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ORIGINAL ARTICLE

Combination of Simple Sequence Repeat, *S*-Locus Polymorphism and Phenotypic Data for Identification of Tunisian Plum Species (*Prunus* spp.)

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Abstract

Plums (*Prunus* spp.) are among the first fruit tree species that attracted human interest. Artificial crosses between wild and domesticated species of plums are still paving the way for creation of new phenotypic variability. In Tunisia, despite a considerable varietal richness of plum as well as a high economic value, the plum sector is experiencing a significant regression. The main reason of this regression is the absence of a national program of plum conservation. Hence, this work was aimed to phenotypically and genetically characterize 23 Tunisian plum accessions to preserve this patrimony. Closely related *Prunus* species from the same subgenus may be differing at two characteristics: ploidy level and phenotypic traits. In this study, single sequence repeat (SSR) markers allowed distinguishing between eighteen diploid accessions and five polyploid accessions, but SSR data alone precluded unambiguous ploidy estimation due to homozygosity. In contrast, *S*-allele markers were useful to identify the ploidy level between polyploid species, but they did not distinguish species with the same ploidy level. Seven out of 12 phenotypic traits were shown to be discriminant traits for plum species identification. Molecular and phenotypic traits were significantly correlated and revealed a powerful tool to draw taxonomic and genotypic keys. The results obtained in this work are of great importance for local Tunisian plum germplasm management.

Keywords Plums · *Prunus* L. · Phenotypic analysis · *S*-Locus · SSR markers

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Abbreviations

DNA	Desoxyribonucleic acid
GSI	Gametophytic self-incompatibility system
PCR	Polymerase chain reaction
SFB	*S*-Haplotype-specific F-box
S-RNase	*S*-Ribonuclease
UPOV	Union internationale de la Protection des Obtentions Variétales

Introduction

The *Prunus* genus, established by Linnaeus (1753), belongs to the subfamily Prunoideae Focke of the Rosaceae family. The most widely adopted classification of *Prunus* is the one of Rehder (1940) that divides the genus into five subgenera based on phenotypic traits: *Amygdalus* L. (almonds and peach), *Cerasus* Miller (sweet and sour cherries), *Laurocerasus* Duhamel (bay-cherries), *Padus* Miller (bird cherries) and *Prunophora* Neck (plums and apricots) (Li et al. 2011; OCDE 2002).

These Eudicots shrubs and trees are characterized by a solitary carpel, a fleshy drupe, five sepals and five petals (Li et al. 2011). In *Prunus*, over 200 species exist, with some of them being especially well known for their cultivated fruits. These have attained a prime position amongst all the temperate fruit crops due to their delicious edible drupes, and many species have an ornamental value as well (Das et al. 2011).

The genus comprises more domesticated temperate fruit species than other genera in the Rosaceae family (e.g. *Malus, Pyrus, Sorbus, Cydonia, Rubus, Fragaria*). In fact, one of the obvious reasons for the abundant domestication of *Prunus* species might have been the coincidence between the location of the *Prunus* variability and the human evolution site and/or of the first ancient high civilizations of human history (OCDE 2002; Li et al. 2011; Zohary et al. 2013).

Within *Prunus* species, plums appear to have been domesticated in the Neolithic (OCDE 2002; Janick 2005; Li et al. 2011; Zohary et al. 2013). In fact, three plum groups are distinguished according to their origins. European plums, generally represented by *Prunus domestica* ($2n=48$), *Prunus cerasifera* ($2n=16$), *Prunus spinosa* L. ($2n=32$) and *Prunus insititia* ($2n=48$) species, are commonly used for processing and fresh market purposes and as plum rootstock (Abdallah et al. 2016). Japanese plums which originated in China and have been cultivated there since ancient times, were introduced into Japan some 200–400 years ago and more recently, they were introduced to California and Italy (OCDE 2002). Japanese plums are the major fresh sweet and juicy plums represented by *Prunus salicina* Lindl. ($2n=16$), and *Prunus simonii* Carr. ($2n=16$) species (Mestre et al. 2017; Reig et al. 2018). Finally, most of the North American plum species are $2n=16$, such as *Prunus americana* Marsh., *Prunus angustifolia* Marsh., *Prunus maritima* Marsh. and *Prunus nigra* Ait. (Chittaranjan and Timothy 2008). Like other *Prunus* species, the basic chromosome number of plum species (x) is 8. However, polyploidy, mediated by interspecific hybridization, took place several times during the evolution of *Prunus* species (García-Verdugo et al. 2013; El Dabbagh 2016).

Traditionally, the characterization of *Prunus* crops has involved phenotypic traits (Zhang 1992; Browicz and Zohary 1996), isozymes (Mowrey and Werner 1990), proteins markers and DNA markers such RAPD, RFLP, AFLP, SSRs, ITS (Arús et al. 1994; Lee and Wen 2001). The availability of new technologies for high-throughput genome and transcriptome analysis, offers new approach to gene labeling and development of molecular markers for marker-assisted selection (MAS) including quantitative (quantitative trait loci, QTL) and qualitative (Mendelian trait loci, MTL) traits (Salazar et al. 2014).

Nowadays, the annual worldwide plum production has attained 11.35 million tons of prune (Salazar et al. 2014). As other fruit crops, artificial crosses between wild and domesticated species of plums, as well as traditional cultivars across the globe, are paving the way for the creation of new phenotypic variability and these constitute raw material for important activities such as passport data generation, conservation (in situ, ex situ, in vitro) in germplasm banks as well as breeding techniques (Das et al. 2011).

In Tunisia, the plum culture can be traced back to ancient times (Nabli 2011). In the Flora of Tunisia (Nabli 2011), it has been noted that most of the Tunisian plum varieties belong to Japanese plum (*P. salicina* and *P. cerasifera* [$2n = 16$]) mostly abundant in northern Tunisia. In addition, two wild plum species (*P. spinosa* and *P. insititia* [$2n = 48$]) have been described in northern and north-western Tunisia (Nabli 2011). Currently, plum ranks fourth among the Tunisian fruit tree species in cultivation. Its production is about 15,500 tons with a yield of 53,650 hg/ha (FAOstat 2016). However, the plum trees area has experienced a notable regression from 4300 ha in 2010 (Abdallah et al. 2016) to 2889 ha in 2016 (FAOstat 2016). Most of this area is used for cultivating introduced varieties, while local varieties are grown only in small orchards. Hence, many Tunisian plum varieties have disappeared especially due to the lack of programs addressed to preserve this local germplasm. Moreover, one of the most important needs for the conservation of Tunisian plum germplasm is the establishment of a taxonomic key to identify the species to which these local varieties belong. For this purpose, this work was aimed to characterize 23 Tunisian plum accessions. Samples of these varieties were collected to identify: (i) the polyploidy level and molecular polymorphism using eight SSR markers and three *S*-alleles introns published in previous studies and (ii) to create a taxonomic key based on distinctive phenotypic traits available in the literature.

Materials and Methods

Plant Material

Twenty-three Tunisian plum samples were used in this study (Table S1). These accessions were collected from local plantations at Bizerte (Northern Tunisia) and at Gabes (Southern Tunisia) (Fig. 1).

Young leaves from each of these accessions were used as the raw material to extract DNA using the cetyl trimethyl ammonium bromide (CTAB) following the

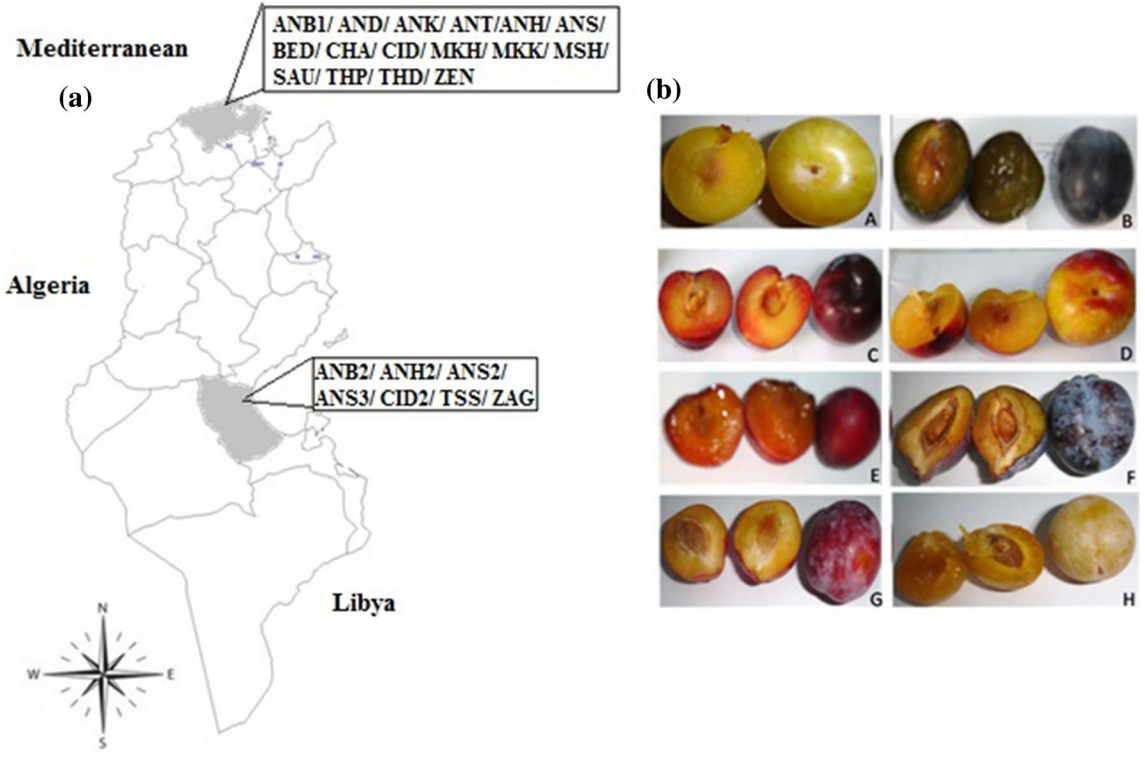

Fig. 1 **a** Map of Tunisia indicate the geographic origin for Tunisian plum accessions, **b** examples of mature fruits' skin and flesh colors of some Tunisian plum varieties. A: TSS. B: CHA. C: CID. D: ANH. E: MSH. F: MKK. G: ZAG. H: ANS

protocol described by Doyle and Doyle (1987). The DNA was quantified using a Nanodrop 1000 and diluted to 10 ng/µL aliquots.

Molecular Markers Amplification and Scoring

SSR Markers Amplification

A set of eight SSR primer pairs was used in this study: BPPCT 007, BPTCT 037 and BPPCT 025 (Dirlewanger et al. 2002), CPSCT 021 (Mnejja et al. 2004), CPDCT 044 (Mnejja et al. 2005), Pchgms-3 (Sosinski et al. 2000), UDP96-001 and UDP96-003 (Cipriani et al. 1999). The forward primers were labelled with 6-FAM fluorescent dye for detection through capillary electrophoresis.

PCR reactions were performed in a final volume of 20 µL containing between 20 and 40 ng of genomic DNA, 2.5 µL of 10×buffer, 4 mM MgCl2, 0.1 mM of each dNTP, 0.2 µM of each primer, and 0.45 units Taq polymerase. Reactions were carried out on a TC 512 thermocycler using the following temperature profile: an initial step of 2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 57 °C and 1 min at 72 °C, and a final step of 5 min at 72 °C (Wünsch et al. 2006).

The amplification products of SSR markers were analyzed by capillary electrophoresis through an ABI 3100 genetic analyser (Applied Biosystems).

S-*Locus* Markers Amplification *S*-Allele amplification was performed using three pairs of consensus primers, namely, PaCons1F/PaCons1R2 (Sonneveld et al. 2005) and PruC2/PCER (Tao et al. 1999; Yamane et al. 2001), specific for the *S*-RNase first and second introns, respectively, and F-Box 50A/F-Box intronR (Vaughan et al. 2006), spanning the SFB gene intron.

PCR reactions were performed in 15 mL total volume reaction mixture containing approximately about 40 ng DNA, 10 µM of each primer, 1.5 µL buffer (10×), 2 mM $MgCl_2$, 0,5 mM of dNTP and 0.45 U of Ex Taq DNA polymerase. The cycling parameters were: an initial denaturation step at 94 °C for 1 min; 35 cycles of 94 °C for 3 min, 56 °C for 1 min, and 72 °C for 3 min, followed by a 7 min final extension at 72 °C.

Amplified PCR products of PaCons1F/PaCons1R2 and F-Box 50A/F-Box intron R, whose sizes are smaller than 500 bp, were analyzed by capillary electrophoresis. The fluorescently labeled products were run in an automated sequencer ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary) using the GENOTYPER 3.7 software. However, the amplification products of PruC2/PCER primers, higher than 500 bp, were electrophoresed through a 2% agarose gel, stained with Cyber Green and visualized over UV light using a 100 bp Plus DNA Ladder as molecular size standard.

Data Analysis

Detected alleles from SSR and *S*-locus genotyping were scored as present (1) or absent (0) matrix.

Based on SSR and *S*-locus profiles defined among the 23 studied plants, the number of alleles per locus (NA), the number of allele per cultivar (Ai), the number of genotypes (Gn), the observed (H_o) and the expected (H_e) heterozygosities were calculated using Arlequin ver. 3.5 (Excoffier et al. 2005). Polymorphism information content (PIC) per each locus and each cultivar was calculated according to the equation described by Botstein and White (1980).

Based on binary matrix, genetic distances were calculated with Maximum Composite Likelihood (Tamura et al. 2011) in Popgene ver. 1.31, then imported to Mega5 to construct UPGMA tree. Numbers on major branches represent bootstrap supports from 2000 replicates.

Evaluation of Phenotypic Parameters

For phenotypic characterization, the Test Guidelines defined by UPOV (2014) for *Prunus* L. species were followed (TG/187 Code UPOV: PRUNU/Geneva–2014).

Twelve phenotypic traits were studied and partitioned in qualitative and quantitative traits (Table 2). Quantitative traits were evaluated in natural conditions including fruit length (cm), shape and color. Measurements were scored for ten fruits of each tree.

For qualitative traits, we used the 'visual' method as described on the UPOV guidelines. This method is based on sensorial observations such as smell, taste and touch. This method was tested by two agronomists who have a large knowledge and expertise with the Tunisian plum varieties characteristics as well as with introduced ones. In addition, three plum samples (Santa Rosa, Angeleno and Black gold), previously characterized by UPOV guidelines, were used as references to compare qualitative traits. These qualitative traits included three parameters: fruit taste classifying each sample as sweet, slightly bitter or bitter), juiciness and acidity (both classified as high, medium or low) (UPOV 2014).

Principal component analysis (PCA) and principal coordinate analyses (PCoA) were conducted using the PAST software (Ver. 3.21) (Hammer et al. 2001).

Results

SSR Marker Analysis

As the ploidy level is crucial to identify Tunisian *Prunus* species, eight SSR loci were screened using primers designed for different *Prunus* species. Ninety-eight alleles were detected with an average of 12.3 alleles by locus (Table 1). The highest number of alleles (16 alleles) was detected in the locus Pchgms-3, whereas the lowest number (9 alleles) was obtained for CPDCT 044, UDP96-003 and BPPCT 025 loci (Table 1). Allele size varied from 87 bp at the locus BPTCT 037–210 bp at the UDP96-001 locus. An average of 16.1 genotypes was discriminated based on SSR fingerprints. The observed heterozygosity (H_o) ranged from 0.55 (BPPCT 025) to 1 (CPSCT 021) and the expected heterozygosity (H_e) ranged from 0.76

Table 1 Diversity statistics for eight nuclear SSR and three *S*-locus markers studied in 23 plum accessions

Locus	SR	NA	NG	H_e	H_o	PIC
SSRs markers						
UDP96-001	177–210	15	14	0.812	0.611	0.800
CPSCT 021	119–153	14	19	0.906	1.000	0.892
Pchgms-3	117–153	16	18	0.842	0.611	0.829
CPDCT 044	113–142	9	13	0.760	0.833	0.748
UDP96-003	152–173	9	15	0.814	0.833	0.801
BPPCT 007	135–157	12	18	0.884	0.888	0.870
BPTCT 037	87–139	14	16	0.788	0.777	0.776
BPPCT 025	118–144	9	16	0.849	0.555	0.836
Average	–	16.12	16.12	0.832	0.763	0.819
S-locus markers						
PruC2-PCER	312–1440	17	14	0.830	1.000	0.817
PaCons1F-PaCons1R2	208–413	15	14	0.811	1.000	0.799
F-Box50A F-Boxintron R	174–196	15	14	0.830	1.000	0.817
Average	–	15.67	14	0.823	1.000	0.811

SR size range of alleles, *NA* number of alleles per locus, *NG* number of genotypes per locus, H_e expected heterozygosity, H_o observed heterozygosity, *PIC* polymorphism information content

(CPDCT 044) to 0.9 (CPSCT 021) (Table 1). The polymorphic information content (PIC) varied from 0.74 (CPDCT 044) to 0.89 (CPSCT 021) (Table 1). The PIC value depicted the discriminatory power of SSR markers. Of the eight primers, the PIC value was higher than 0.5 which indicates a good discriminating ability.

The number of alleles per cultivar (Ai), shown in Table S1, ranged from 73 (BED) to 50 (SNR) with an average of 62 alleles per cultivar. The observed heterozygosity (H_o) varied from 0.99 (CHA) to 0.42 (BLG). This important genetic variability resulted in high PIC values, which ranged from 0.92 (BED) to 0.48 (AGL) giving an average of 0.72 (Table S1).

Among the 98 amplified SSR alleles, 27 alleles were specific to eighteen accessions. These samples amplified 1 or 2 alleles per locus, which may be indicative that they belong to diploid *Prunus* species. On the other hand, 25 SSR alleles were specific to five plum accessions (CHA, MKH, MKK and MSH and ZEN) for which multiallelic profiles were obtained. Pchgms-3 and UDP96-001 loci amplified the highest allele's number (5) in CHA, MKH and ZEN. However, remaining SSR loci amplified up to four alleles per polyploid sample. Such results suggest that these accessions may belong to polyploid species. However, the ploidy level of these accessions cannot be assessed based on SSR data solely since homozygosity may not allow the determination of the copy number of alleles (Halász et al. 2017). The genotypes characterized by five alleles in CHA, MKH and ZEN are likely to be pentaploids or hexaploids, while the genotypes carrying up to four alleles in MKK and MSH may be tetraploid, pentaploid or hexaploid species.

S-Alleles Markers Analysis

Plum is a strictly self-incompatible species, and its sexual incompatibility is governed by the highly polymorphic *S*-locus which contains at least two genes (Abdallah et al. 2016). Thus, PCR analysis using primers from conserved regions of *S*-RNase and SFB genes might be exploited for the estimation of genetic variation.

A total of 47 alleles were identified with an average of 5.66 alleles by primer. The allele number varied between 17 (PruC2-PCER) and 15 (PaCons1F- PaCons1R2 and F-Box50A F-Boxintron R) and the allele size ranged from 174 bp (F-Box50A F-Boxintron R) to 1440 bp (PruC2-PCER). These alleles were classified in 14 genotypes for each primer.

The observed heterozygosity (H_o) showed a high value for the three markers ($H_o = 1$) and expected heterozygosity (H_e) ranged between 0.811 (PaCons1F-PaCons1R2) and 0.830 (PruC2-PCER and F-Box50A F-Boxintron R) (Table 1). The polymorphism information content (PIC) ranged from 0.799 (PaCons1F-PaCons1R2) to 0.817 (PruC2-PCER and F-Box50A F-Boxintron R) with an average of 0.811. Such result highlights the important polymorphism of used *S*-locus primers (Table 1).

As shown in Fig. 2, PCR reactions using PruC2-PCER primer pairs showed two ploidy levels. Eighteen varieties, previously identified as diploid samples by SSR markers, amplified two bands per accession (labeled 1 and 2 and ranging between 350 bp and 1440 bp). On the other hand, the five polyploid samples (CHA, ZEN, MKH, MKK and MSH) showed also polyploid profiles with genotypes carrying four to six bands (labeled from 1 to 6 ranging between 300 bp and 1600 bp) for each variety (Fig. 2).

To confirm this result, a second range of PCR amplifications were conducted using PaCons1F- PaCons1R2 (Sonneveld et al. 2005) and F-Box50A F-Box intron

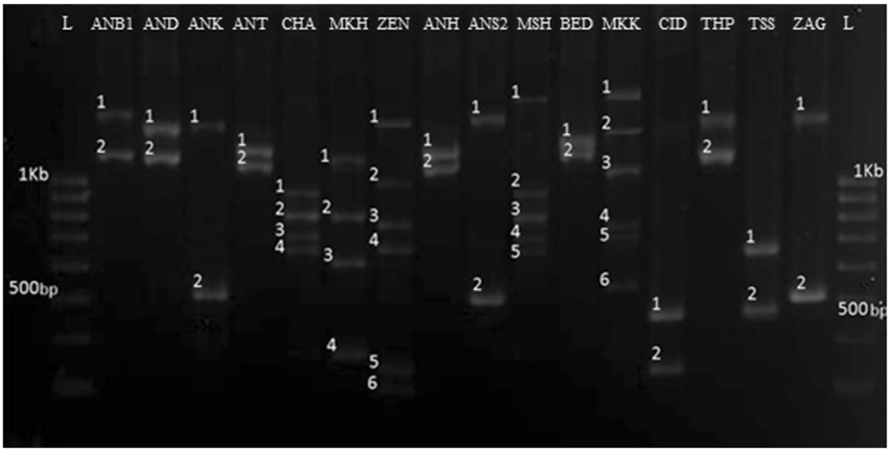

Fig. 2 A 2% agarose gel of PCR amplification using primer set PruC2-PCER of analyzed samples. *L* 100 bp size standard

R (Vaughan et al. 2006) primers and amplification products were analyzed by capillary electrophoresis. Twice the number of alleles was amplified using PaCons1F-PaCons1R2 primers (8 alleles) than F-Box50A F-Boxintron R primer (4 alleles). Results analysis confirmed the diploidy level of the 18 varieties previously mentioned and suggest that these samples belong to *Prunus salicina* $2x=16$ (Darlington and Ammal Janaki1945), *Prunus cerasifera* $2x=16$ (Janick and Moore 1975) or *Prunus simonii* $2x=16$ (Carrière 1872). On the other hand, polyploid profiles were identified for the five other samples. Although the number of amplified bands per sample is different between primers, it exceeds, however, five alleles. This result suggests that these samples carry hexaploid genomes and belong to *Prunus domestica* ($2n=48$) or *Prunus insititia* ($2n=48$).

Analysis of Combined SSR and *S*-locus Markers

Phylogenetic Analysis

Based on genetic distance of combined data (SSR and *S*-locus), the UPGMA dendrogram shows two main groups (Fig. 3). The first one (I) clustered diploid accessions and the second group (II) gathered the five hexaploid accessions. These results highlight the effectiveness and usefulness of SSR and *S*-locus markers to discriminate plum accessions with different ploidy levels.

Tree analysis shows that plum cultivars are clustered independently from their geographic origin (North and South of Tunisia). Moreover, accessions having the same nomenclature such 'Aouina Hamra 1-2', 'Aouina Safra 1-2-3' and 'Ain Bagra 1-2', do not cluster together, highlighting an important intra-varietal polymorphism.

Principal Coordinate Analysis (PCoA)

PCoA was conducted and showed the same topology than the dendrogram which revealed clear differentiation between diploid and hexaploid accessions for both SSRs and *S*-locus markers. For SSR markers, 38.4% of variability is represented in the first two axes (Fig. 4a). For *S*-locus markers, 55.12% of variability is represented in the first two axes. The discrimination between putative diploid and hexaploid accessions was also clear. However, some putative diploid samples were represented superposed (Fig. 4b). This result may suggest that these samples share the same *S*-alleles and/or the same *S*-genotypes.

Phenotypic Characterization

Quantitative Traits

Fruit shape has been considered as a discriminant trait between *Prunus* species (UPOV 2014). Based on the rate *R* (length/width) and the position of the large part of each fruit, different fruit shapes could be differentiated in plums. Hence, the analyzed samples showed medium to large round-shaped fruits, since their diameters

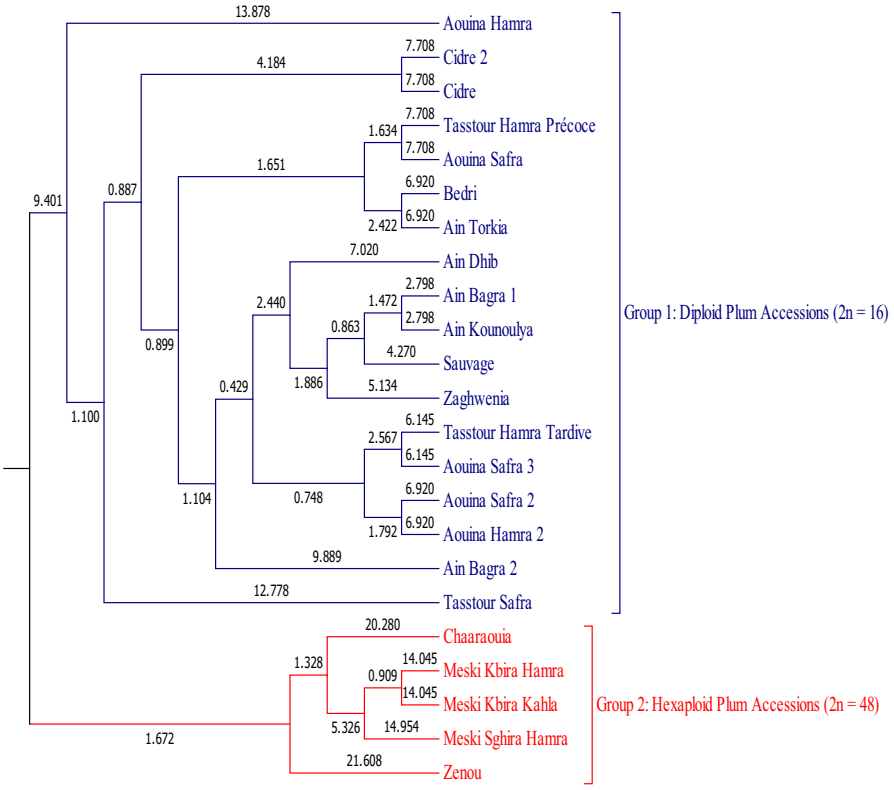

Fig. 3 UPGMA consensus trees of 23 accessions of Tunisian plum based on combined data (SSR and S-locus markers)

ranged from 6.5 to 10.5 cm. In addition, these accessions showed fruits whose colors varied from yellow to reddish (Table 2).

Putative polyploid accessions showed ovate or obovate fruit shapes. Additionally, these fruits showed smaller sizes than putative diploid plums with colors varying from green to purple (Table 2). Five parameters were not discriminant and showed the same result for almost all samples, namely, drooping trees, brown to reddish brown shoots, elliptic leaf blade, medium flower size and circular stones.

Qualitative Traits

Three plum samples (Santa Rosa, Angeleno and Black gold), previously characterized by UPOV technical guidelines, were used as references to compare qualitative traits.

Putative diploid accessions shared a combination of traits (Table 2). Most of these studied accessions have moderately to strongly sweet flavor, with a low to medium acidity and a strong juiciness. These traits were tested at the full ripening stage of each variety.

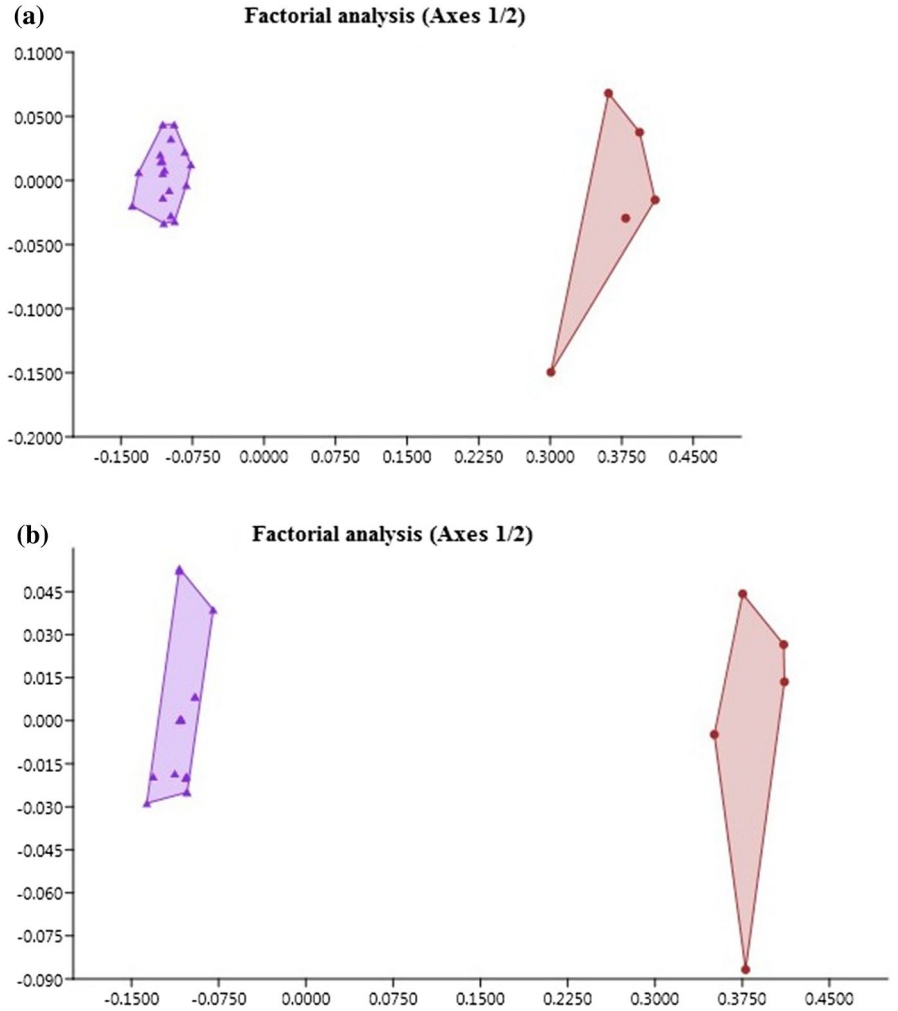

Fig. 4 Organization of Tunisian plum genetic diversity; principal coordinates analysis using: **a** SSR markers data, **b** S-locus markers data. Red dots represent the five putative polyploid plums and purple triangles represent the eighteen putative diploid plums

Putative polyploid accessions were bitter or slightly bitter and had higher acidity and lower juiciness than putative diploid accessions (Table 2).

Principal Component Analysis (PCA)

The PCA was conducted based on 12 phenotypic traits. Result shows 71.11% of total variation using the first two principal components (Table 3). As it can be seen in Fig. 5, two groups were distinguished: a first group gathered putative diploid accessions and a second one clustered possible hexaploid accessions.

Table 2 Quantitative and quantitative measurements of Tunisian plum accessions

Sample	*R* (length/width)	Shape	Skin color	Flesh color	Juiciness	Acidity
AGL	7	2	1	1	2	3
ANB_1	10.5	1	1	1	1	2
ANB_2	10.5	1	1	1	1	2
AND	7	2	1	1	2	3
ANH	9	1	1	2	2	3
ANH_2	9	1	1	2	2	3
ANK	8	1	1	1	3	2
ANS	9	1	2	3	1	3
ANS_2	8	1	2	1	1	3
ANS_3	9	1	2	1	1	3
ANT	6.5	1	1	1	3	2
BED	7	1	1	1	3	2
BLG	7.5	2	1	1	2	3
CHA	5	3	3	2	3	2
CID	6.5	1	4	4	1	3
CID_2	6.5	1	4	4	1	3
MKH	7	3	1	2	3	2
MKK	7	4	5	2	3	2
MSH	4	5	4	5	2	2
SAU	7	1	1	2	1	1
SNR	10	1	1	1	1	2
THD	8	1	1	5	1	2
THP	8	1	6	6	2	3
TSS	9	1	2	2	1	3

Table 2 (continued)

Sample	*R* (length/width)	Shape	Skin color	Flesh color	Juiciness	Acidity
ZAG	8	1	1	2	2	3
ZEN	4	5	6	2	3	1
	–	1: Round 2: Obcordate 3: Obovate 4: Ovate 5: Elliptic	1: Medium red 2: Yellowish green 3: Dark blue 4: Dark red 5: Black 6: Purplish	1: Medium red 2: Yellow 3: Orange yellow 4: Orange 5: Dark red 6: Whitish	1: High 2: Medium 3: Low	1: High 2: Medium 3: Low

Sample	Sweetness	Habit of the tree	Color of a one-year-old shoot	Shape of leaf blade	Diameter of flower	Shape of stone
AGL	1	4	4	2	3	3
ANB_1	2	4	4	2	3	3
ANB_2	2	4	4	2	3	3
AND	1	4	4	2	3	3
ANH	1	4	4	2	3	3
ANH_2	1	4	4	3	3	3
ANK	4	4	3	2	3	3
ANS	2	4	4	2	3	3
ANS_2	1	4	2	3	3	3
ANS_3	1	4	2	2	3	3
ANT	1	4	3	2	3	2
BED	1	4	3	3	3	3
BLG	1	4	4	2	3	3
CHA	2	4	2	2	3	2
CID	1	4	4	2	3	3

Table 2 (continued)

Sample	Sweetness	Habit of the tree	Color of a one-year-old shoot	Shape of leaf blade	Diameter of flower	Shape of stone
CID_2	1	4	4	2	3	3
MKH	1	3	4	3	3	3
MKK	2	4	4	2	3	3
MSH	1	4	4	2	1	2
SAU	3	3	4	3	3	3
SNR	2	4	4	2	3	3
THD	2	4	4	2	3	3
THP	1	4	4	2	3	3
TSS	2	3	4	3	3	3
ZAG	1	4	4	2	3	3
ZEN	3	4	2	3	2	2
	1: High 2: Medium 3: Low 4: Medium with special aroma	1: Upright 2: Semi-upright 3: Spreading 4: Drooping	1: Greyish brown 2: Yellow brown 3: Brown 4: Reddish brown	1: Ovate 2: Elliptic 3: Obovate	1: Small 2: Medium 3: Large	1: Narrow elliptic 2: Medium elliptic 3: Circular 4: Broad ovate

Table 3 Relative contribution of each variable to the variation provided by the first three axis of the principal component analysis

Component	Axis 1	Axis 2	Axis 3
% Variation	53.753	22.493	9.040
% Cumulated	53.753	76.246	85.286
Contributing of variable to the definition of the PCA axis			
R (length/width)	35.578	12.131	27.262
Skin color	33.519	11.199	39.463
Shape	17.935	10.856	2.685
Flesh color	10.441	46.299	15.471

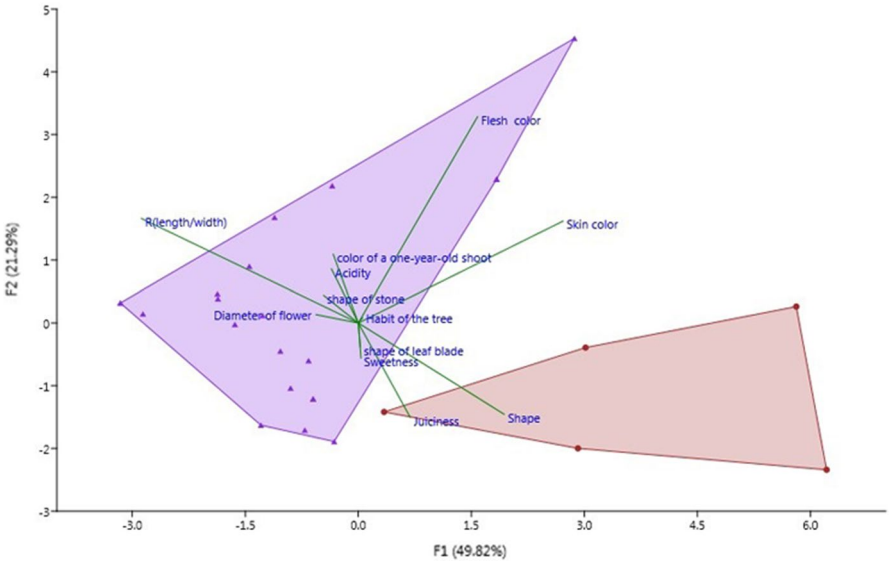

Fig. 5 Dispersion of Tunisian hexaploid and diploid plum trees in the biplot (1–2) of the principal component analysis, accounting for 71.11% of the global variability based on total of phenotypic traits. Red dots represent the five putative polyploid plums and purple triangles represent the eighteen putative diploid plums

The first axis explained 49.82% of the total variation. It had a large positive loading for juiciness, shape, skin color and flesh color; while negative loadings were obtained for *R* (length/width). This component separated the putative hexaploid varieties from the remaining accessions. The second axis, explained 21.29% of the total variation. It was positively correlated with flesh color, and negatively correlated with juiciness and shape.

Correlations Between Phenotypic and Molecular Data

Overall, 12 phenotypic parameters and 11 molecular markers (8 SSRs + 3 *S*-locus markers) were used to characterize our studied accessions. Hence, the Spearman test

was calculated between the Euclidean distance matrix based on phenotypic traits and molecular (SSRs and *S*-locus) markers. The Spearman test shows a significant value of 0.34*** ($p < 0.05$) and confirms that these two approaches are correlated and complementary to characterize Tunisian plum germplasm. In addition, *S*-locus and phenotypic results showed also a significant value of 0.30** ($p < 0.05$). Furthermore, SSR markers were significantly correlated with phenotypic results and showed a value of 0.28** ($p < 0.05$).

Discussion

In the present study, Tunisian plum germplasm showed a high genetic diversity with an average of 12.3 and 15.7 alleles per locus for SSR and *S*-locus, respectively. The same allele number was identified by Halàsz et al. (2017) using six SSR markers (98 alleles) in *P. spinosa* ($2n = 32$) and *P. insititia* ($2n = 48$) with 19.5 average allele number per locus. In the same work, 23 *S*-alleles were obtained in polyploid samples. The comparatively low number of *S*-alleles found in our study may be due to the different number of primers used.

SSR markers allowed to distinguishing between 18 putative diploid accessions and five putative polyploid accessions. The successful separation between two groups is due to the appropriate choice of used primers which have been proved adequate to distinguish between diploid and polyploid samples. Loci BPTCT007, BPPCT025 and BPPCT037 were screened for European plums and *P. cerasifera* genotypes (Halász et al. 2017); while CPDCT 044, CPSCT018 and CPSCT021 loci were extensively analyzed in Japanese plums, *P. spinosa* and *P. insititia* (Halász et al. 2017). Pchgms-3, UDP96-001 and UDP96-003 loci worked more properly in peaches (*P. persica*) and amplified between 4 and 6 alleles per sample (Wünsch et al. 2006; Halász et al. 2017). A combination of these loci also allowed identification of clonal diversity within *P. lusitanica* subspecies (García-Verdugo et al. 2013).

However, the ploidy level of the analyzed accessions cannot be assessed based on SSR data alone, since homozygosity or fixed heterozygosity in some polyploids may preclude the determination of the copy number of alleles (García-Verdugo et al. 2013). Hence, additional markers and/or approaches would be needed to unequivocally determine ploidy levels. i.e., cytological or flow cytometry analysis was needed to accomplish the SSR fingerprints in apple cultivars (Galli et al. 2005) and *Prunus* species (García-Verdugo et al. 2013; Fernández-Cruz et al. 2014).

Unlike SSR loci, the advantage of the *S*-locus resides on the absence of homozygous individuals. This locus controls gametophytic self-incompatibility system in Angiosperms which tends to prevent inbreeding between two individuals which have at least one common *S*-allele (Kao and Tsukamoto 2004). Hence, the *S*-locus introns primers, especially PruC2-PCER and PaconsF1/PaconsR1, spanning the *S*-RNase first and second introns, respectively, have been identified as powerful markers to separate diploid and polyploid accessions. Although these *S*-primers amplified full diploid genotypes, they amplified, however, up to six alleles per polyploid sample and confirmed that polyploid samples belong to hexaploid plum. The unexpectedly low number of *S*-alleles in some polyploid analyzed accessions can

be the consequence either of imperfect amplification or multiple appearance of the given allele (Kota-Dombrovska and Lacis 2013). In fact, it is still unclear whether an *S*-allele may appear in multiple copies in a polyploidy (Halász et al. 2017).

PCoA analysis of SSR and *S*-locus results highlighted the effectiveness of SSR in assessing diversity between varieties. However, the *S*-locus markers seem to be more powerful at the inter-species than at the intra-species level. Unlike SSR markers, the *S*-locus tends to create species-specific *S*-alleles. Hence, SSR and *S*-locus markers have been revealed in this study as complementary markers to discriminate between species with different ploidy levels. However, they do not distinguish species with same chromosome number (such *P. domestica* and *P. insititia*, both with $2n=48$).

For this purpose, phenotypic analyses were conducted. Although phenotypic markers are sometimes considered as prone to equivocal interpretations as well as time consuming (Eldridge et al. 1992), they have been, however, used for a long time as crucial elements in species characterization attempts (Khadivi-Khub and Anjam 2014). In fact, the five subgenera identified by Rehder (1940) in *Prunus* (*Amygdalus, Cerasus*, *Laurocerasus*, *Padus* and *Prunophora*) have been basically determined by how the leaves are rolled up in the bud, whether the flowers are organized in cymes or in racemes and finally by phenotypic characteristics of the generative organs, i.e. the size and color of flowers, fruit, stone and seed traits (OCDE 2002).

Five of the parameters measured in our study (i.e. habit of the tree, color of a one-year-old shoot, leaf blade shape, flower diameter and stone shape) presented relatively similar values indicating the high degree of homoplasy described in *Prunus* (Zeinalabedini et al. 2008). However, seven other parameters seem useful to distinguish between species and have been identified as discriminant traits. Hence, we assume that the right phenotypic marker must be discriminant with a low degree of homoplasy and must be marginally affected by environmental and ecological factors.

Among diploid plum species, *P. cerasifera*, a European plum, is known as a big and bushy tree with small acid fruits whose color is generally pale (yellow-green). In fact, this species is well known as rootstock but of little interest as a fruit crop (Das et al. 2011). Yet, the majority of analyzed accessions presented large sweet fruits. For accessions 'TSS' and 'ANS', despite their pale color (yellowish green skin and yellow flesh), their fruits presented however bigger size and lower acidity than myrobolan (*P. cerasifera*). Thus, quantitative and qualitative analyses limited the plausibility of the hypothesis that analyzed varieties belonged to *P. cerasifera*. This result is somewhat coherent with the assumption that the majority of Tunisian plum accessions belong to Japanese plum (Nabli 2011). On this point, it has been noted that Japanese plums have been brought back and widely implanted in warm North African countries where European plum does not adapt. i.e., the first varieties have been tested successfully in Algeria since 1895 (Liu et al. 2005).

Among Japanese plum, two diploid species are known: *Prunus salicina* and *Prunus simonii*. Hence, to distinguish between these species, two criteria should be estimated: the tree size and leaves size (length and width) (OCDE 2002; Das et al. 2011). In fact, *Prunus simonii* has a smaller tree size with larger leaves than those displayed by *Prunus salicina* (OCDE 2002). However, these parameters are influenced by environmental conditions. i.e. leaf traits in populations located in humid

and semi-humid regions that receive greater annual rainfall had greater mean leaf dimensions than those located in drier areas (Khadivi-Khub and Anjam 2014). On the other hand, it was admitted that *P. simonii* is an ancient hybrid between apricots and plums (known in some revues as 'the apricot plum') (Zhengy et al. 2003). It has thus a larger fruit than other Japanese plum with a very special flavor due to the presence of 1 or 2 large nectaries on either side of petiole (Gu and Bartholomew 2003). These two criteria characterize the accession 'ANK' which is very appreciated in Tunisia because of its special aromatized nectars. Thus, we assume that both fruit size and aromatized flavor are discriminant criteria to distinguish *P. simonii*. Hence, remaining accessions share the characters of large roundish fruit, a high juiciness and a low acidity. These traits strictly define Japanese *P. salicina* species (Zhengy et al. 2003).

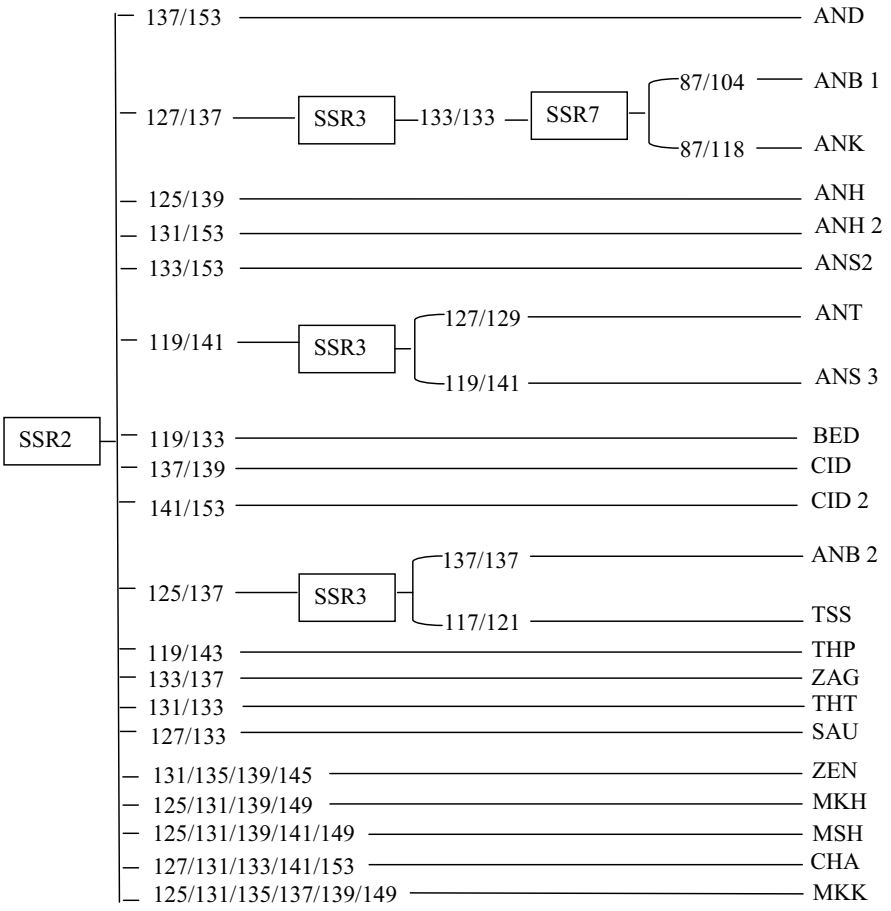

Fig. 6 Genotypic key of 23 Tunisian plum tree genotypes based on 3 microsatellites loci fingerprints SSR2 = CPSCT 021; SSR3 = Pchgms-3; SSR7 = BPTCT 037. The values written in square indicated SSRs loci. Normal writing values indicated allele sizes

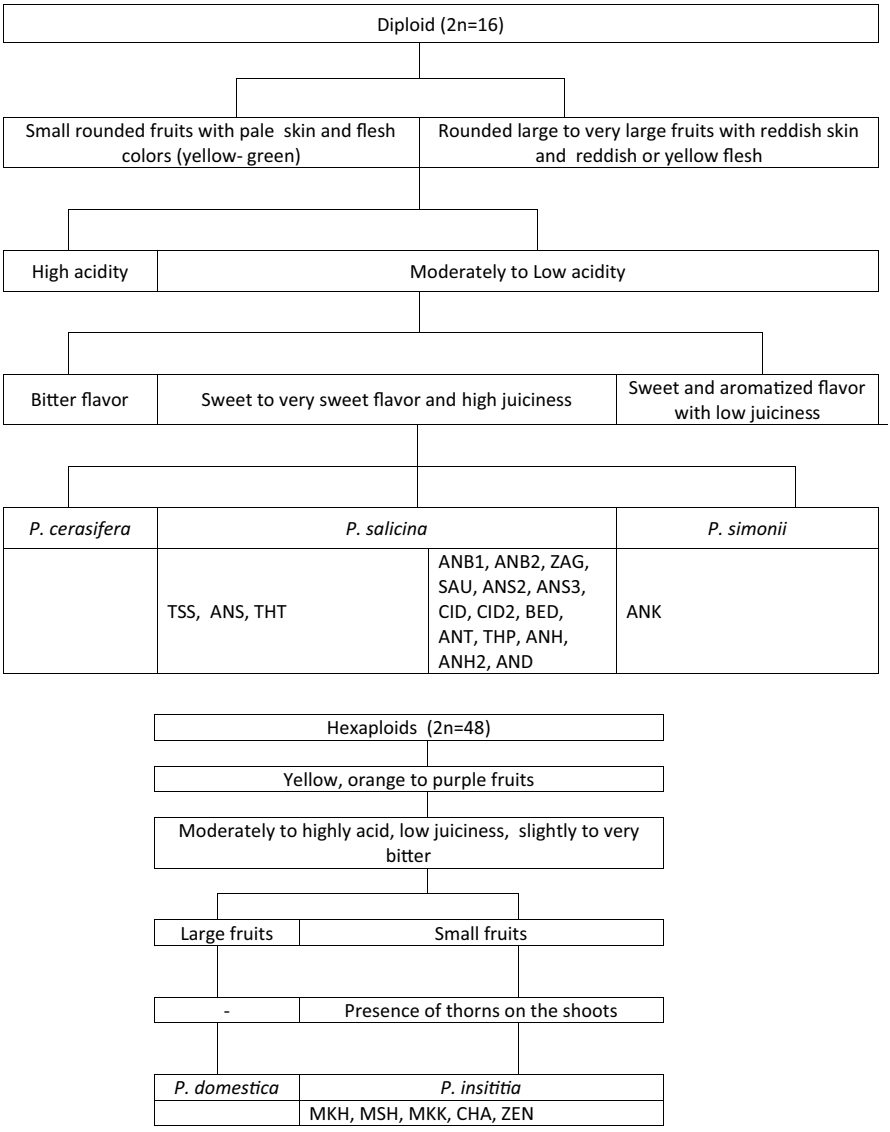

Fig. 7 Taxonomic key of Tunisian plum species using the ploidy level and phenotypic traits

On the other side, five samples carrying putative hexaploid genotypes have been identified. These accessions may belong to either *Prunus domestica* ($2n=48$) or *Prunus insititia* ($2n=48$). In the Flora of Tunisia, two wild plum species have been previously described: *P. spinosa* abundant in Ouled Ali, Aïn Soltane (jendouba), Aïn Drahem, route de Sidi Mechrig, Kef Enesour et Sejnane; and *P. insititia* L. abundant in Northern Tunisia in forests of Bizerte, and the North-west region, in Ghardimaou and Tamera (Nabli 2011).

Although it was not described in the Flora of Tunisia, the presence of *P. domestica* should not be however ruled out, because it was admitted that *P. insititia* could be a subspecies of *P. domestica* (Zhengy et al. 2003). This close genetic relationship makes difficult to distinguish between them. In fact, *P. insititia* and *P. domestica* have fruits whose color ranges between yellow to purple (Gu and Bartholomew 2003). However, it has been mentioned that *P. insititia* fruits are smaller than those of *P. domestica*. On the other hand, it was always noticed that *P. insititia* is a wild form which is characterized by the presence of thorns on the shoots (Zhengy et al. 2003). This criterion could be considered as a discriminant trait between these species. Yet, the analysis of CHA, ZEN, MKH, MKK and MSH accessions showed greyish brown shoots with medium to long length and the presence of thorns along shoots. This result confirmed that the analyzed polyploid plum varieties belong to *P. insititia*.

Genetic and Taxonomic Keys

Three SSRs loci, with 98 alleles and 129 genotypes, have been proved suitable to draw an identification key in order to characterize the studied plums (Fig. 6). The key showed that the 129 obtained genotypes, distinguished all the studied species with a discriminating power of 100%. The locus CPSCT 021 generated 19 genotypes, while Pchgms-3 and BPTCT 037 gave 18 and 16 genotypes, respectively.

The combination of ploidy level and phenotypic parameters allows determining to which species belong the 23 studied Tunisian plum accessions (Fig. 7). Such a result is of importance for local Tunisian plum germplasm management since it shows the high genetic variability of this patrimony and indicates that a core collection of Tunisian plums can be established to preserve it.

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Author Contributions GB performed the experiments and statistical analyses, developed the genetic analyses and wrote the manuscript. DA provided some plant material discussed and corrected the content. SBM provided some plant material and discussed the content. HBT gave additional information regarding plum and corrected the content. ASH offered experimental instructions, supervised and provided editorial advice.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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