini and Mars, 2008). According to Carraut (1986), local Tunisian pears can be divided into two main groups: Arbi (or Ajmi) and Meski. Some of these, particularly Meski group, have been clonally propagated by farmers for a very long time. During the last decades, most farmers have stopped rejuvenating their pear trees as a consequence of outbreaks of fire blight (*Erwinia amylovora*) (Rhouma et al., 2014), and/or changing production to an economically more profitable crops such as olive, peach and almond. Hence, many old pear cultivars have completely disappeared, possibly leading to a severe genetic erosion of the local germplasm. Therefore, it is urgent to establish strategies for conservation of pear cultivars, starting with the identification and characterization of remaining traditional accessions.

Phenotypic diversity in pear has been studied using morphological characters, mainly on fruits and leaves (Katayama and Uematsu, 2006; Pereira-Lorenzo et al., 2012; Ait Said et al., 2013). More accurate information can be better obtained with DNA fingerprinting techniques, especially SSR markers (Kimura et al., 2002; Bao et al., 2007; Ferradini et al., 2017; Xue et al., 2017). A previous SSR-based study on local pears in Tunisia (Brini et al., 2008) revealed a significant diversity and an originality of the local genetic heritage.

The main objective of this study is to characterize Tunisian low chilling pear accessions using SSR markers, and to investigate genetic variability among sets of local accessions of *P. communis* and *P. syriaca*, thereby, to estimate the genetic relatedness among them. An additional objective is to identify obvious duplicates and instances of erroneous labeling among the studied accessions.

2. Materials and methods

2.1. Plant material and DNA extraction

A total of 61 pear accessions (individuals) were analyzed in this study (Table 1), including 49 Tunisian accessions, nine from Europe for comparison, and three wild forms of *Pyrus syriaca* (2 accessions) and *Pyrus calleryana* (1 accession) obtained from the pear germplasm collection at the Higher Agronomic Institute of Chott-Mariem (Tunisia). Tunisian pear accessions were collected during 2014–2015, from various orchards in six different geographical locations situated in the Central-East of Tunisia: Sidi Khlifa (N 36°22′, E 9°55′), Sidi Bou Ali (N 35°57′, E 10°28′), Moknine (N 35°37′, E 10°54′), Mahdia (N 35°19′, E 10°34′), Menzel Fersi (N 35°67′, E 10°82′) and Jammel (N 35°34′, E 10°40′).

Young leaves were dried and kept in silica gel until DNA extraction. Total genomic DNA was extracted from 70 to 90 mg of dried foliar tissues, at the Swedish University of Agricultural Sciences (SLU), Alnarp, using the Qiagen Dneasy™ Plant Mini Kit according to the manufacturer's protocol with minor modifications. DNA concentration was estimated with a Nanodrop ND-1000 spectrophotometer (Saveen Werner, Sweden). The quality of DNA was also checked by electrophoresis in a 1.2% agarose gel. The DNA of the European cultivars was provided by the Department of Plant Breeding-Balsgård (Sehic et al., 2012), the Swedish University of Agricultural Sciences.

2.2. PCR and microsatellite analysis

Twelve primer pairs (Table 2) were selected out of 17 nuclear SSR loci recommended for germplasm management by the European Cooperative Program for Plant Genetic Resources (ECPGR) (Evans et al., 2009). Forward primers were fluorescently labeled on their 5′ end using one of the following fluorescent compounds: FAM, NED or HEX, to enable analysis on automated sequencers.

PCR amplifications were carried out in a final volume of 12.5 μL containing 10–15 ng of genomic DNA, 1 μL l × buffer (Dream Taq buffer, Thermo Fisher Scientific), 0.2 mmol·L^{-1} dNTP, 1 mmol·L^{-1} $MgCl_2$ and 0.25 U *Taq* DNA polymerase (Thermo Fisher Scientific). Three different concentrations of primers were used: 0.5 μmol·L^{-1} for CH01d09, CH01f07a, CH03d12, CH03g07, EMPc117, GD96 and GD147, 1.0 μmol·L^{-1} for CH01d08, CH02b10, CH04e03, and CH05c06, and 1.5 μmol·L^{-1} for EMPc11. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 10 cycles of 94 °C for 30 s, 55 °C for 45 s, with a decrease of annealing temperature of 0.5 °C per cycle, and 72 °C for 1 min, followed by 25 cycles at the annealing temperature of 50 °C and extension at 72 °C for 1 min, and a final step of 15 min at 72 °C. PCR products were checked by 1.5% agarose gel in a 1 × TBE buffer. Labelled amplification products were multiplexed in four panels and detected by capillary electrophoresis on Genetic Analyzer 3500 (Applied Biosystems) at University of Copenhagen (Denmark).

2.3. Data analyses

The size of the amplified alleles was determined using the Gene-Marker ver. 2.2.0 software (SoftGenetics, State College, Pennsylvania). In order to ascertain how informative these ten SSR loci, calculations were performed only on the correctly-identified diploid samples, excluding triploids and duplicates.

Table 1 Denominations, codes and geographic origins of studied *Pyrus* accessions

Pyrus species	Geographic origin	Group	Accession (denomination)	Code
Pyrus communis	Tunisia	Sidi Khlifa	Meski Arteb Sidi Khlifa	MTB1
			Radsi Sidi Khlifa	RDS1
			Meski Bouguedma Sidi Khlifa	MBG1
			Arbi 1 Bouficha	ABF1
			Arbi 2 Bouficha	ABF2
		Sidi Bou Ali	Arbi 1 Sidi Bou Ali	ASB1
			Arbi 2 Sidi Bou Ali	ASB2
			Arbi 3 Sidi Bou Ali	ASB3
			Soukri Sidi Bou Ali	SKR
			Meski Arteb Sidi Bou Ali	MTB2
			Double Meski Sidi Bou Ali	DMK1
		Moknine	Moknine 4	MOK4
			Moknine 6	MOK6
			Moknine 7	MOK7
			Moknine 7A	MOK7A
			Moknine 10	MOK10
		Menzel Fersi	Mostfi	MSF
			Arbi Manzel Fersi	AMF
			Chemi	CHM
			Tourki	TRK
			Meski Bouguedma1 MF	MBG3
			Meski Bouguedma2 MF	MBG4
			Meski Ahrech	MHR
			Makkaoui	MKW
		Jammel	Fayeli	FYL
			Arbi Mili	ARJ1
			Arbi Chiheb	ARJ2
			Arbi Touzen	ARJ3
			Arbi GRD	ARJ4
			Arbi Cherif	ARJ5
			Arbi GL	ARJ6
			Jrani1	JRN1
			Jrani2	JRN2
			Malti	MLT
			Radsi	RDS2
			Meski Bouguedma 5	MBG5
			Meski Bouguedma 6	MBG6
		Mahdia	Mahdia 5	MAH5
			Mahdia 6	MAH6
			Mahdia7	MAH7
			Bkalta 5	BKL5
			Meski Mahdia	MSK
			Ambri	AMB
			Double Meski Mahdia	DMK2
			Souri	SRA
			Arbi Mahdia	ARM
			Meski Bouguedma Mahdia	MBG2
			Mahdia 11	P1A1
			Mahdia 12	P2A2
	Europe	Europe	Bonne Louise	BLS
			Carola	CRL
			Clapp's Favorit	CLF
			Clara Frijs	CRF
			Conference	CON
			Seigneur dÉsperen	ESH
			Göteborgs Diamant	GDM
			Hergogin Elsa	HEL
			Blodpäron	RKT
Pyrus spp.	Tunisia	Other *Pyrus*	*Pyrus syriaca* 1	PSI
			Pyrus syriaca 2	PG2
			Pyrus calleryana	PG5

The software GenAlEx 6.41 (Peakall and Smouse, 2006) was used to estimate genetic diversity parameters for each microsatellite locus: the number of alleles per locus (N_a), allele frequencies (p), observed heterozygosity (H_o) and expected heterozygosity (H_e). Gene diversity, often referred to as expected heterozygosity (H_e) was calculated according to Nei (1978). We calculated the percentage of polymorphic alleles within each group (%P) and Shannon's index of diversity (I) and Nei's (1972) genetic distance using the same software. PIC (Polymorphic Information Content) was measured according to the formula of Anderson et al. (1993). The deviation from the Hardy-Weinberg equilibrium and linkage disequilibrium were

Table 2 Characteristics of the 12 microsatellites markers used in the study

Genome origin	SSR locus	Linkage group	Primer sequence (5′ - 3′)	Reference
Apple	CH01d08	15	F: CTCCGCCGCTATAACACTTC; R: TACTCTGGAGGGTATGTCAAAG	Liebhard et al., 2002
	CH01d09	12	F: GCCATCTGAACAGAATGTGC; R: CCCTTCATTCACATTTCCAG	
	CH01f07a	10	F: CCCTACACAGTTTCTCAACCC; R: CGTTTTTGGAGCGTAGGAAC	
	CH03d12	6	F: GCCCAGAAGCAATAAGTAAACC; R: ATTGCTCCATGCATAAAGGG	
	CH03g07	3	F: AATAAGCATTCAAAGCAATCCG; R: TTTTTCCAAATCGAGTTTCGTT	
	CH04e03	11	F: TTGAAGATGTTTGGCTGTGC; R: TGCATGTCTGTCTCCTCCAT	
	CH05c06	16	F: ATTGGAACTCTCCGTATTGTGC; R: ATCAACAGTAGTGGTAGCCGGT	
	CH02b10	2	F: CAAGCAAATCATCAAAGATTCAAG; R: CAAGTGGCTTCGGATAGTTG	Gianfranceschi et al., 1998
	GD147	13	F: TCCCGCCATTTCTCTGC; R: AAACCGCTGCTGCTGAAC	Hokanson et al., 1998
	GD 96	17	F: CGGCGGAAAGCAATCACCT; R: GCCAGCCCTCTATGGTTCCAGA	Hokanson et al., 1998
Pear	EMPc11	11	F:GCGATTAAAGATCAATAAACCCATA; R: AAGCAGCTGGTTGGTGAAAT	Fernández-Fernández et al., 2006
	EMPc117	7	F: GTTCTATCTACCAAGCCACGCT; R: CGTTTGTGTGTTTTACGTGTTG	

tested using Genetix 4.04 program (Belkhir et al., 1996–2004). To evaluate population subdivision, *F*-statistics, the three fixation indices were calculated according to the formula of Weir and Cockerham (1984): F_{IS} (inter-individual), F_{ST} (sub-populations) and F_{IT} (total population). Total gene diversity (H_T) and the genetic differentiation among populations (G_{ST}) were estimated using Genetix 4.04. Additionally, Factorial correspondence analysis (FCA) was performed in order to obtain more synthetic representation of the organization of molecular variability. The mean value of gene flow (N_m) for all loci was estimated by POPGENE 1.3 software (Yeh et al., 1999) according to Slatkin and Barton (1989): $N_m = 0.25\ (1 - F_{ST})/\ F_{ST}$.

Dice genetic similarity coefficient values (Dice, 1945) were used to visualize genetic relationships among the accessions via the unweighted pair-group method with arithmetic average (UPGMA) cluster analyses, using XLSTAT software package (http://www.xlstat.com). For population structure analysis, Bayesian model-based clustering program STRUCTURE version 2.3.3 (Pritchard et al., 2000) was used to infer to how many clusters (k) were the most appropriate for interpreting the data. The program presumes Hardy-Weinberg equilibrium (HWE) and linkage equilibrium within clusters. The original data matrices were imported into the program and ten independent replicates ran for each k value were performed. The optimum number of populations (k) was calculated using the ad hoc measure of Δk of Evanno et al. (2005). The ad hoc quantity is based on the second order rate of change of the likelihood function implemented in the program Structure Harvester version 0.6.94 website (Earl and von Holdt, 2012).

3. Results

3.1. SSR loci polymorphism and genetic diversity

Among the 61 accessions analyzed, 16 were considered putative triploids and 3 duplicates. Thus, primer polymorphism as well as genetic diversity were evaluated on the 42 diploid samples. Using ten SSR primers, 95 different alleles were identified with fragment size ranging from 86 (CH05c06) to 288 bp (CH01d08) (Table 3). The number of alleles per locus varied from 5 to 14 with an average of 9.5. Both of CH04e03 and CH03d12 markers amplified the minimum number of alleles (5 and 6 respectively), whereas the maximum number was observed at CH01d09 with 14 alleles (Table 3). The discriminative power of each SSR was assessed by calculating its PIC (Polymorphic Information Content). CH01d09 and CH01f07a loci provided the highest informativeness since they exhibited the highest PIC values (0.90 and 0.89 respectively). In contrast, the lowest PIC value (0.42) was observed for the CH04e03 locus (Table 3).

According to allelic frequencies, we classified 27 alleles (approx 28%) as rare due to their low frequency ($P < 0.03$), 54 as common alleles (frequencies from 0.03 to 0.20) and 14 were considered as most frequent alleles ($P > 0.2$) (Table 3). All loci showed at least one rare allele. Also, 81 out of the 95 alleles (approx 85.3%) occurred at a frequency of 0.2 or less indicating high allelic diversity (Table 4). A total of 13 unique alleles were found, most of them were detected in *P. syriaca* and *P. calleryana*. Meski Bouguedma 6 (MBG6), Radsi 2 (RDS2), Mahdia 7 (MAH7), and two Swedish cultivars: Blodpäron (RKT) and Carola (CRL) presented also unique alleles. With regard to the Shannon's index (*I*), CH01d09 showed the highest value (2.44) whereas CH04e03 recorded the lowest value (0.79) as expected (Table 4).

We have identified 16 putative triploid accessions (10 accessions from Jammel; 5 from Mahdia and 1 from Sidi Khlifa) out of 61 (26.2%). An examination of their SSR profiles demonstrated some mislabeling cases: 'Arbi Mahdia' (ARM) and 'Souri' (SRA) from Mahdia region were synonyms. 'Arbi Mili' (ARJ1) and 'Arbi GRD' (ARJ4), 'Fayeli' (FYL) and 'Arbi GL' (ARJ6) from Jammel, could be also synonyms, whereas 'Jrani 1′ (JRN1) and 'Jrani 2′ (JRN2) from the same group were homonyms. Meski Bouguedma from Sidi Khlifa (MBG1) and Meski Bouguedma 5 from Jammel (MBG5) presented similar fingerprints. They could be synonymous accessions from two different geographic groups. As per the diploid samples, three pairs of genetically identical accessions have been found: Arbi 2 Sidi Bou Ali (ASB2) and Arbi 3 Sidi Bou Ali (ASB3), Moknine 7 (MOK7) and Moknine 7A (MOK7A), and Mahdia 6 (MAH6) and Mahdia 7 (MAH7). Surprisingly Meski Bouguedma1 MF (MBG3) and Meski Bouguedma2 MF (MBG4) from Menzel Fersi were revealed as homonymous although they have many similar phenotypic traits. The unknown accessions Mahdia 11 (P1A1) and Mahdia 12 (P2A2) were highly similar to European cultivars.

The expected heterozygosity (H_e) ranged from 0.192 (CH04e03) to 0.752 (CH01d09) with a mean of 0.622. The observed heterozygosity (H_o) varied from 0.056 (CH04e03) to 0.822 (EMPc11) with a mean of 0.534 (Table 4). Indeed, significant heterozygote deficiency was registered for the EMPc117, CH04e03, CH01f07a, CH01d09 and CH03d12 loci suggesting a genetic deviation from Hardy-Weinberg equilibrium. The F_{ST} mean value calculated for all SSR loci was 0.179. F_{ST} was the highest for CH04e03 (0.540) and the lowest for CH01d08 (0.100) (Table 4). The inbreeding

Table 3 Polymorphism analysis of 42 *Pyrus* diploid accessions with 10 SSR markers

Locus	Size range/bp	A	Ae	Rare alleles ($P < 0.03$)	Common alleles ($P = 0.03$–0.2)	Most frequent alleles ($P > 0.2$)	PIC
CH01d08	242–288	7	3.86	1	5	1	0.81
CH01d09	122–158	14	4.36	3	10	1	0.90
CH01f07a	177–209	12	4.41	1	10	1	0.89
CH03d12	91–124	6	2.76	1	3	2	0.71
CH03g07	228–266	10	2.83	5	3	2	0.70
CH04e03	177–205	5	1.33	2	2	1	0.42
CH05c06	86–109	7	2.93	1	3	3	0.75
EMPc11	137–160	13	3.66	5	7	1	0.83
EMPc117	87–119	10	3.54	2	7	1	0.81
GD147	118–155	11	3.36	6	4	1	0.76
Total	86–288	95	33.00	27	54	14	–
Mean	–	9.5	3.30	2.7	5.4	1.4	0.76

Note: A. Average number of alleles per locus; Ae. Average number of effective alleles per locus; PIC. Polymorphic Information Content.

Table 4 The genetic parameters revealed by SSR primers for the 42 analyzed *Pyrus* accessions

Locus	Range of allele frequency	H_o	H_e	H_T	F_{IS}	F_{ST}	F_{IT}	G_{ST}	I	N_m
CH01d08	0.021–0.271	0.740	0.731	0.812	- 0.012	0.100	–0.037	–0.025	1.729	2.252
CH01d09	0.021–0.153	0.521	0.752	0.904	0.308	0.167	0.474	0.008	2.441	1.244
CH01f07a	0.026–0.207	0.504	0.712	0.886	0.292	0.197	0.421	0.035	2.394	1.017
CH03d12	0.021–0.443	0.373	0.614	0.711	0.392	0.136	0.470	–0.018	1.520	1.584
CH03g07	0.007–0.458	0.502	0.575	0.699	0.127	0.178	0.220	0.043	1.670	1.155
CH04e03	0.007–0.738	0.056	0.192	0.416	0.710	0.540	0.775	0.432	0.791	0.213
CH05c06	0.020–0.376	0.773	0.638	0.745	−0.212	0.144	0.123	0.015	1.472	1.485
EMPc11	0.007–0.327	0.822	0.692	0.826	−0.188	0.163	0.119	0.075	2.140	1.287
EMPc117	0.021–0.349	0.422	0.639	0.814	0.341	0.214	0.393	0.002	2.092	0.917
GD147	0.007–0.432	0.632	0.674	0.756	0.062	0.109	0.138	–0.013	1.719	2.045
Mean	–	0.534	0.622	0.757	0.141	0.179	0.310	0.037	1.797	1.151

Note: H_o. Observed heterozygosity; H_e. Expected heterozygosity; H_T. Total gene diversity; F_{IS}. Inter-individual fixation indice (inbreeding index); F_{ST}. Coefficient of genetic differentiation; F_{IT} Total population fixation indice; G_{ST}. Genetic differentiation among groups; I. Shannon's Information Index; N_m. Gene flow. The same below.

Table 5 Genetic diversity indices for the geographic groups of *Pyrus* accessions

Number	Group	H_o	H_e	Mean number of alleles/ locus	F_{IS}	%P	I
1	Sidi Khlifa	0.550	0.616	3.9	0.246	90.00	1.142
2	Sidi Bou Ali	0.437	0.591	3.5	0.390	100.00	1.047
3	Moknine	0.450	0.597	3.8	0.376	90.00	1.120
4	Menzel Fersi	0.508	0.684	5.2	0.324	100.00	1.398
5	Jammel	0.500	0.617	3.4	0.412	100.00	1.160
6	Mahdia	0.468	0.639	4.4	0.366	90.00	1.271
7	Europe	0.732	0.671	5.4	−0.032	100.00	1.383
8	Other Pyrus	0.600	0.528	3.3	0.132	80.00	0.977
	Mean	0.530	0.62	4.1	0.280	94.00	1.187

Note: %P. Percentage of polymorphic alleles within each group.

coefficient (F_{IS}) varied from −0.212 (CH05c06) to 0.710 (CH04e03). In fact, CH04e03 showed the highest value of F_{IS} since it exhibited the lowest allelic richness along with the smallest values of H_o and H_e. In contrast, CH05c06, EMPc11 and CH01d08 loci demonstrated, a heterozygote excess compared with the expectations from Hardy-Weinberg Equilibrium (HWE). The genetic differentiation G_{ST} ranged from −0.025 (CH01d08) to 0.432 (CH04e03) with an average of 0.037. The mean value of gene flow ($N_m = 1.151$) for all loci (Table 4) is considered moderate. In spite of the same location at linkage group 11, the gene flow (N_m) at locus EMPc11 (1.287) is higher than CH04e03 (0.213) locus.

3.2. *Genetic diversity among geographic groups*

At the population level, the highest H_e value over 10 loci (0.684) and the highest value for Shannon Index (1.398) were detected in group Menzel Fersi, while the lowest values were found in other group ($H_e = 0.528$, $I = 0.977$) (Table 5). H_o values varied from 0.437 in the Sidi Bou Ali group to 0.732 in the Europe group. Values of F_{IS} were positive for all the groups except for the group of Europe (–0.032) and the highest value (0.412) was revealed by the group of Jammel. This suggested that all groups, except the Europe group, had deficiency in heterozygotes compared with panmictic equilibrium. The mean value of F_{IS} over the 8 groups was 0.280. The percent of polymorphism (%P) ranged from a minimum of 80% to a maximum of 100% for the Sidi Bou Ali, Menzel Fersi, Jammel and Europe groups with an average of 94% for all groups.

3.3. *Multivariate analyses*

To illustrate a synthetic representation of the genetic variability distribution among accessions, Factorial correspondence analysis was performed (Belkhir et al., 1996-2004). The three dimensional scatter plot of FCA coordinate for the first, second and the third factors, which explain, respectively, 34.59%, 18.60% and 17.58% of the total diversity (70.77%), showed a pattern of genetic

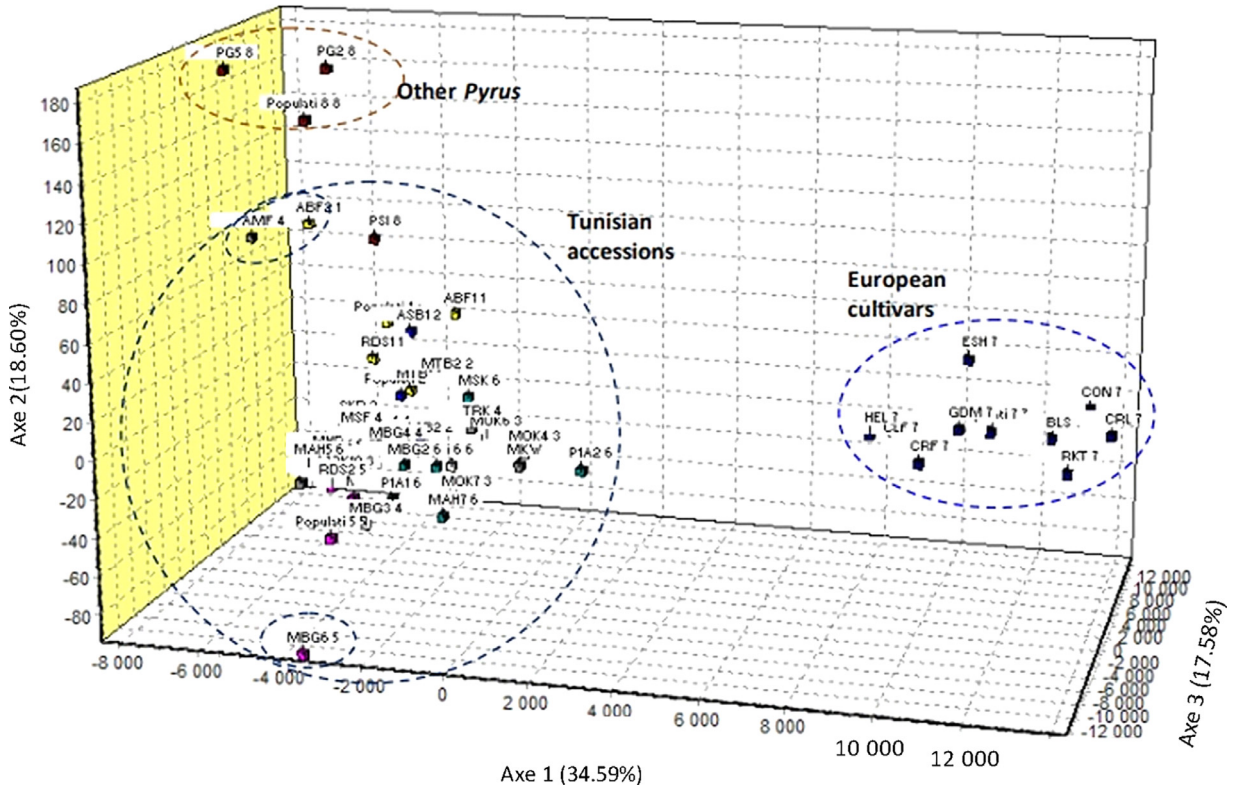

Fig. 1 Factorial correspondence analysis for eight *Pyrus* groups based on data for 10 SSRs in Tunisian, European and other pear

Table 6 Nei's genetic distance values between different *Pyrus* groups

	Sidi Khlifa	Sidi Bou Ali	Moknine	Menzel Fersi	Jammel	Mahdia	Europe
Sidi Bou Ali	0.364	0					
Moknine	0.624	0.373	0				
Menzel Fersi	0.498	0.439	0.173	0			
Jammel	0.886	0.653	0.295	0.217	0		
Mahdia	0.736	0.333	0.242	0.239	0.351	0	
Europe	1.140	0.881	0.498	0.547	0.854	0.537	0
Other *Pyrus*	0.902	0.598	0.532	0.382	0.488	0.438	0.981

diversity (Fig. 1). The cultivars originating from Europe (group 7) were clearly differentiated from the Tunisian accessions and were grouped in the positive parts of the three axes. Additionally, the wild accessions of *Pyrus* spp. (group 8) were somehow isolated from the local groups except *Pyrus syriaca* (PSI). Within Tunisian groups (groups from 1 to 6), the FCA did not show a clear geographic segregation. Arbi Bouficha 2 (ABF2) and Arbi Menzel Fersi (AMF) characterized by a late ripening period were grouped together. Meski Bouguedma 6 (MBG6) was quite distant to other accessions (Fig. 1). This isolation is may be due to its unique allele identified at locus CH04e03 (allele 188). In addition, SSR profiles revealed that the allele '87′ at locus EMPc117 was only detected in Meski Bouguedma 6 (MBG6) and Mahdia 7 (MAH7). Furthermore the allele '103′ at locus CH05c06 was shown no more than that in MBG6 and Bonne Louise (BLS). According to the FCA analysis, groups of local pears might be considered as genetically similar.

Nei's genetic distances were reported in Table 6. Values ranged from 0.173 between Moknine and Menzel Fersi, to 1.14 between Europe and Sidi Khlifa. The distances between local groups were narrow (0.217 between Jammel and Menzel Fersi, 0.239 between Mahdia and Menzel Fersi) indicating a strong similarity.

To interpret the genetic relationships among diverse *Pyrus* accessions, an UPGMA cluster analysis based on the Dice's coefficient (Dice, 1945) was performed. UPGMA dendrogram showed two main clusters and two branches at the lowest similarity value of 0.2 (Fig. 2). The separation of the two main clusters I and II was with a similarity value of 0.3. The first cluster was divided into two groups: Ia and Ib at a similarity value of 0.33 as well as for the second cluster (IIa and IIb). Cluster I included the majority of the Tunisian accessions. Some accessions originating from the same province (same group) were genetically distant like Radsi Sidi Khlifa (RDS1) and Meski Arteb Sidi Khlifa (MTB1). However other accessions from distant locations showed a strong similarity. It seems that Chemi (CHM) and Moknine 10 (MOK10) were the closest pair of accessions with the highest similarity value of 0.96. In fact, all these results showed an overlapping of local accessions and corroborate the FCA results. Indeed, the Tunisian cultivars are grouped in Ia, Ib and IIa subclusters, while the subcluster IIb is only composed of foreign cultivars from Europe which were included for comparison. Meski Bouguedma 6 (MBG 6) accession from Jemmel was obviously diverged from the other accessions and formed a separated branch on its own.

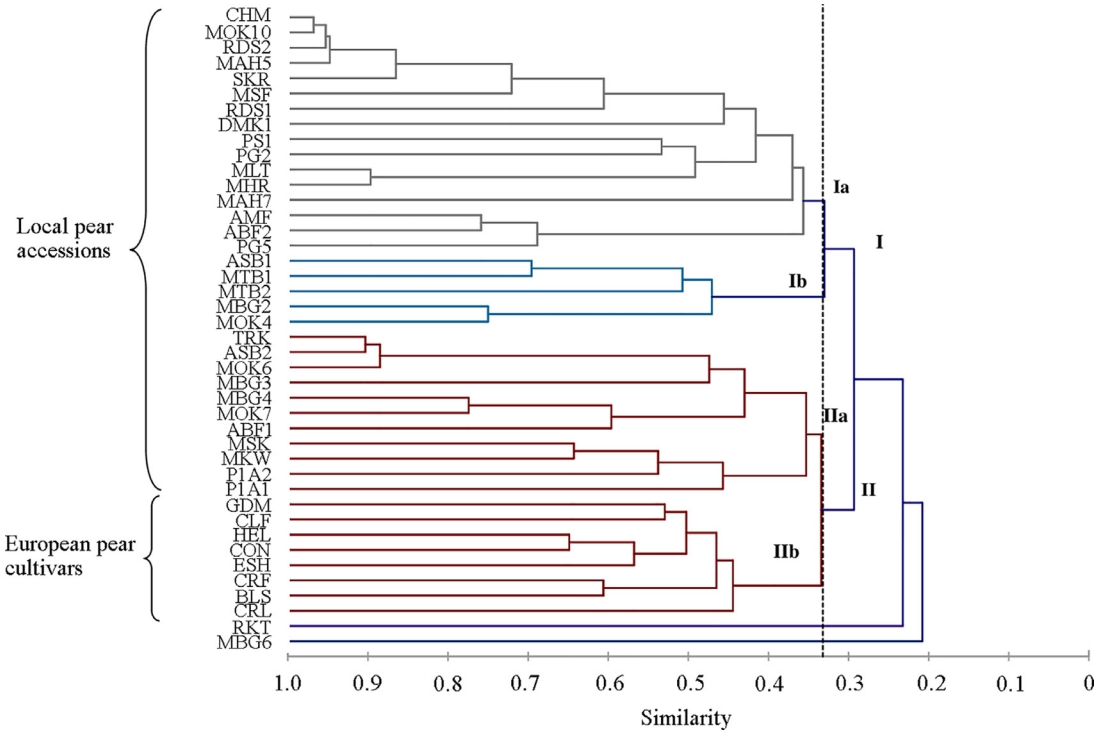

Fig. 2 Dendrogram of *Pyrus* accessions generated by unweighted pair-group method (UPGMA) cluster analysis

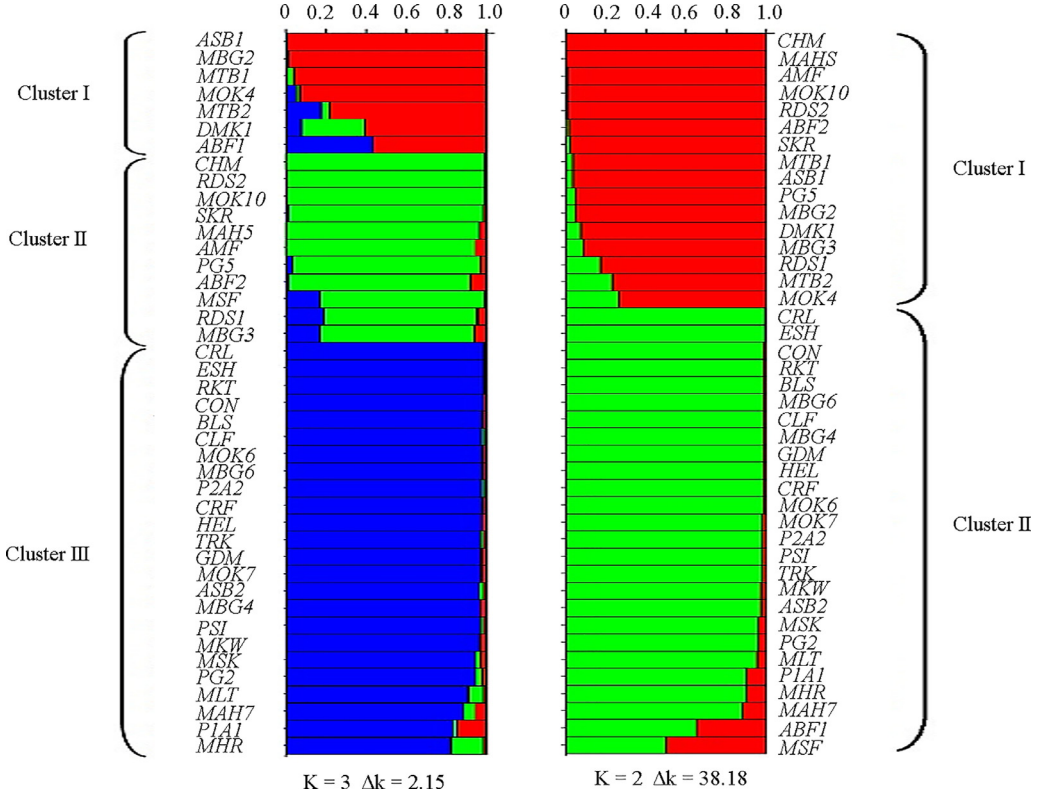

Fig. 3 Genetic structure of 42 *Pyrus* accessions as inferred by STRUCTURE based on ten SSRs

Single vertical line represents a single accession and different colors indicate different clusters, Segments of each vertical line (0; 0.2; 0.4…) represent extent of admixture in an individual and the proportion of its genome in k clusters.

3.4. Bayesian clustering analysis

Using a model-based Bayesian clustering, the estimated log probability of the data [ln Pr(x/k)] was computed by STRUCTURE harvester and the highest value was at $k = 8$ (data not shown). Furthermore, an ad hoc quality based on the second order rate of the likelihood function (Evanno et al., 2005) indicating the highest peak at $k = 2$ ($\Delta k = 38.18$) then at $k = 3$ ($\Delta k = 2.15$) (Fig. 3). The optimum model considered at $k = 2$ suggested the existence of two main clusters. The first one (Cluster I) included 38% of all accessions, and it combined autochthones accessions coming from the six local groups. The second overlapping-cluster (Cluster II) was composed of all European cultivars, with the remaining local accessions and the two accessions of *P. syriaca*. The existence of two major groups of pear accessions ($k = 2$) strongly confirmed the UPGMA cluster analysis (Fig. 2). At $k = 3$, the Tunisian accessions corresponding to Cluster I when $k = 2$ were split into two clusters. At this level ($k = 3$), accessions belonging to Sidi Khlifa and Sidi Bou Ali groups were sorted together in the first cluster (Cluster I). In the second cluster (Cluster II) accessions from Menzel Fersi, Mahdia and Moknine groups banded together. All European cultivars and the remaining Tunisian accessions were assembled in Cluster III (Fig. 3). The Bayesian-structure analysis showed that indigenous accessions were grouped according to their pedigree and no clear distinction based on their geographic origin was detected.

4. Discussion

4.1. SSR loci polymorphism and genetic diversity

Our study is based on the screening of 42 pear accessions where 85.3% of the 95 alleles occurred at a frequency of 0.2 or less, indicating high allelic diversity. This was due to genetic variability of plant material and the high level of polymorphism of the markers. Brini et al. (2008) used seven SSR markers to assess genetic relationships in Tunisian pear cultivars including 25 local and 6 foreign cultivars, and they found 36 alleles altogether with an average of 7 alleles per locus. Therefore, we got a higher number of alleles (95) when comparing both works. The difference in allele number can be explained by a lower number of samples compared with our samples and the different SSR markers used. Sehic et al. (2012) used the same set of SSR loci that we used to fingerprint 86 samples of European pear maintained in the Swedish pear collection at Balsgård and reported a lower number of alleles (76) but a higher expected and observed heterozygosity ($H_e = 0.78$, $H_o = 0.74$). In general, the high level of genetic diversity in view of a high level of heterozygosity is due to the cross pollinating nature and self incompatibility of pear (Zisovich et al., 2009; Nashima et al., 2015).

In the study of Sehic et al. (2012) as well as ours, the two loci, CH02b10 and GD96, were deleted for poor amplification and unreliable SSR profiles. Yet, CH02b10 showed good amplification in the study of Akçay et al. (2014) of Anatolian pear germplasm, showing 19 alleles and $H_o = 0.637$. The SSR primers used in this study, except CH04e03, were considered as informative for pear genetic analysis (PIC ≥ 0.7) and the results proved their effectiveness to detect polymorphism in pear. Therefore they can be used for cultivar identification and assessing genetic diversity of pear germplasm. In addition, PIC values obtained in this work were higher than those reported by Wolko et al. (2010) and Sisko et al. (2009) in different pear collections. The most informative locus was CH01d09 ($PIC = 0.9$; $I = 2.44$) as reported by Sehic et al. (2012), while the least informative one was CH04e03 ($PIC = 0.42$; $I = 0.79$). In fact, CH04e03 locus was monomorphic for the groups Sidi Khlifa, Moknine, Mahdia and the wild pears. Dissimilarly, CH04e03 showed both high *PIC* value (0.84) and Shannon's Information Index value ($I = 1.87$) in a similar study of Himalayan *Pyrus* (Rana et al., 2015). It was noticeable that there is a great variation in SSR profiles of accessions of the same group as well as accessions growing in different regions and localities. The moderate gene flow ($N_m = 1.151$) could be explained by the relative proximity of geographical areas of studied accessions and the easy exchange of plant material. In addition, the dispersal of pollen between neighboring localities by pollinating insects, mainly by bees, can also increase gene flow.

Compared with Ferreira dos Santos et al. (2011), the percentage of triploids in our work is quite higher. In total, we counted 16 putative triploid accessions from 3 different groups; most of them were found in Jammel region. In this region, triploid accessions showed wider distribution and more ecological amplitude than diploids. Lin and Fang(1994) provided an evidence for triploidy and tetraploidy in *Pyrus* spp. Puskás et al. (2016) confirmed 23 accessions out of 188 Romanian and German accessions of pear as triploids by flow cytometry. Wang et al. (2015) reported that, triploid and tetraploid offspring may result from crosses between mutant buds and normal diploid pear plants, which will benefit polyploidy breeding. Thus, triploids may arise from unreduced female or male gametes (Sugiura et al., 2000; Garkava-Gustavsson et al., 2008). Triploid fingerprints were quite different from the alleles detected in diploids.

4.2. Genetic relationships among geographic groups and accessions

Based on our molecular data, *Pyrus syriaca* shared several alleles with a number of local accessions from different groups which is not the case with *Pyrus calleryana*. Thus, *P. syriaca* could be related to local cultivated accessions (*P. communis*). This may indicate an exchange of genetic information between these two species through natural hybridization: since *P. communis* and *P. syriaca* were known to form inter-specific hybrids with no difficulty (Zisovich et al., 2010). In fact, *P. syriaca* originates from Northeastern Africa and is naturally distributed in the mountains of North Africa and other Mediterranean areas (Challice and Westwood, 1973; Mir Ali et al., 2007). In contrast, *P. calleryana* is geographically distant as it is native to China (Rubtsov, 1944; Liu et al., 2012). This hypothesis matched the results shown in the FCA where *P. syriaca* 1 (PSI) was nearby local accessions. UPGMA dendrogram revealed that the two accessions of *P. syriaca* 1 and 2 (PSI and PG2) were close to Malti (MLT) and Meski Ahrech (MHR) (similarity coefficient of 0.5). Moreover, STRUCTURE analysis corroborated these results by clustering *P. syriaca* (PSI and PG2) with the same Tunisian accessions (MLT, MHR) and some others. Ferradini et al. (2017) reported also that *P. syriaca* was clustered with some Italian cultivars and confirmed that *P. syriaca* is genetically close to *P. communis*. The hybridization between these two species offered an opportunity of transferring important agronomic qualities from the wild species to the cultivated ones.

According to multivariate analysis, Meski Bouguedma 6 (MBG6) branched outside of the local groups. Based on the SSR profiles, this accession (MBG6) had already shown high resemblance with putative triploid accessions belonging all together to the same group (Jammel). Then we can assume that MBG6 may possibly be their progenitor.

Genetic relationships among local groups using Nei's genetic distance indicated strong similarity. This result was consistent with the FCA pattern where traditional accessions agglomerated distantly from both European and wild pear groups. The narrowest Nei's genetic distance was noted between Menzel Fersi and Moknine (0.173). This may due to the geographic proximity between these two districts that are the closest ones (15 km). Hence the exchange of plant material occurred easily. Sidi Khlifa is a small region while Sidi Bou Ali is a big one where growing pear has taken place for long time ago. Several samples from those two localities shared common or almost identical alleles. This observation pointed out the consequences of a founder effect. It is supposed that farmers from Sidi Khlifa often brought landraces from Sidi Bou Ali area.

Within the last ten years, we have noticed a total disappearance of some cultivars previously investigated and described by Brini and Mars (2008) like Fayouni, Nahli and Meski Asfer. Hence, an urgent need for conservation and preservation of the germplasm is required as soon as possible.

4.3. *Bayesian clustering analysis*

In the present study, Bayesian genetic structure analysis generated 2 main groups ($k=2$) with admixture regardless of geographical distribution especially for the Tunisian accessions. Similar study conducted by Kumar et al. (2017) on 214 pear accessions of various species (Asian, European and interspecific hybrids) showed that the most significant number of clusters was $k=2$. Furthermore, Rana et al. (2015) assigned five clusters with an extent admixture in a Bayesian clustering analysis of Indian *Pyrus* accessions. Nonetheless, a Bayesian population analysis carried on Turkish pear accessions inferred 6 groups showing a consistency with their origin locality (Akçay et al., 2014). Xue et al. (2017) found also that the population structure of 67 Asian pear landraces in Tibet is subdivided into five clusters strongly matched to their geographic sites.

5. Conclusion

In conclusion, exploring the genetic diversity and relationships in *Pyrus* germplasm is essential to germplasm preservation and improvement, which could be adapted to the rapid climatic change or to the development of new cultivars with resistance to diseases and pests. This work showed that traditional Tunisian pear cultivars harbored rich genetic diversity. At an intra- and inter-species level, our study showed a strong distinction of gene pool of Tunisian pear compared with European and wild pear. Meanwhile, local pear groups showed an overlapping and mixed grouping which demonstrated a gene flow among them. We analyzed a part of the Tunisian pear germplasm in the Center of the country. The high diversity encouraged further work with additional cultivars collected from Northern and Southern areas.

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