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Fingerprinting of On-Farm Conserved Local Tunisian Orange Cultivars (*Citrus sinensis* (L.) Osbeck) Using Microsatellite Markers

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This study aims to illustrate the diversity among local orange genotypes. Collecting missions targeting the traditional culture areas were organized by the National Gene Bank. Twenty-five cultivars have been screened, some of them have not yet been included in established orange collections. A powerful set of seven SSR Primer pairs was used to indentify forty-four alleles and forty-four genotypes. Polymorphic information content varies from 0.55 to 0.78 with an average of 0.66. Some mislabeling cases such as synonymy and homonymy have been clarified and genetic relationships among cultivars based on genetic distances have been revealed showing two major clusters. Finally, on the basis of multilocus genotyping, an identification key was established to unambiguously distinguish 23 well-defined genotypes (resolving a power of 92%).

Keywords: SSR, *Citrus sinensis*, genotyping, diversity, genetic relationships

INTRODUCTION

Citrus genus belongs to *Rutaceae* family which includes around 160 genera and 1800 species widely distributed in the tropics (Pfeil and Cris, 2008). Its distribution started in Southeast Asia and spread globally through ancient sea routes at least 4000 years ago (Gmitter and Hu, 1990). However, despite the popularity of some *Citrus* cultivars, their phylogenetic relations are still controversial (Penjor et al., 2013). Indeed, two different taxonomic approaches are adopted for *Citrus* species. Swingle and Reece (1967) recognized 16 species while Tanaka (1977) divided *Citrus* genus into 162 species. Recently, the availability of nuclear and chloroplast reference genomes of *Citrus* species (Carbonell-Caballero et al., 2015) helped to better elucidate their phylogenetic relationships. Three true species are considered: *Citrus medica* L.(citrons), *Citrus maxima* (Burm.) Merr. (pummelos) and *Citrus reticulata* Blanco (mandarins) (Barret and Rhodes, 1976). Some studies confirmed the three taxa hypothesis (Carbonell-Caballero et al., 2015), while other researchers consider *Citrus micrantha* Wester to be the fourth ancestral taxon (Ollitrault et al., 2013). *Citrus* genus includes economically important species such as *Citrus reticulata* Blanco (tangerine and mandarin), *Citrus limon* L. (lemon), *Citrus grandis* Osbeck (pummelo) and *Citrus paradisi* Macfadyen (grapefruit). *Citrus sinensis* Osbeck L. (sweet orange) represents the majority

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of *Citrus* production and it is considered one of the most important cultivated fruit trees worldwide with 66.8 million tons produced in 2016 (FAOSTAT, 2017). Citrus fruits were introduced to Tunisia probably when Andalusians arrived in the 10th century and citrus industry and trade began in the 20th century when a great diversification of the crop took place with a diverse collection of cultivars (Hannachi et al., 2014). The 'Cap-Bon' region is a peninsula holding the greatest diversity in orange cultivars combining adaptation to local conditions and high organoleptic fruit quality (Ellouze et al., 2011). Unfortunately, some local orange cultivars have been abandoned over the last years. Indeed, agricultural fields are resorbed in favor of urban areas as traditional family-farms are generally small and low profitable. Moreover, introduced cultivars substitute some well-adapted local ones and the threat of viral diseases and abiotic stress in the Mediterranean area is raising. Furthermore, mislabeling problems are still occurring since fruit traits are the only basis for cultivar denomination. All those factors may generate genetic erosion of some local cultivars. Thus, a strategy of evaluation and characterization of the local orange germplasm becomes necessary and the use of powerful methods to fingerprint the different genotypes becomes an imperative for better valorization of the local plant material. Previous studies reported morphometric characters of Tunisian *Citrus sinensis* germplasm (Saddoud et al., 2013) and a recent survey highlighted the potential of the sweet orange Tunisian cultivars when analyzing the individual and total carotenoid contents as well as color attributes (Ben Abdelaali et al., 2018). However, horticultural traits are highly influenced by environmental conditions. Molecular markers, being stable and independent from the environment or cultural practices, are an option to overcome this problem. AFLP (Amplified Fragment Length Polymorphism) markers have already been used for Tunisian oranges (Saddoud et al., 2014). Recently, the microsatellite markers (SSR) were also used to characterize some local cultivars, nonetheless, these studies present some limitations regarding the origin of the cultivars involved and/or the choice of molecular markers. Indeed, the microsatellite markers are considered markers of choice for evaluating the evolutionary and genealogical relationships among plant germplasm; they permit diversified studies, QTL mapping, molecular breeding and comparative mapping (Liu et al., 2013). Their desirable genetic features as their co-dominance inheritance, wide distribution in the genome, high polymorphism, reproducibility, multi-allelic nature and chromosome-specific location made them noticeably important (Biswas et al., 2011). Considering the desirable attributes of the microsatellite markers, we investigated 25 cultivars to elucidate the genetic diversity structure among the Tunisian orange germplasm using microsatellite markers in order to resolve the mislabeling problems, the confusion occurring in plant nurseries and the disagreements connected with the fact that the same cultivars are known under different names (synonymy), whereas different cultivars are known under the same name and their phenotypic diversity is underestimated visually (homonymy).

MATERIAL AND METHODS

PLANT MATERIAL

Twenty-two Tunisian sweet orange cultivars were studied (Table 1). Washington navel, Moro and Double Fine Améliorée oranges were also included in this study for comparative purposes. These cultivars are grown in Cap-Bon (North-East of Tunisia) and exhibit not only large pomological diversity (Saddoud et al., 2013) but also interesting variability in the contents of health promoting compounds (Ben Abdelaali et al., 2018).

DNA ISOLATION

DNA was extracted from young leaves according to Saghai-Maroof et al. (1984) protocol with some modifications. The quality and integrity of the DNA were verified using electrophoresis on 1% agarose gel and the concentration of the extracted DNA was estimated by spectrophotometry (Sambrook et al., 1989).

PRIMERS AND PCR ASSAYS

Seven microsatellite primers previously isolated by Ahmad et al. (2003) and identified as CMS4, CMS14, CMS19, CMS20, CMS23, CMS30 and CMS47 were used (Table 2). Every forward primer was 5'-tailed with an M13 fluorescence-labeled universal primer (Boutin-Ganache et al., 2001). PCR amplifications were performed in 50 µL reaction mixture containing 50–150 ng DNA (aproximately 2 µL), 8 pM reverse primer, 2 pM forward-tailed primer, 0.2 mM dNTPs, 1 U Taq DNA polymerase (QBIO gene, France), 10 µL enzyme buffer, 1.5 mM $MgCl_2$ and 29.5 µL MilliQ water. The amplifications were performed in a thermocycler (C1000, Bio Rad, California, USA) programmed as follows: initial denaturation at 94°C for 5' followed by 30 cycles, the first series of denaturation at 94°C for 30 s followed by an annealing series at 52–56°C, depending on the primers combination, to finish by an extension series at 72°C for 45 s. Finally, an

TABLE 1. Denominations and geographical origins of *Citrus sinensis* cultivars selected for this study.

No.	Cultivar	Code	Collection site	Grin Global Code
1	Bourouhin	BRH	Gobba Kbira	NBGTUN784ARB
2	Washington Navel*	WNN	Gobba Kbira	NBGTUN773ARB
3	Meski Malti	MEM	Bouargoub	NBGTUN785ARB
4	Meski Ahmer	MEH	Bouargoub	NBGTUN782ARB
5	Meski Ansli	MES	Khlidia	NBGTUN761ARB
6	Meski Arbi	MEA	Khlidia	NBGTUN751ARB
7	Meski Boujnab	MBM	Bouargoub	NBGTUN748ARB
8	Meski Sifi	MEI	Gobba Kbira	NBGTUN753ARB
9	Malti Trabelsi	MTR	Gobba Kbira	NBGTUN991ARB
10	Malti Twil	MTW	Gobba Kbira	NBGTUN807ARB
11	Malti Abiadh	MSW	Gobba Kbira	NBGTUN833ARB
12	Malti Boujnab	MBB	Morneg	NBGTUN813ARB
13	Malti Mdawer	MDW	Menzel Bouzalfa	NBGTUN835ARB
14	Ballerin	BAL	Gobba Kbira	NBGTUN774ARB
15	Beldi Abiadh	BAB	Bouargoub	NBGTUN989ARB
16	Maltaise Petit-Pierre	MPP	Gobba Kbira	NBGTUN799ARB
17	Double fine améliorée*	DFM	Takelsa	NBGTUN755ARB
18	Maltaise demi-sanguine	MDM	Gobba Kbira	NBGTUN778ARB
19	Malti Lsen Asfour	MLS	Hammamet	NBGTUN988ARB
20	Malti Ahmer	MSS	Gobba Kbira	NBGTUN775ARB
21	Chemi	CHE	Gobba Kbira	NBGTUN780ARB
22	Moro*	MRO	Bouargoub	NBGTUN792ARB
23	Boukhobza	BKH	Gobba Kbira	NGBTUN771ARB
24	Beldi Ahmer	BAH	Bouargoub	NGBTUN990ARB
25	Sakasli	SAK	Gobba Kbira	NGBTUN749ARB

* Introduced varieties (for comparison).

extension step of 10' at 72°C was carried out. PCR products were separated in an automatic capillary sequencer ABI Prism 3130 DNA sequencer (Applied Biosystems, Hitachi, Tokyo, Japan). While doing the PCR, negative and positive controls were used in order to check the absence of contamination and the right and reproducible amplification, respectively. Positive controls (Moro, Double fine améliorée and Washington Navel) had a known genotype (previously identified). For each SSR amplified, we checked whether we obtained the same genotypes for the positive controls and so we confirmed the reproducibility of the results.

DATA ANALYSIS

Data analysis was carried out using GENETIX 4.05.2 and GENEALEX 6.1 software to evaluate the polymorphism level, allelic diversity (number of alleles, mean number of alleles per locus),

TABLE 2. Characteristics of primers tested on the selected sweet orange cultivars.

Locus code	Repeat Motif	Primer sequence	AT(°C)
CMS4	$(CT)_{11}(AT)_6$ $(CA)_6$	F: CCTCAAACCTTCCAATCC R:CTGTAAAGTACATGCATGTTGG	52
CMS14	$(CA)_{14}$ $(GA)_9$	F: FGGCTTCTCTTCTACTAGAACGG R: FGGCTTCTCTTCTACTAGAACGG	52
CMS19	$(TCA)_{11}$ $(TC)_{14}$	F: GGCTTTTGCCCAATGATG R: GTTGACCTAAAAGGGGGGAG	52
CMS20	$(CA)_{13}$ $(AT)_5$	F: CTATGTGACAGCACTGATGG R: TTTCCTATCTCTCTTGAGACAT	52
CMS23	$(CA)_{12}$	F: TTTCCTATCTCTCTTGAGACAT R: AACACCCCTTGCAGGGAG	56
CMS30	$(CT)_9$ $(CA)_9$	F: GGTGTTCACACACACAACCC R: GGATCCTCCACCATCTCGTA	52
CMS47	$(CTT)_{14}$	F: GTGGAGAGGGAGAGGAG R: CGAGATGCCAATTCAAATCA	52

F – forward primer, R – reverse primer, AT – annealing temperature

allelic frequencies and observed (H_o) and expected (H_e) heterozygosity (NEI, 1978). The genetic relationships among the studied cultivars and the cophenetic correlations were analyzed by NTSYS-PC software version 2.02 Rholf (1998) using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973).

RESULTS

POLYMORPHISM ANALYSIS AND GENETIC DIVERSITY ESTIMATION

The SSR primers tested on twenty-five Tunisian sweet orange cultivars exhibited more than 6 alleles per locus, ranging from 4 (CMS14) to 10 (CMS23) alleles that sized from 98 bp (CMS23) to 244 bp (CMS20). All of the used SSR markers were polymorphic and amplified a total of 44 alleles. As shown in Table 3, allelic frequencies varied from 0.02 for CMS 4, CMS19 and CMS23 to 0.56 for CMS14. Polymorphic information content (PIC) exhibited the average value equal to 0.66 varying from 0.55 for CMS14 to 0.78 for CMS23. The expected heterozygosity ranged between 0.55 (CMS14) and 0.78 (CMS23) with an average of 0.66 and the observed heterozygosity from 0.44 (CMS19) to 1.00 (CMS47) with an average of 0.78. The fixation index (F_{is}) varied from -0.59 to 0.43 for the loci CMS47 and CMS19, respectively. Indeed, the majority of the tested loci allowed to see a high level of heterozygocity, except for CMS19 locus.

Fifteen specific alleles detected in only one cultivar in the population were identified in some local cultivars. CMS23 and CMS19 markers had the highest number of specific alleles and CMS23 locus permitted to record the highest number of allele-specific cultivars (Table 4). 'Baldi abiadh' (BAB) showed 2 specific alleles with CMS23 and CMS20 while 'Baldi Ahmer' (BAH) exhibited 3 specific alleles with CMS14, CMS19 and CMS23 loci. Additionally, 'Meski Malti' (MEM) cultivar showed two specific alleles for CMS4 and CMS19. Other cultivars like 'Maltaise sanguine', (MSS), 'Washington Navel' (WNN), 'Malti Lsen Asfour' (MLS), 'Ballerin' (BAL), 'Malti Boujnab' (MBB), 'Meski Ansli' (MES) and 'Boukhobza' (BKH) presented one specific allele.

GENETIC RELATIONSHIPS

Data of SSR markers generated from twenty-five orange cultivars with seven primers were used to evaluate similarity. The highest similarity (1) was observed between 'Meski Arbi' (MEA) and 'Meski Sifi' (MEI). The two pairs 'Malti Ahmer' (MSS) – 'Chemi' (CHE) and 'Beldi Abiadh' (BAB) – 'Malti Ahmer' (MSS) were genetically most diverse having the same similarity value of 0.25 (Table 5). The average similarity recorded across all genotypes was 0.62. The cophenetic coefficient (r) obtained from the SSR analysis was estimated as 0.812, which is considered a good fit for UPGMA clustering. The dendrogram showed separation of the studied cultivars into two main clusters A and B

Table 3. Summary of the genetic parameters revealed by used primers for the Tunisian *Citrus sinensis* germplasm.

Locus	Alleles number	Alleles size	Alleles frequencies	Genotypes number	PIC	H_{exp}	H_{obs}	Fis
CMS4	5	161–185	0.02–0.43	4	0.64	0.64	0.86	-0.32
CMS14	4	134–228	0.04–0.56	4	0.55	0.55	0.72	-0.28
CMS19	9	131–182	0.02–0.42	8	0.75	0.75	0.44	0.43
CMS20	6	118–244	0.04–0.48	7	0.69	0.68	0.88	-0.25
CMS23	10	98–156	0.02–0.34	11	0.78	0.78	0.76	0.05
CMS30	5	141–171	0.04–0.50	5	0.61	0.6	0.84	-0.38
CMS47	5	162–175	0.04–0.44	5	0.62	0.62	1	-0.59
Total	44			44				
Mean	6.29	–		–	0.66	0.66	0.78	-0.16

H_{obs} – observed heterozygosity, H_{exp} – expected heterozygosity, PIC – polymorphic information content, Fis – fixation index.

Table 4. Specific alleles found in some Tunisian *Citrus sinensis* cultivars.

Locus	Cultivars with specific alleles
CMS4	Meski Malti (1), Maltaise Sanguine (1)
CMS14	Malti Lsen Asfou (1), Baldi Ahmer (1)
CMS19	Meski Malti (1), Baldi Ahmer (1), Boukhobza (2)
CMS20	Chemi (1), Baldi Abiadh (1)
CMS23	Baldi Ahmer (1), Baldi Abiadh (1), Ballerin (1), Malti Boujnab (1)
CMS30	Meski Ansli (1)

( ) Number of specific allele.

with a similarity value of 0.47 and two major clades (I, II) were formed in cluster B (Fig. 1).

'Baldi Abiadh' (BAB) and 'Malti Mdawer' (MDW) formed cluster A diverging from all other cultivars at a similarity coefficient of 0.57. Within cluster B, 'Chemi' cultivar (CHE) individually formed a distinct clade (I) separated from the rest of cultivars at a distance of 0.53. 'Baldi Ahmer' (BAH) was grouped with 'Boukhobza' (BKH) and 'Malti Abiadh Boujnab' (MBB) and separated from other genotypes with a similarity value of 0.54. 'Bourouhin' (BRH) and 'Meski Ahmer' (MEH) joined other cultivars at 0.79 and were grouped at a similarity level of 0.78, despite their morphologic and biochemical differences. BRH was characterized by large navel fruits (Saddoud et al., 2013), while MEH had a discriminating pinkish hue. Three Malti cultivars ('Meski Malti' (MEM), 'Malti Ahmer' (MSS) and 'Malti Lsen Asfour' (MLS)) were separated individually, each of them at respective similarity values of 0.6; 0.63 and 0.66. The most widely distributed in Tunisia Malti cultivar ('Maltaise demi-sanguine' (MDM)) formed a sub-group with 'Malti Abiadh' (MSW) showing a Dice coefficient equal to 0.72 and merging with a similarity value of 0.71. 'Malti Trabelsi' (MTR) and 'Double fine améliorée' (DFM) were very close presenting a Dice coefficient equal to 0.96, while 'Maltaise Petit-Pierre' (MPP) joined this sub-group at a similarity coefficient close to 0.87. The last group contains nine cultivars. 'Washington Navel' was separated individually from the rest of the cultivars. Four Meski (low-acid) cultivars ('Meski Ansli' (MES), 'Meski Arbi' (MEA), 'Meski Sifi' (MEI) and 'Meski Boujnab' (MBM) exhibited a high level of similarity, particularly MEA and MEI, which were considered probably identical with 100% of similarity.

TABLE 5. DICE similarity coefficient matrix for the studied orange cultivars.

	BRH	**WNN**	**MEM**	**MES**	**MEH**	**MEA**	**MEI**	**MBM**	**MSW**	**BAB**	**MBB**	**MDW**	**MPP**	**MTW**	**MTR**	**BAL**	**DFM**	**MDM**	**MSS**	**MRO**	**MLS**	**SAK**	**BKH**	**BAH**	**CHE**
BRH	0																								
WNN	0.61	0																							
MEM	0.45	0.58	0																						
MES	0.72	0.74	0.60	0																					
MEH	0.78	0.64	0.38	0.50	0																				
MEA	0.80	0.81	0.69	0.92	0.58	0																			
MEI	0.80	0.81	0.69	0.92	0.58	**1**	0																		
MBM	0.72	0.81	0.60	0.84	0.58	0.92	0.92	0																	
MSW	0.75	0.61	0.54	0.72	0.52	0.80	0.80	0.80	0																
BAB	0.52	0.40	0.38	0.58	0.27	0.58	0.58	0.50	0.43	0															
MBB	0.52	0.48	0.57	0.50	0.45	0.58	0.58	0.50	0.52	0.36	0														
MDW	0.36	0.50	0.60	0.60	0.28	0.60	0.60	0.60	0.45	0.57	0.38	0													
MPP	0.66	0.53	0.54	0.64	0.43	0.72	0.72	0.64	0.66	0.52	0.78	0.36	0												
MTW	0.69	0.78	0.66	0.81	0.56	0.88	0.88	0.88	0.69	0.56	0.64	0.58	0.76	0											
MTR	0.64	0.66	0.69	0.76	0.41	0.84	0.84	0.76	0.72	0.5	0.66	0.52	0.88	0.88	0										
BAL	0.69	0.71	0.58	0.74	0.56	0.81	0.81	0.81	0.69	0.56	0.64	0.50	0.76	0.92	0.81	0									
DFM	0.58	0.61	0.63	0.72	0.34	0.8	0.80	0.80	0.75	0.43	0.60	0.34	0.83	0.84	0.96	0.76	0								
MDM	0.56	0.66	0.32	0.61	0.41	0.69	0.69	0.61	0.72	0.41	0.58	0.34	0.72	0.74	0.76	0.74	0.72	0							
MSS	0.48	0.74	0.43	0.53	0.58	0.61	0.61	0.69	0.48	**0.25**	0.41	0.43	0.48	0.74	0.61	0.66	0.64	0.61	0						
MRO	0.64	0.74	0.69	0.76	0.50	0.84	0.84	0.84	0.64	0.58	0.5	0.60	0.64	0.88	0.76	0.81	0.72	0.61	0.61	0					
MLS	0.48	0.59	0.43	0.61	0.33	0.69	0.69	0.76	0.56	0.41	0.41	0.43	0.56	0.81	0.69	0.74	0.72	0.53	0.61	0.69	0				
SAK	0.69	0.78	0.66	0.81	0.56	0.88	0.88	0.88	0.69	0.56	0.56	0.58	0.69	0.92	0.81	0.85	0.76	0.66	0.66	0.96	0.74	0			
BKH	0.48	0.44	0.52	0.46	0.41	0.53	0.53	0.46	0.48	0.33	0.66	0.43	0.64	0.59	0.61	0.59	0.56	0.61	0.38	0.46	0.38	0.51	0		
BAH	0.30	0.38	0.36	0.48	0.26	0.56	0.56	0.56	0.58	0.34	0.60	0.27	0.60	0.61	0.64	0.61	0.66	0.56	0.40	0.48	0.56	0.53	0.48	0	
CHE	0.52	0.40	0.47	0.50	0.27	0.58	0.58	0.50	0.52	0.54	0.45	0.28	0.69	0.56	0.66	0.56	0.60	0.50	**0.25**	0.66	0.50	0.64	0.50	0.43	0

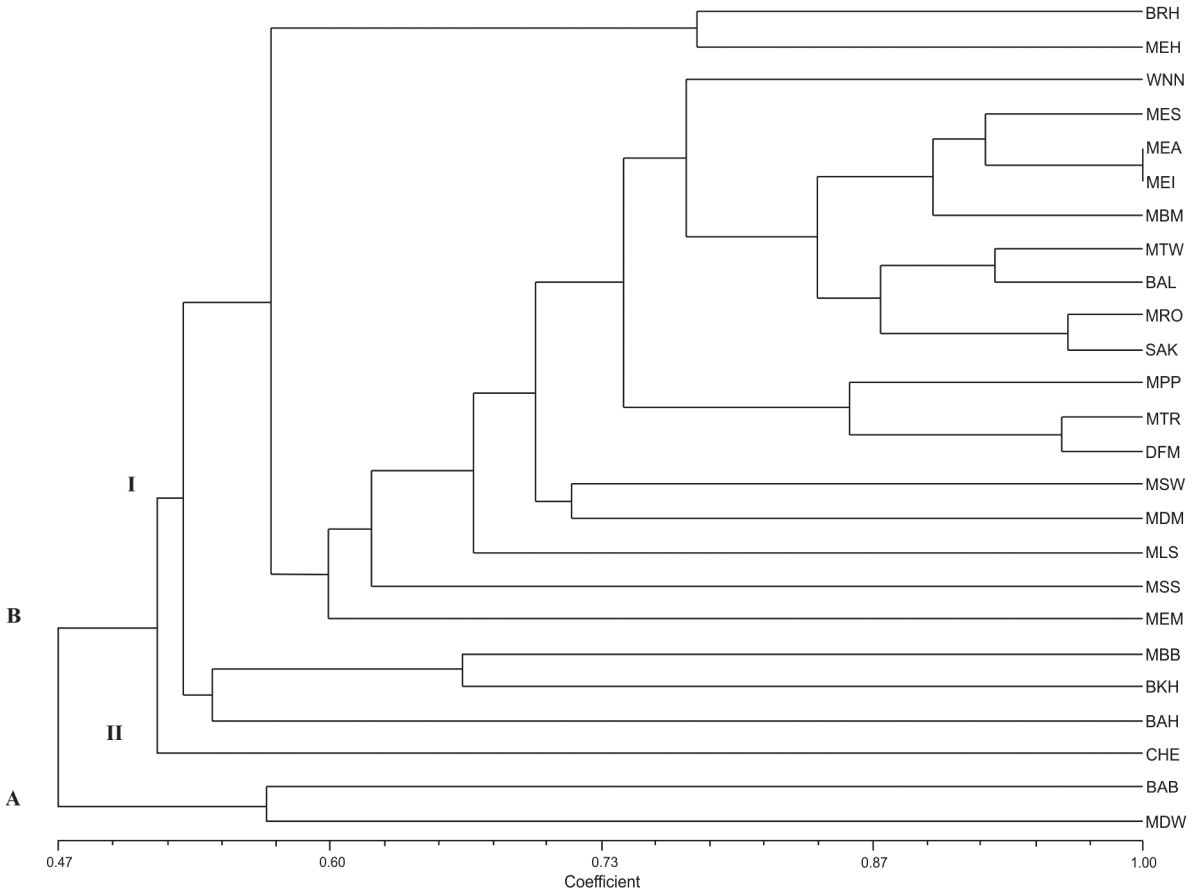

Fig 1. UPGMA dendrogram of orange cultivars based on Dice similarity coefficient (1945). Matrix correlation: $r = 0.81261$ (= normalized Mantel statistic Z). Approximate Mantel t-test: $t = 5.313$. Prob. random Z < obs. Z: $P = 1.0000$.

IDENTIFICATION KEY

An identification key was established on the basis of the obtained multilocus genotypes (Fig. 2). It is based on the classification of the loci in a descending order according to their numbers of alleles. The cultivars are subsequently ordered by grouping together those that have the same fingerprint until the allelic combination specific to each cultivar is obtained. The 7 loci were used to establish this key (Fig. 2). The multilocus genotypes of the different cultivars are shown in Table 6. The alleles were labeled from A to G from CMS23 to CMS14, respectively, and each letter is indexed 1 to n depending on the number of alleles. The key exhibited a power of 92% permitting to differentiate 23 cultivars out of 25. Indeed, only two Meski cultivars ('Meski Arbi' (MEA) and 'Meski Sifi' (MEI)) were characterized by identical multilocus fingerprints. Eight genotypes could be identified on the basis of unique or specific alleles with CMS23 locus. Except for MEI and MEA cultivars, the remaining ones could also be differentiated from one another by the rest of the markers.

DISCUSSION

The results of the tested SSR primers on twenty-five Tunisian sweet orange cultivars reveal the efficiency of SSR markers to distinguish the citrus genotypes at an intra-species level. Generally, all PIC values were higher than 0.5, which indicates the strength of the used markers. Similar PIC values were recorded for Iranian acid limes (0.766 and 0.72 for CMS19 and CMS4) (Sharafi et al., 2016). Meanwhile, Singh et al. (2016) obtained lower values for exotic mandarin genotypes (0.56 and 0.22 for CMS30 and CMS47, respectively).

The observed heterozygosity excess was mentioned in previous works for locus CMS4

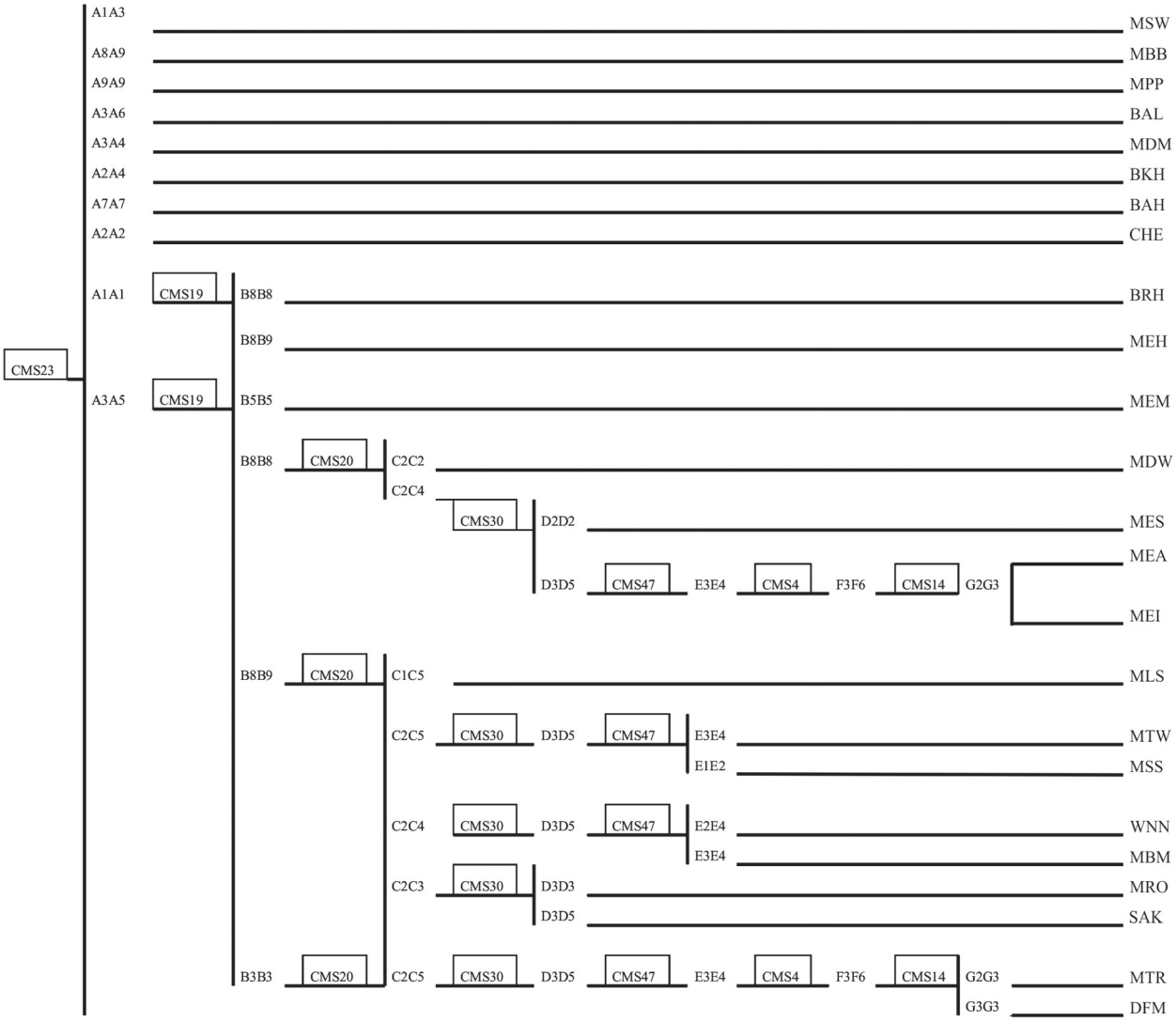

Fig 2. Identification key of 25 cultivars of *Citrus sinensis* representing Tunisian sweet orange germplasm, based on multilocus genotypes.

and CMS23 (Shahsavar et al., 2007) and the observed heterozygosity deficiency for locus CMS19 was reported by Sharafi et al. (2016). This deficiency might be caused by co-dominant nature of microsatellites. The high average of the observed heterozygosity indicates the importance of diversity among Tunisian orange cultivars. High averages were reported in previous works on *Citrus sinensis* when analyzed with microsatellites (Snoussi et al., 2012). Barrett and Rhodes (1976) reported that the important heterezygosity in *Citrus* may be due to diverse phylogenetic origin of the cultivated species derived from the citron (*Citrus medica* L.), mandarin (*Citrus reticulata* Blanco) and pumello (*Citrus grandis* L. Osbeck). In fact, recent studies focused on a draft genome of sweet orange supported the previous observations of the high heterozygous diploid sweet orange genome. Based on this character, authors confirmed the most accepted hypothesis that *Citrus sinensis* may be the result of an interspecific hybridization with pummelo (*Citrus grandis*) and mandarin (*Citrus reticulata*) (Xu et al., 2013).

Both local 'Baldi' cultivars showed more that 2 specific alleles, however, those two cultivars are considered by farmers commercially unattractive

Table 6. SSR genotypes revealed for Tunisian orange cultivars.

Locus	CMS4	CMS14	CMS19	CMS20	CMS23	CMS30	CMS47
Number of genotypes	4	4	8	7	11	5	5
Number of alleles	5	4	9	6	10	5	5
BRH	F2F5	G2G3	B8B8	C2C4	A1A1	D3D5	E3E5
WNN*	F2F5	G2G3	B8B9	C2C4	A3A5	D3D5	E2E4
MEM	F4F4	G2G3	B5B5	C2C2	A3A5	D3D3	E3E4
MEH	F2F5	G2G3	B8B9	C2C4	A1A1	D3D5	E1E5
MES	F2F5	G2G3	B8B8	C2C4	A3A5	D2D2	E3E4
MEA	F2F5	G2G3	B8B8	C2C4	A3A5	D3D5	E3E4
MBM	F2F5	G3G3	B8B9	C2C4	A3A5	D3D5	E3E4
MEI	F2F5	G2G3	B8B8	C2C4	A3A5	D3D5	E3E4
MTR	F2F5	G2G3	B3B3	C2C5	A3A5	D3D5	E3E4
MTW	F2F5	G2G3	B8B9	C2C5	A3A5	D3D5	E3E4
MSW	F2F5	G3G3	B7B7	C2C4	A1A3	D3D5	E3E4
MBB	F2F5	G2G3	B2B2	C2C5	A8A9	D3D5	E3E4
MDW	F1F1	G3G3	B8B8	C2C2	A3A5	D1D4	E3E4
BAL	F2F5	G2G3	B8B9	C2C5	A3A6	D3D5	E3E4
BAB	F2F5	G2G3	B8B8	C6C6	A10A10	D1D4	E3E4
MPP	F2F5	G2G3	B3B3	C2C5	A9A9	D3D5	E3E4
DFM*	F2F5	G3G3	B3B3	C2C5	A3A5	D3D5	E3E4
MDM	F2F5	G2G3	B7B7	C2C5	A3A4	D3D5	E2E4
MLS	F2F5	G1G1	B8B9	C1C5	A3A5	D3D5	E3E4
MSS	F3F5	G2G3	B8B9	C2C5	A3A5	D3D5	E1E2
CHE	F2F5	G2G3	B3B3	C1C3	A2A2	D3D3	E3E4
MRO*	F2F5	G2G3	B8B9	C2C3	A3A5	D3D3	E3E4
BKH	F1F1	G2G3	B1B4	C2C5	A2A4	D3D5	E3E4
BAH	F2F5	G4G4	B2B6	C2C5	A7A7	D3D5	E3E4
SAK	F2F5	G2G3	B8B9	C2C3	A3A5	D3D5	E3E4

* Introduced varieties (for comparison)
Alleles: CMS4: F1: 161, F2: 170, F3: 172, F4: 177, F5: 185. CMS14: G1: 134, G2: 152, G3: 164, G4: 288. CMS19: B1: 131, B2: 137, B3: 143, B4: 158, B5: 162, B6: 168, B7: 170, B8: 172, B9: 182. CMS20: C1: 118, C2: 128, C3: 148, C4: 159, C5: 162, C6: 224. CMS 23: A1: 98, A2: 106, A3: 109, A4: 113, A5: 117, A6: 131, A7: 142, A8: 150, A9: 154, A10: 156. CMS30: D1: 141, D2: 146, D3: 154, D4: 163, D5: 171. CMS 47: E1: 162, E2: 167, E3: 171, E4: 173, E5: 175.

due to their high acidity rates and they are rarely found in Tunisian orchards. Additionally, 'Meski Malti' (MEM) cultivar showed also two specific alleles and it also exhibited specific alleles in a previous study (Mhajbi et al., 2016). Barkley et al. (2006) suggested that accessions displaying unique alleles may represent wild germplasm, wild derivatives or hybrid accessions. It is worth noticing that microsatellite markers that amplified unique alleles in specific genotypes could be employed

in *Citrus* improvement as well as the selection of parental *Citrus* cultivars used in breeding programs (Ghanbari et al., 2009; Jannati et al., 2009; Zerihun et al., 2009).

A hypothesis of synonyms could be forwarded to explain the results obtained for 'Meski Sifi' (MEI) and 'Meski Arbi' (MEA) cultivars. However, the latter oranges may be different cultivars representing a very high level of similarity detectable after a larger and higher discriminative markers use. Sakasli and Boukhobza cultivars were believed to be two denomination growers attributed to the same cultivars, nevertheless, a polymorphism level exists within those two genetically different cultivars. Homonymous confusions are a result of the traditional nomenclature system which is mainly based on morphological characters as it is the case for 'Malti Lsen Asfour' (MLS) (bird tongue) referring to its juice vesicles. Indeed, this orange was believed to be the same as Maltaise demi-sanguine cultivar, however, our results show that they are genetically different and they share only 7 alleles from a total of 18. Those results are in agreement with the findings of Mahjbi et al. (2016) where two Sakasli and two 'Maltaise demi-sanguine' cultivars where found to be genetically different while analyzed by SSR markers. With the use of this set of markers, we could identify cases of synonymy and homonymy or misidentification. The detection of these 'onyms' will not only help to determine the true extent of genetic diversity in Tunisian germplasm, but could also be useful for more accurate estimation and characterization of cultivars.

The identification key had a power of 92% to precisely distinguish twenty-three cultivars. It is worth mentioning that the scored rate is very similar to the percentage found for other fruit crops as the date palm (Zehdi et al., 2012), apricot (Krichen et al., 2006), fig (Chatti et al., 2008) and almond (Gouta et al., 2012). Thus, it is clear that it is possible to differentiate specifically all cultivars based on their multilocus genotypes and that the different tested loci helped to establish the genetic fingerprints of the studied cultivars.

CONCLUSION

This study evidenced once more the effectiveness of SSR markers in identification of *Citrus sinensis* (L.) Osbeck cultivars and the establishment of a powerful identification key using few high polymorphic markers. Our results will be part of a national database useful for better management of the local citrus germplasm and national agrodiversity protection by establishing a local catalog and creating an established orange collection. Hence, it will be of a great interest not only for the description, registration and certification of the plant material but also for the development of new cultivars via breeding programs exploiting the particular organoleptic quality of Tunisian citrus and their adaptation traits. New markets could be targeted then, that would help to assure the profitability of some neglected local cultivars, hence their active conservation and valorization.

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