

Assessment of genetic diversity of Tunisian orange, *Citrus sinensis* (L.) Osbeck using microsatellite (SSR) markers

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ABSTRACT. *Citrus* are one of the most cultivated crops in the world. Economically, they are very important fruit trees in Tunisia. Little is known about the genetic diversity of the Tunisian *Citrus* germplasm. Exploring this diversity is a prerequisite for the identification and characterization of the local germplasm to circumvent and controlling genetic erosion caused by biotic and abiotic stress to aid its conservation and use. In the present study, we explored the genetic diversity of 20 Tunisian orange cultivars [*Citrus sinensis* (L.) Osbeck] and established their relationships by using seven simple sequence repeat (SSR) loci. In total, 37 alleles and 44 genotypes were scored. The sizes of alleles ranged from 90 to 280 bp. The number of alleles per locus was from 4 to 7, with an average of 5.28. Polymorphic information content value changed from 0.599 to 0.769 with an average of 0.675. Analysis of the genotypes revealed a heterozygote deficiency across all the genotypes. The observed heterozygosity varied from 0 to 1 (average of 0.671).

Cluster analysis showed that three groups could be distinguished and the polymorphism occurred independently of the geographical origin of the studied orange cultivars. The detected SSR genotypes allowed the establishment of an identification key with a discriminating power of 100%. Multivariate analysis and the neighbor-joining phylogenetic tree indicated a narrow genetic base for the orange cultivars. The usefulness of SSR markers for orange fingerprinting and evaluation of the genetic diversity in the Tunisian germplasm are discussed in this paper.

Key words: SSR; *Citrus sinensis* (L.) Osbeck; Molecular polymorphism; Identification key

INTRODUCTION

Citrus are one of the most widely cultivated crops in the world and are economically very important fruit trees in Tunisia. The genus *Citrus* L. is native to China, eastern India, and Southeast Asia; it belongs to the subfamily *Aurantioidea* of the *Rutaceae* family (Webber, 1967). *Citrus* trees are typically grown in tropical and subtropical regions. The differences between the *Citrus* species are both morphological and pomological including variations in size, shape, color, and flavor of the fruit, resulting in disparate classification systems based on these characteristics. For example, taxonomist Tanaka listed 162 species while others have suggested only three ancestral species of *Citrus*, namely, *C. maxima* (L.) Osbeck (pummelo), *C. reticulata* Blanco (mandarin), and *C. medica* L. (citron). All the other species are considered to be introgression of these three ancestral forms (Swingle and Reece, 1967). The genus *Citrus* L. is diverse, and composed of many species that can hybridize easily (e.g., sweet orange is probably an ancient hybrid of mandarin and pummelo). The majority of *Citrus* species are diploid, with nine pairs of chromosomes ($2n = 2x = 18$; Gmitter et al., 2007). While the reproductive system of *Citrus* L. is hallmarked by diversity, the majority of its species reproduce by pollination (Roose et al., 1998). Polyembryony, while common among some genus, is relatively rare among the *Citrus* L. genotypes and is believed to be acutely limited by the introduction of genetic exchange in seedlings. Higher frequencies of polyembryony occur along the lines of maternal genotypes, but self-incompatibility has also played a role in producing a primarily monoembryonic population.

In Tunisia, about 21.000 hectares of land were planted with 6.4 million trees of the different *Citrus* L. varieties in 2010 (GIFruits, 2010) compared to 16.100 hectares in 1999 (Najar et al., 2005), resulting in a total increase of 26%. The region of Cap Bon, which represents 71% of the total area for *Citrus* cultivation, remains the most important citrus region of the country. The main varieties grown in this region are the Maltaise, Acidless, Navel, Valencia Late, Clementine, Lemon, Sour Orange, and Wilking Mandarin (Najar et al., 2005). In the past, several techniques using molecular markers were used to characterize and identify the *Citrus* varieties. Starting with the comparison of isozymes in the 1970s (Torres et al., 1978), researchers adopted restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD) in the 1980s and early 1990s. More recently, amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR and SSR), chloroplast DNA markers (cpSSR), and inter-retrotransposon amplified polymorphisms (IRAP) have been used to identify and categorize the *Citrus* varieties (Fang and Roose, 1997; Bretó et al., 2001; Sankar and Moore, 2001; Barkley et

al., 2006; Deng et al., 2007; Pang et al., 2007; Hvarleva et al., 2008; Jannati et al., 2009). These studies revealed a high allelic diversity and an excess of heterozygotes for some groups of ancestral species, especially in oranges and grapefruit. The diversity of *Citrus* L. genus is credited (in part) to the presence of transposable elements, especially retrotransposons as reported by Bretó et al. (2001). It is important to note that the chloroplast genome of the sweet orange has recently been sequenced completely by Bausher et al. (2006). In recent years, the expressed sequence tags (ESTs) have been identified by several research groups from a number of *Citrus* species (Terol et al., 2007). The relatively small size of the *Citrus* genome has made the preparation of its bacterial artificial chromosome library (BAC) possible, unlike in the case of other species. Several BAC libraries have been developed for *Citrus* varieties and related families (Yang et al., 2001). Little is known about the genetic diversity of the Tunisian *Citrus* germplasm. Exploring the genetic diversity of sweet orange, a fruit tree economically very important in Tunisia, is crucial for the identification and characterization of the local germplasm to circumvent and controlling genetic erosion and control genetic erosion caused by biotic and abiotic stress (monovarietal culture and diseases) and for starting conservation and improvement programs. The present study was conducted with the following objectives: assessment of the molecular polymorphism and characterization of 20 Tunisian sweet orange cultivars of *Citrus sinensis* (L.) Osbeck by using microsatellite markers, (ii) elucidation of genetic relationships among the varieties, (iii) establishment of an identification key useful in *Citrus* breeding programs.

MATERIAL AND METHODS

Twenty cultivars of Tunisian oranges *C. sinensis* (L.) Osbeck, were examined, including eight from the Technical Center of *Citrus* located in Zaouiet djedidi, two varieties collected from the orchards in the Mornag region, eight varieties from the orchards in the Gobba (Cap Bon) region, and two others from the orchards in the Soliman region (Table 1). The varieties consisted of eight blood oranges, eight sweet oranges, two navel oranges, and two Valencia oranges.

Table 1. List of the studied orange cultivars (*Citrus sinensis* (L.) Osbeck).

Cultivar name	Code	Locality	Group	Species (Tanaka name)
Chime 1	CH1	Soliman	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Chem 2	CH2	Soliman	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Meski Arbi 1	MSA1	Mornag	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Meski Arbi 2	MSA2	Mornag	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise Douce 1	MD1	Gobba	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise Douce 2	MD2	Gobba	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Meski Ansli 1	MAN1	Gobba	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Meski Ansli 2	MAN2	Gobba	Sweet orange	<i>Citrus sinensis</i> (L.) Osbeck
Sakasli Arbi 1	SKA1	Gobba	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Sakasli Arbi 2	SKA2	Gobba	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Moro Arbi 1	MOR1	Gobba	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Moro Arbi 2	MOR2	Gobba	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Washington Navel 1	WN1	Zaouiet jdidi	Navel orange	<i>Citrus sinensis</i> (L.) Osbeck
Washington Navel 2	WN2	Zaouiet jdidi	Navel orange	<i>Citrus sinensis</i> (L.) Osbeck
Valencia Late 1	VL1	Zaouiet jdidi	Valencia orange	<i>Citrus sinensis</i> (L.) Osbeck
Valencia Late 2	VL2	Zaouiet jdidi	Valencia orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise ½ Sanguine 1	MDS1	Zaouiet jdidi	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise ½ Sanguine 2	MDS2	Zaouiet jdidi	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise Barlerin 1	MB1	Zaouiet jdidi	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck
Maltaise Barlerin 2	MB2	Zaouiet jdidi	Blood orange	<i>Citrus sinensis</i> (L.) Osbeck

DNA was extracted from lyophilized young leaves (0.5 g) according to the protocol of Dellaporta et al. (1983) with minor modifications. The quality and integrity of the DNA was tested and it was purified by analytical electrophoresis on a 1% agarose gel. The concentration of the extracted DNA was estimated spectrophotometry (GeneQuant, Pharmacia, France).

PCR assays

For identifying the microsatellite markers, seven pairs of primers described by Kijas et al. (1997) were tested. The characteristics of these primers, namely, TAA15, TAA27, TAA41, CAC23, CAC15, CAC33, and CAC39 are given in Table 2. Polymerase chain reactions (PCRs) were performed in a 25- μ L reaction mixture containing 20-30 ng DNA (approximately 1 μ L), 2 pM each primer, 20 mM dNTPs, 1.25 U Taq DNA polymerase (QBIO gene, France), 2.5 μ L enzyme buffer (10X), 3 mM MgCl₂, and 25 μ L MilliQ water, QSF. The amplifications were performed in a thermocycler (TC-512 Techne resistance, Burlington, NJ, USA) programmed to execute the following steps: initial denaturation for 4 min at 94°C followed by 32 cycles of denaturation for 1 min at 94°C, annealing (specific for each primer) at 55°-60°C for 30 s, and extension at 72°C for 1 min and one cycle at 72°C for 4 min. To increase the resolution of bands in electrophoresis and to accomplish a better separation of alleles, the migration patterns of PCR products were observed on a 10% polyacrylamide gel.

Table 2. Characteristics of the primers tested in *Citrus sinensis* (L.) Osbeck.

Locus code	Repeat motif	Sequence of primers	AT (°C)
TAA15	TAA	F: GAAAGGGTTACTTGACCAGGC R: CTTCCAGCTGCACAAGC	55
TAA27	TAA	F: GGATGAAAAATGCTCAAAATG R: TAGTACCCACAGGGAAGAGAGC	60
TAA41	TAA	F: AATGCTGAAGATAATCCGCG R: TGCCTTGCTCTCCACTCC	55
CAC23	CAC	F: ATCACAATTACTAGCAGCGCC R: TTGCCATTGTAGCATGTTGG	55
CAC15	CAC	F: TAAATCTCCACTCTGCAAAGC R: GATAGGAAGCGTCGTAGACCC	55
CAC33	CAC	F: GGTGATGCTGCTACTGATGC R: CAATTGTGAATTGTGATTCCG	60
CAC39	CAC	F: AGAAGCCATCTTCTGTCTGC R: AATTCAGTCCCATTCCATCC	55

F = forward primer; R = reverse primer; AT = annealing temperature.

Data analysis

The average number of alleles per locus, allele frequencies, observed heterozygosity, expected heterozygosity, and fixation index (Weir and Cockerham, 1984) were calculated by GenAlEx software version 6.51 (Peakall and Smouse, 2006). The polymorphic information content (PIC) was estimated using the PowerMarker V3.25 software (Liu and Muse, 2005) using the following formula established by Botstein et al. (1980):

$$(PIC)=1-\sum f_i^2 \quad (\text{Equation 1})$$

where f_i^2 was the frequency of the i^{th} allele.

To represent the differences among the cultivars and to construct a phylogenetic tree, the simple matching distances were calculated using the equation:

$$d_{ij} = 1 - 1/L \sum_{l=1}^L m_l / \pi \quad (\text{Equation 2})$$

where d_{ij} was the dissimilarity between units i and j , L was the number of loci, π was the ploidy, and m_l was the number of matching alleles for locus l . Unweighted neighbor-joining phylogenetic tree was constructed (Saitou and Nei, 1987) with 1000 bootstrap replicates from the genetic distance matrix generated using DARwin software version 5.0.158 (Perrier and Jacquemoud-Collet, 2006). The factorial correspondence analysis (FCA) was applied to the data matrix form of the allele frequencies using the GENETIX software version 4.04 (Belkhir, 2001).

RESULTS

Polymorphism analysis

The SSR primers tested in the Tunisian cultivars of *Citrus sinensis* (L.) Osbeck revealed a total of 37 alleles and 44 genotypes. The size of amplified fragments ranged from 90 bp for the locus TAA41 to 280 bp for the locus CAC23 (Table 3). The number of alleles ranged from four alleles for the loci CAC15 and CAC33 to seven for the locus TAA41, with an average of 5.28. The allelic frequencies ranged from 0.025 for the allele 141 of locus TAA15, alleles 90 and 185 of locus TAA41, alleles 237 and 270 of locus CAC23, and allele 200 of locus CAC39 to 0.475 for the allele 280 of locus CAC23 (Table 3). Additionally, we identified specific alleles in the Tunisian cultivars, namely, allele 237 of locus CAC23, alleles 174 and 190 of locus CAC15, and the allele 200 of locus CAC39 that characterized the accession ‘Maltaise Douce’ (MD) 2. The observed heterozygosity ranged from 0 for the locus CAC15 to 1 for the locus CAC23. The locus CAC23 appeared to contribute most to the genetic diversity and had the highest number of heterozygotes observed for all the studied cultivars. The expected heterozygosity ranged from 0.660 for the locus CAC23 to 0.799 for TAA41 (Table 3). The average expected heterozygosity for all the loci (0.707) was higher than the average observed heterozygosity for the seven loci (0.671). This heterozygous deficiency could be a consequence of the breeding practices that employ the selection of the best horticultural characters and their maintenance by vegetative propagation. The fixation index (Fis) ranged from -0.515 for the locus CAC23 to 1 for CAC15, with an average of 0.051 for all the studied microsatellite loci. A significant heterozygote deficiency was registered for the TAA41, CAC33, and CAC15 loci. This heterozygous deficiency could be explained by the regime of reproduction by vegetative propagation. It is important to note that an excess of heterozygotes was determined for the TAA15, TAA27, and CAC23 loci. The PIC ranged from 0.599 for the locus CAC33 to 0.769 for TAA41, with an average of 0.657, indicating a high level of polymorphism for all the loci. This result showed that the locus TAA41 contributed most to the polymorphism observed for all the cultivars studied.

Table 3. Diversity statistics for seven SSR markers studied in 20 Tunisian cultivars of *Citrus sinensis* (L.) Osbeck.

Locus	Hexp	Hobs	PIC	Number of alleles	Allele size	Allelic frequencies	Number of genotypes	Fis	P value
TAA15	0.738	0.95	0.697	6	141-204	0.025-0.375	8	-0.288	0.003*
TAA27	0.691	0.8	0.645	6	175-230	0.05-0.45	7	-0.157	0*
TAA41	0.799	0.55	0.769	7	90-185	0.025-0.275	9	0.311	0.006*
CAC23	0.660	1	0.602	5	237-280	0.025-0.475	4	-0.515	0.015*
CAC15	0.675	0	0.612	4	148-190	0.05-0.4	4	1	0*
CAC33	0.664	0.45	0.599	4	132-168	0.05-0.425	5	0.322	0.014*
CAC39	0.724	0.95	0.678	5	160-200	0.025-0.4	7	-0.313	0.069 ^{ns}
Total	-	-	-	37	-	-	44	-	0.036*
Mean	0.707	0.671	0.657	5.285	-	-	-	0.051	

Hobs = observed heterozygosity; Hexp = expected heterozygosity; PIC = polymorphic information content; Fis = fixation index. *Values were different from 0 at a high significant level ($P < 0.001$); ns = not significant.

Genetic diversity and its structure

Genetic relationships

To evaluate the relationship among the cultivars of orange, a neighbor-joining phylogenetic tree based on the simple matching distance was constructed (Figure 1). The genetic distances ranged from 0.142 to 0.928, with an average of 0.574. A low genetic distance was recorded between the two trees of cultivar 'Chemi' (CH), which explained the high genetic proximity between them. The highest genetic distance was recorded between the two cultivars 'Meski Ansli' (MAN) 2 and 'Washington Navel' (WN) 2, which explained their genetic divergence. The topology of the tree highlighted the formation of three major clusters. The first cluster (I), supported by bootstrap values ranging from 59 to 64%, was formed by nine cultivars including five from the region of Soliman and Gobba; the rest of the cultivars were from the locality of Zaouiet djedidi. The second cluster (II), which was supported by bootstraps values ranging from 50 to 56%, included 10 cultivars, of which six cultivars were from the Mornag and Gobba localities and four cultivars were from Zaouiet djedidi. The third cluster (III) was formed only by the cultivar 'Sakasli Arbi' (SKA) 1 from the regions of Gobba. The topology of dendrogram showed that the cluster I was subdivided into two subclusters. The second sub-cluster (I-2) was formed by two the varieties WN1 and WN2. The first subcluster (I-1) was composed of the cultivars CH1, CH2, MD1, MD2, 'Maltaise Barlerin' (MB) 1, MB2, and SKA2. Similarly, the cluster II was subdivided into two sub-clusters; the first, labeled, II-1, included the cultivars 'Valencia Late' (VL) 1, 'Moro Arbi' (MOR) 2, 'Valencia Late' (VL) 2, MOR1, and 'Maltaise ½ Sanguine' (MDS) 2; the second labeled (II-2) was formed by the cultivars MAN2, MAN1, MDS1, MSA1, and MSA2. The NJ tree underlined the presence of a low variability in the DNA of the orange genotypes. This diversity occurred independently of the geographical origin of the varieties.

Multivariate analysis

Factorial correspondence analysis was applied on the microsatellite data obtained from the 20 tested cultivars (Figure 2). The three-dimensional scatter plot of the FCA coordinates for the first, second, and the third factors explained 20.57, 13.68, and 10.27% of the diversity, respectively, with 44.52% of the total inertia, demonstrating a good structuring of the genetic diversity. The projection of individuals on the three-dimensional plot of the FCA indicated the genetic approximation of the cultivars MAN2, MAN1, MDS1, MDS2, MSA1, and MSA2. It

also revealed a detachment from the other cultivars and on the basis of markers 141, 151, and 175 bp of the locus TAA15, 197 and 230 bp of the locus TAA27, 100, 110, and 122 bp of the locus TAA41, 190 bp of the locus CAC39, 247 bp of the locus CAC23, 152 bp of the locus CAC33, and 174 bp marker of the locus CAC15. The dispersion of the cultivars in the FCA plan underscored the approximation of the cultivars VL1, MOR2, VL2, MOR1, CH1, CH2, MD1, MD2, MB1, MB2, SKA1, and SKA2 and their discrimination based on the markers 162, 190, and 204 bp of the locus TAA15, 90, 155, and 168 bp of the locus TAA41, 175, 185, 210, and 220 bp of the locus TAA27, 132, 142, and 168 bp of the locus CAC33, 148 and 159 bp of the locus CAC15, 160, 180, and 200 bp of the locus CAC39, and 280 bp of the locus CAC23. On the other hand, a genetic detachment of both the feet of cultivar WN from the other cultivars was detected on the basis of markers 190 and 237 bp of the CAC15 and CAC23 loci, respectively. These results demonstrated a low inter-varietal genetic diversity level with the exception of two trees for the cultivar WN that differentiated it from the other cultivars based on the specific alleles, 190 and 237 bp.

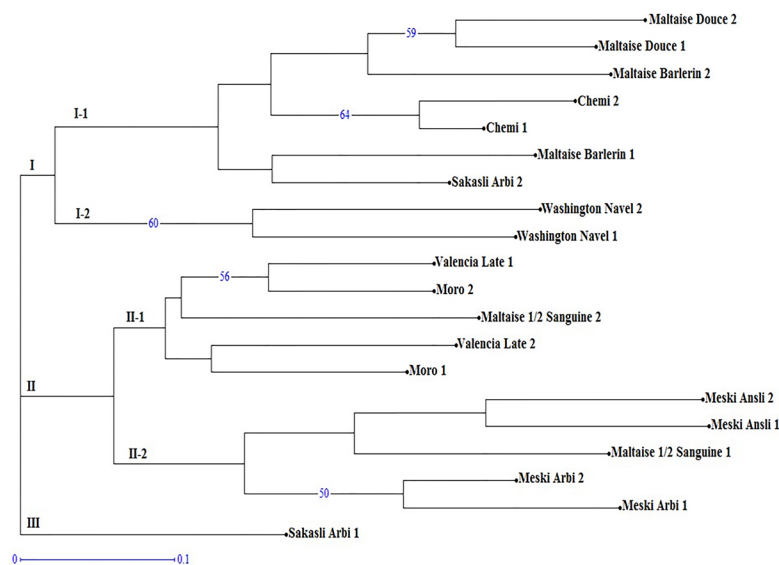


Figure 1. Neighbor-joining tree based on the simple matching dissimilarity matrix of seven SSR markers from 20 orange cultivars. The numbers on the tips indicate 1000 bootstrap values expressed as percentages and are shown for all the clusters when $\geq 50\%$.

Identification key

The use of the detected genotypes led to the establishment of an identification key for the 20 Tunisian cultivars of orange on the basis of the 37 alleles and the 44 genotypes revealed in the present study (Table 4). The key, thus constructed, showed that the 44 genotypes obtained, distinguished all the studied cultivars, which gave a discriminating power of 100% (Figure 3). With the combinations of different genotypes, it could theoretically be possible to obtain $8 \times 7 \times 9 \times 4 \times 4 \times 5 \times 7$ ($= 282240$) multi-locus genotypes of the Tunisian orange cultivars described in this work. The locus TAA41 generated three genotypes, while TAA15 gave 13 genotypes, TAA27, two genotypes, and CAC39 two other genotypes. Four SSR markers were sufficient to establish the

identification key: TAA41, TAA15, TAA27, and CAC39. Furthermore, these four markers could theoretically include $3 \times 13 \times 2 \times 2 (= 156)$ possible genotypes, suggesting that the most polymorphic marker, TAA15, may differentiate 13 genotypes in the 20 orange cultivars initially tested (65%).

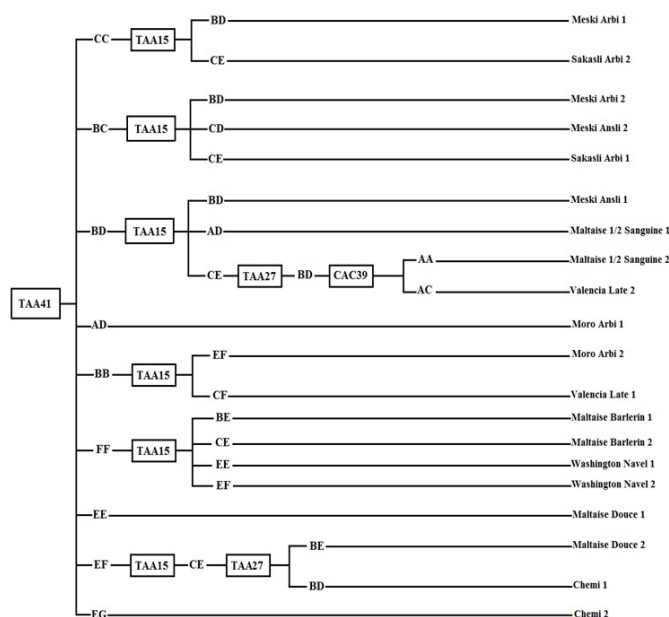


Figure 2. Identification key of the 20 Tunisian oranges [*Citrus sinensis* (L.) Osbeck] studied, based on the 7 microsatellite loci.

Table 4. SSR genotypes revealed for 20 Tunisian oranges (*Citrus sinensis* (L.) Osbeck).

Locus	TAA15	TAA27	TAA41	CAC23	CAC15	CAC33	CAC39
Number of genotypes	(N = 8)	(N = 7)	(N = 9)	(N = 4)	(N = 4)	(N = 5)	(N = 7)
Number of alleles Cultivar	6	7	7	5	4	4	5
Meski Arbi 1	BD	CD	CC	BE	BB	BB	CD
Meski Arbi 2	BD	BD	BC	BE	BB	BD	CD
Meski Ansl 1	B	CF	BD	BE	CC	BD	CD
Meski Ansl 2	CD	CF	BC	BE	CC	AC	CD
Maltaise ½ Sanguine1	AD	BD	BD	BE	CC	AC	AC
Maltaise ½ Sanguine2	CE	BD	BD	BE	CC	BD	AA
Moro Arbi 1	CE	BD	AD	BE	BB	BB	AC
Moro Arbi 2	EF	BD	BB	BE	BB	BD	AC
Valencia Late 1	CF	BD	BB	BE	AA	BD	AC
Valencia Late 2	CE	BD	BD	BD	AA	BB	AC
Sakasli Arbi 1	CE	BD	BC	CE	AA	BB	AD
Sakasli Arbi 2	CE	BD	CC	CE	AA	AA	CD
Maltaise Barlerin 1	BE	DD	FF	CE	AA	AA	CD
Maltaise Barlerin 2	CE	AA	FF	CE	AA	AD	BC
Maltaise Douce 1	CE	BE	EE	CE	AA	AA	BC
Maltaise Douce 2	CE	BE	EF	CE	AA	AD	BE
Chemi 1	CE	BD	EF	CE	BB	AA	BC
Chemi 2	CE	AD	EG	CE	BB	AA	BC
Washington Navel 1	EE	DD	FF	CE	DD	BB	AC
Washington Navel 2	EF	DD	FF	AE	BB	BB	AB

Alleles: TAA15: A: 141, B: 151, C: 162, D: 175, E: 190, F: 204; TAA27: A: 175, B: 185, C: 197, D: 210, E: 220, F: 230; TAA41: A: 90, B: 100, C: 110, D: 122, E: 155, F: 168, G: 185; CAC23: A: 237, B: 247, C: 260, D: 270, E: 280; CAC15: A: 148, B: 159, C: 174, D: 190; CAC33: A: 132, B: 142, C: 152, D: 168; CAC39: A: 160, B: 170, C: 180, D: 190, E: 200.

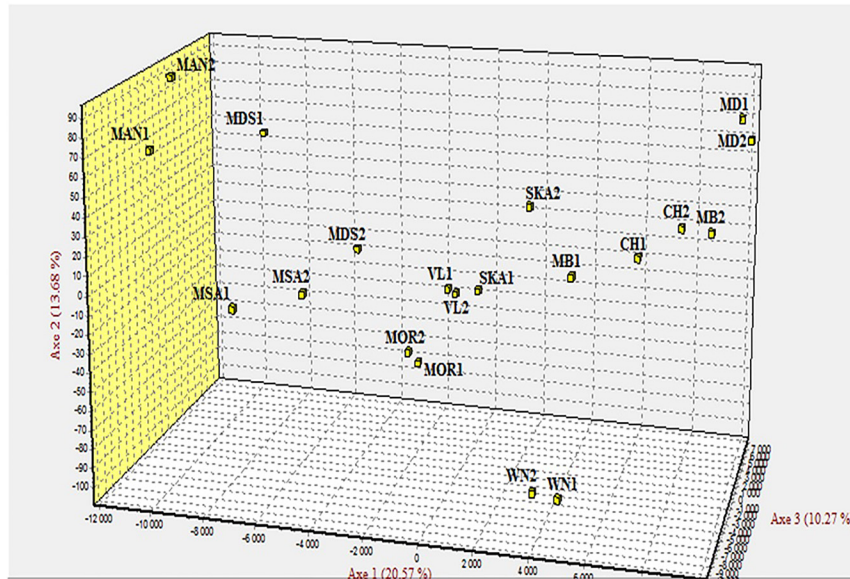


Figure 3. Dispersion of the 20 Tunisian oranges [*Citrus sinensis* (L.) Osbeck] on the three-dimensional plot of the factorial correspondence analysis (FCA;44.52% of total inertia).

DISCUSSION

The results obtained in the present study demonstrate that the microsatellite markers used to assess the genetic diversity of *C. sinensis* growing in Tunisia were polymorphic and informative allowing the identification of 37 alleles and 44 profiles. The determination of allele frequencies of each locus revealed different distributions of these frequencies for the 20 studied orange cultivars. Using the same microsatellite loci for 23 Iranian cultivars of *Citrus*, Jannati et al. (2009) generated 49 alleles. Furthermore, the revelation of the loci TAA15, TAA41, and CAC23 in the varieties of sweet orange and mandarin detected 21 alleles (Golein et al., 2005). The use of seven loci for 370 accessions of *Citrus* L. genus generated 86 alleles (Barkley et al., 2006) confirming the utility and power of microsatellite markers in both the analysis of genetic diversity and in phylogenetic studies. It is also important to note that the three loci, TAA15, TAA27, and CAC23 considered in this study were amplified in the Egyptian *Citrus* accessions belonging to eight different species with a high level of polymorphism (Hussein et al., 2003). In addition, the genetic diversity observed in this study was similar to that obtained by Hvarleva et al. (2008) in cultivated local *Citrus* in Cyprus. Indeed, the use of four loci SSR in 51 accessions of *Citrus* gave a total of 18 alleles with an average of 4.5 alleles per locus. Heterozygous deficiency was estimated for all the 20 cultivars studied (Hobs = 0.671; Hexp = 0.707; Fis = 0.051), which was consistent with the results previously reported by Hvarleva et al. (2008). This result could be explained by the modality of reproduction by vegetative propagation (Bretó et al., 2001). Moreover, the PIC values obtained in this study were considerably higher than those reported by Jannati et al. (2009) using SSR markers. All these results confirm the effectiveness of microsatellite markers in the exploration of the genetic diversity of local germplasm.

Globally, the varieties considered in this study showed a low level of polymorphism reflecting a low genetic diversity at the inter-varietal level and consequently a narrow genetic base for the Tunisian germplasm of *C. sinensis*. A similar result was obtained by Golein et al. (2005) using SSR markers of sweet orange and mandarin cultivars. This could be explained by the appearance of somatic mutations (Barkley et al., 2006) or by natural hybridization frequently observed for *Citrus* L. genus, as suggested by Ferrante et al. (2010). Moreover, several studies have shown that sweet orange varieties are monophyletic, resulting from a single ancestor through mutation and selection of desirable clones (Fang and Roose, 1997). The low genetic diversity among the orange cultivars suggests that the observed morphological polymorphism might be associated with somatic mutations, which were confirmed by the molecular markers (Novelli et al., 2006). In the present, it was possible to differentiate the two trees of Washington navel cultivar, WN1 and WN2 by SSR specific alleles. These results confirm the effectiveness of the SSR markers in genotyping and the identification of cultivars, as was done previously on the date palm (Zehdi et al., 2012), apricot (Krichen et al., 2006), fig (Saddoud et al., 2007; Chatti et al., 2010), olive (Taamalli et al., 2008), and almond (Gouta et al., 2012) by SSR markers.

In conclusion, our results clearly confirmed the usefulness of microsatellite markers in detecting genetic diversity and their power in the identification and characterization of *C. sinensis* trees. To the best of our knowledge, these results provide the first identification key for *C. sinensis* genotypes which offers an interesting tool for breeders to better manage the pre-breeding materials and the choice of parents for further crossing experiments. It should also resolve the confusion in the identification of cultivars, clones, and variants in the *Citrus* collections, and assist in the management of *Citrus* core collections in Tunisia.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

- Barkley NA, Roose ML, Krueger RR and Federici CT (2006). Assessing genetic diversity and population structure in a *citrus* germplasm collection utilizing simple sequence repeat markers (SSRs). *Theor. Appl. Genet.* 112: 1519-1531. <http://dx.doi.org/10.1007/s00122-006-0255-9>
- Bausher MG, Singh ND, Lee SB, Jansen RK, et al. (2006). The complete chloroplast genome sequence of *Citrus sinensis* (L.) Osbeck var 'Ridge Pineapple': organization and phylogenetic relationships to other angiosperms. *BMC Plant Biol.* 6: 21-32. <http://dx.doi.org/10.1186/1471-2229-6-21>
- Belkhir K (2001). Genetix version 4.0, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UPR 9060, Université de Montpellier II, Montpellier.
- Botstein D, White RL, Skolnick M and Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32: 314-331.
- Bretó MP, Ruiz C, Pina JA and Asíns MJ (2001). The diversification of *Citrus clementina* Hort. ex Tan., a vegetatively propagated crop species. *Mol. Phylogenet. Evol.* 21: 285-293. <http://dx.doi.org/10.1006/mpev.2001.1008>

- Chatti K, Baraket G, Ben Abdelkrim A, Saddoud O, et al. (2010). Development of molecular tools for characterization and genetic diversity analysis in Tunisian fig (*Ficus carica*) cultivars. *Biochem. Genet.* 48: 789-806. <http://dx.doi.org/10.1007/s10528-010-9360-1>
- Dellaporta SL, Wood J and Hicks JB (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Report.* 1: 19-21. <http://dx.doi.org/10.1007/BF02712670>
- Deng Z, Malfa SL, Xie Y, Xiong X, et al. (2007). Identification and evaluation of chloroplast uni- and trinucleotide sequence repeats in *Citrus*. *Sci. Hortic. (Amsterdam)* 111: 186-192. <http://dx.doi.org/10.1016/j.scienta.2006.10.004>
- Fang DQ and Roose ML (1997). Identification of closely related *Citrus* cultivars with inter-simple sequence repeat markers. *Theor. Appl. Genet.* 95: 408-417. <http://dx.doi.org/10.1007/s001220050577>
- Ferrante SP, Lucretti S, Reale S, De patrizio A, et al. (2010). Assessment of the origin of new *Citrus* tetraploid hybrids (2n = 4x) by means of SSR markers and PCR based dosage effects. *Euphytica* 173: 223-233. <http://dx.doi.org/10.1007/s10681-009-0093-3>
- GIFruits (2010). Données sur le secteur des arbres fruitiers en Tunisie. Available at [www.gifruit.nat.tn].
- Gmitter FG, Jr., Chen C, Rao MN and Soneji JR (2007). *Citrus* Fruits. In: Genome mapping and molecular breeding in plants. Vol. 4, Fruits and nuts (Kole C, eds.). Springer-Verlag, Heidelberg, 265-279.
- Golein B, Talaie A, Zamani Z, Ebadi A, et al. (2005). Assessment of genetic variability in some Iranian sweet oranges (*Citrus sinensis* [L.] Osbeck) and mandarins (*Citrus reticulata* Blanco) using SSR Markers. *Int. J. Agric. Biol.* 2: 167-170.
- Gouta H, Ksia E, Buhner-Zaharieva T, Mliki A, et al. (2012). Development of an SSR-based identification key for Tunisian local almonds. *Sci. Agric.* 69: 108-113.
- Hussein EHA, Abd-alla SMM, Awad NA and Hussein MS (2003). Genetic analysis in some *Citrus* accessions using microsatellites - and AFLP-based markers. *Arab J. Biotechnol.* 6: 203-222.
- Hvarleva T, Kapari-Isaia T, Papayiannis L, Atanassov A, et al. (2008). Characterization of *Citrus* cultivars and clones in Cyprus through microsatellite and RAPD analysis. *Biotechnol. Biotechnol. Equip.* 22: 787-794. <http://dx.doi.org/10.1080/13102818.2008.10817554>
- Jannati M, Fotouhi R, Pourjan Abad A and Salehi Z (2009). Genetic diversity analysis of Iranian citrus varieties using microsatellite (SSR) based markers. *J. Hortic. For* 1: 120-125.
- Kijas JMH, Thomas MR, Fowler JCS and Roose ML (1997). Integration of trinucleotide microsatellites into a linkage map of *Citrus*. *Theor. Appl. Genet.* 94: 701-706. <http://dx.doi.org/10.1007/s001220050468>
- Krichen L, Mnejja M, Arus P, Marrakchi M, et al. (2006). Use of microsatellite polymorphisms to develop an identification key for Tunisian apricots. *Genet. Resour. Crop Evol.* 53: 1699-1706. <http://dx.doi.org/10.1007/s10722-005-1220-1>
- Liu K and Muse SV (2005). PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* 21: 2128-2129. <http://dx.doi.org/10.1093/bioinformatics/bti282>
- Najar A, Duran-Vila N, Khlij A and Bové JM (2005). Virus and virus-like diseases of *Citrus* in Tunisia. *Sixteenth IOCV Conf. Short Commun.* 484-486.
- Novelli VM, Cristofani M, Souza AA and Machado MA (2006). Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* (L.) Osbeck). *Genet. Mol. Biol.* 29: 90-96. <http://dx.doi.org/10.1590/S1415-47572006000100018>
- Pang XM, Hu CG and Deng XX (2007). Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. *Genet. Resour. Crop Evol.* 54: 429-436. <http://dx.doi.org/10.1007/s10722-006-0005-5>
- Peakall R and Smouse PE (2006). GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* 6: 288-295. <http://dx.doi.org/10.1111/j.1471-8286.2005.01155.x>
- Perrier X and Jacquemoud-Collet JP (2006). DARwin software. <http://darwin.cirad.fr/darwin>. Accessed on Monday, June 1, 2014.
- Roose ML, Schwarzacher T and Heslop-Harrison JS (1998). The chromosomes of *Citrus* and *Poncirus* species and hybrids: identification of characteristic chromosomes and physical mapping of rDNA loci using *in situ* hybridization and fluorochrome banding. *J. Hered.* 89: 83-86. <http://dx.doi.org/10.1093/jhered/89.1.83>
- Saddoud O, Chatti K, Salhi-Hannachi A, Mars M, et al. (2007). Genetic diversity of Tunisian figs (*Ficus carica* L.) as revealed by nuclear microsatellites. *Hereditas* 144: 149-157. <http://dx.doi.org/10.1111/j.2007.0018-0661.01967.x>
- Saitou N and Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
- Sankar AA and Moore GA (2001). Evaluation of inter-simple sequence repeat analysis for mapping in *Citrus* and extension of the genetic linkage map. *Theor. Appl. Genet.* 102: 206-214. <http://dx.doi.org/10.1007/s001220051637>
- Swingle WT and Reece PC (1967). The botany of *Citrus* and its wild relatives. In: *The Citrus industry Vol 1* (Reuther W, Webber HJ, Batchelor LD, eds.). University of California Press, Berkeley, 190-430.

- Taamalli W, Guena F, Bassi D, Daoud D, et al. (2008). SSR marker based DNA fingerprinting of Tunisian olive (*Olea europaea* L.) varieties. *J. Agron.* 7: 176-181. <http://dx.doi.org/10.3923/ja.2008.176.181>
- Terol J, Conesa A, Colmenero JM, Cercos M, et al. (2007). Analysis of 13000 unique Citrus clusters associated with fruit quality, production and salinity tolerance. *BMC Genomics* 8: 31. <http://dx.doi.org/10.1186/1471-2164-8-31>
- Torres AM, Soost RK and Diederhufen U (1978). Leaf isozymes as genetic markers in *Citrus*. *Am. J. Bot.* 65: 869-881. <http://dx.doi.org/10.2307/2442183>
- Webber HJ (1967). History and development of the *Citrus* industry. In: The *Citrus* industry Vol 1 (Reuther W, Webber HJ, Batchelor LD, eds.). University of California Press, Berkley, 1-39.
- Weir BS and Cockerham C (1984). Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370. <http://dx.doi.org/10.2307/2408641>
- Yang ZN, Ye XR, Choi S, Molina J, et al. (2001). Construction of a 1.2-Mb contig including the citrus tristeza virus resistance gene locus using a bacterial artificial chromosome library of *Poncirus trifoliata* (L.) Raf. *Genome* 44: 382-393. <http://dx.doi.org/10.1139/gen-44-3-382>
- Zehdi S, Cherif E, Rhouma S, Santoni S, et al. (2012). Molecular polymorphism and genetic relationships in date palm (*Phoenix dactylifera* L.): The utility of nuclear microsatellite markers. *Sci. Hortic. (Amsterdam)* 148: 255-263. <http://dx.doi.org/10.1016/j.scienta.2012.10.011>