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Genetic Diversity and Population Structure of Japanese Plum-Type (Hybrids of *P. salicina*) Accessions Assessed by SSR Markers

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Abstract: Japanese plum (*Prunus salicina* Lindl.) is widely distributed in temperate zones across the world. Since its introduction to USA in the late 19th century, this species has been hybridized with up to 15 different diploid *Prunus* species. This high level of introgression has resulted in a wide range of traits and agronomic behaviors among currently grown cultivars. In this work, 161 Japanese plum-type accessions were genotyped using a set of eight Simple Sequence Repeats (SSR) markers to assess the current genetic diversity and population structure. A total of 104 alleles were detected, with an average of 13 alleles per locus. The overall Polymorphic Informative Content (PIC) value of SSR markers was 0.75, which indicates that these SSR markers are highly polymorphic. The Unweighted Pair Group Method with Arithmetic (UPGMA) dendrogram and the seven groups inferred by Discriminant Analysis of Principal Components (DAPC) revealed a strong correlation of the population structure to the parentage background of the accessions, supported by a moderate but highly significant genetic differentiation. The results reported herein provide useful information for breeders and for the preservation of germplasm resources.

Keywords: Japanese plum; DAPC; genetic structure; simple sequence repeat; microsatellites

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1. Introduction

Japanese plum (*Prunus salicina* Lindl.) belongs to the *Prunus* genus in the Rosaceae family [1], which includes around 430 species [2]. This crop was originated approximately in 300 B.C. in the Yangtze River basin in China, where wild populations can be currently found [3,4]. Japanese plum was introduced to Japan from China more than 2000 years ago [5]. In the late 19th century, it was introduced to California (USA) from Japan, so it was called "Japanese plum" [2,6]. Now, this crop is widely distributed in temperate zones across the world [4].

In California, Luther Burbank started Japanese plum modern breeding by intercrossing *P. salicina* with *Prunus simonii* Carr. and other native American diploid plums in order to improve its adaptation to local conditions [7]. A number of cultivars were released from these hybridizations, such as "Beauty", "Burbank", "Duarte", "Eldorado", "Formosa", "Santa Rosa", and "Wickson", some of which are currently available and widely grown [2,5,6]. In these hybrids, *P. salicina* contributed to the improvement of fruit traits of size, flavor, color, and storability; *P. simonii* contributed to firm flesh and strong flavor; and the native American species such as *Prunus americana* Marsh. or *Prunus besseyi* Bailey contributed to disease resistance, tough skin, and aromatic quality [8]. In the southern United States, some of these cultivars were hybridized with the local *Prunus angustifolia*

Marsh., obtaining cultivars such as "Bruce" and "Six Weeks". Later, breeding programs in the Southern Hemisphere used *Prunus cerasifera* Ehrh. as the parent to create early and cold-hardy hybrids, such as "Methley" in South Africa or "Wilson" in Australia [9].

At present, an important renewal of plant material is underway due to the introduction of a number of new Japanese plum-type cultivars from different breeding programs across the world. These efforts share goals such as productivity, fruit size and quality, extension of the harvest season, and adaptation to growing areas [6,10]. As result of breeding activity, the Community Plant Variety Office of the European Union (CPVO) registered 149 new Japanese plum cultivars from 1995 to 2020 [11]. The term "Japanese plum" now includes a heterogeneous group of interspecific hybrids [6] and few cultivars currently grown are pure *P. salicina*. The high variability generated by the interspecific crosses of *P. salicina* with up to 15 other *Prunus* species is reflected in the different behavior observed in the modern commercial cultivars [12–16].

The use of molecular markers for studies of diversity and population genetics on fruit tree species is steadily increasing [6] because they can be linked to specific alleles [17]. Genetic diversity can be analyzed using a wide array of molecular markers, such as Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNA (RAPDs), Amplification Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSR) [6], and Single Nucleotide Polymorphisms (SNPs) [18,19]. During the past 20 years, SSR markers have emerged as a powerful tool for this type of study because they are highly informative, polymorphic, and codominant, and present transferability among close species [20,21].

Initial work with SSR markers in pome and stone fruits was carried out on the identification and the establishment of genetic relationships of apple (*Malus* × *domestica* Borkh.) genotypes [22–25]. The first SSR markers in *Prunus* species were developed in peach [*Prunus persica* (L) Batsch], verifying their transferability to other *Prunus* species [26]. Currently, most SSR markers available derive from cherry [27–30] and peach [26,31–33], although a small number have been developed in apricot [34] and Japanese plum [35]. They have been used to analyze the genetic diversity and to improve the management of plant genetic resources in almond [36–39], apricot [40,41], European plum [42,43], peach [44], and sweet cherry [45–47].

The significant variability observed in Japanese plum cultivars led to early diversity studies to estimate genetic relationships using isoenzymes [48], RAPDs [49,50], and SSR developed in Japanese plum [35] and other *Prunus* species [46,51–55]. However, the genetic diversity of the cultivars currently grown globally is unknown, because the previous studies were mainly focused on traditional cultivars. This study aims to determine: (i) the current genetic diversity, (ii) genetic relationships among cultivars, and (iii) population structure of a set of 161 Japanese plum-type accessions released from breeding programs from Israel, South Africa, Spain, and the United States.

2. Materials and Methods

2.1. Plant Material

A total of 161 Japanese plum-type accessions, comprising traditional and modern commercial cultivars, advanced selections, and six reference genotypes of *P. salicina*, *P. cerasifera* and *P. simonii* from 27 breeding programs were evaluated. The plant material was obtained from different germplasm collections: the Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX-La Orden) located in Badajoz (42 accessions); the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) located in Zaragoza (74 accessions); the Asociación de Fruticultores de la Comarca de Caspe (AFRUCCAS) located in Caspe, Zaragoza (2 accessions); and the Viveros Mariano Soria located in La Almunia de Doña Godina, Zaragoza (43 accessions) (Table 1).

Table 1. Japanese plum-type accessions analyzed in this study.

Accessions	Origin	Accessions	Origin
606	Reedley Nursery, USA	P006	Provedo, Spain
A001	Unknown	P007	Provedo, Spain
A002	Unknown	Pioneer	ARC Infruitec, South Africa
Abundance	Imported from Japan	Plum Late	Unknown
African Pride	ARC Infruitec, South Africa	Prime Time	Wuhl, USA
African Rose	ARC Infruitec, South Africa	Queen Ann	USDA, USA
Alpha	Selected in New Jersey	Queen Rosa	USDA, USA
Ambra	Unknown	Red Beaut	Reedley Nursery, USA
Angeleno	Garabedian, USA	Redheart	Reedley Nursery, USA
AU Amber	Auburn University, USA	Royal Diamond	Kitahara Farms, USA
AU Road Side	Auburn University, USA	Royal Garnet	Reedley Nursery, USA
AU Rosa	Auburn University, USA	Royal Zee	Zaiger, USA
Autumn Giant	Zaiger, USA	Rubirosa	Zaiger, USA
Black Amber	USDA, USA	Ruby Crunch	ARC Infruitec, South Africa
Black Beaut	Reedley Nursery, USA	Ruby Queen	USDA, USA
Black Diamond	Superior Farming Co, USA	Ruby Star	ARC Infruitec, South Africa
Black Egg	Ben Dor, Israel	Ruby Sweet	USDA, USA
Black Gold	Superior Farming Co, USA	S001	Stargrow, South Africa
Black Late	Unknown	S002	Stargrow, South Africa
Black Ruby	USDA, USA	S003	Stargrow, South Africa
Black Satin	Zaiger, USA	S004	Stargrow, South Africa
Black Splendor	USDA, USA	S005	Stargrow, South Africa
Black Star	Unknown	S006	Stargrow, South Africa
Burmosa	USDA, USA	S007	Stargrow, South Africa
Byrongold	USDA, USA	S008	Stargrow, South Africa
Constante	Unknown	S009	Stargrow, South Africa
Crimson Glo	Zaiger, USA	S010	Stargrow, South Africa
D001	Unknown	S011	Stargrow, South Africa
D002	Unknown	S012	Stargrow, South Africa
D003	Unknown	S013	Stargrow, South Africa
D004	Unknown	S014	Stargrow, South Africa
D42	Ben Dor, Israel	S015	Stargrow, South Africa
Dapple Jack	Zaiger, USA	S016	Stargrow, South Africa
Earlamoon	Ben Dor, Israel	S017	Stargrow, South Africa
Earliqueen	Zaiger, USA	S018	Stargrow, South Africa
Early Fortune	Azienda Agricola Martelli, Italy	S019	Stargrow, South Africa
Ebony Rose	Zaiger, USA	S020	Stargrow, South Africa
Eldorado	Terry, USA	S021	Stargrow, South Africa
Emerald Drop	Zaiger, USA	S022	Stargrow, South Africa
Extremagold	Unknown	S023	Stargrow, South Africa
Formosa	Fancher Creek Nursery, USA	S024	Stargrow, South Africa
Fortune	USDA, USA	S025	Stargrow, South Africa
Freedom	USDA, USA	S026	Stargrow, South Africa
Friar	USDA, USA	S027	Stargrow, South Africa
Frontier	USDA, USA	S028	Stargrow, South Africa
Gaia	Azienda Agricola Martelli, Italy	S029	Stargrow, South Africa
GF81	INRA, Francia	S030	Stargrow, South Africa
Golden Globe	Zaiger, USA	S031	Stargrow, South Africa
Golden Japan	Imported from Japan	S032	Stargrow, South Africa
Golden Kiss	ARC Infruitec, South Africa	S033	Stargrow, South Africa
Golden Plumza	Vivai F.lli Zanzi, Italy	S034	Stargrow, South Africa
Green Sun	Chamberlin, USA	S035	Stargrow, South Africa
Grenadine	Zaiger, USA	S036	Stargrow, South Africa
HD	Ben Dor, Israel	S037	Stargrow, South Africa
Hiromi Red	Zaiger, USA	S038	Stargrow, South Africa
Honey Crisp	Unknown	S039	Stargrow, South Africa
Honey Down	Stargrow, South Africa	S040	Stargrow, South Africa
Honey Lucas	Unknown	S041	Stargrow, South Africa
Honey Moon	Stargrow, South Africa	S042	Stargrow, South Africa

Table 1. *Cont.*

Accessions	Origin	Accessions	Origin
Honey Star	Stargrow, South Africa	Santa Rosa	Burbank, USA
Honey Sweet	INRA, Francia	Sapphire	ARC Infruitec, South Africa
Howard Sun	Agri Sun Nursery, USA	Simka	Coche D Simonian, USA
Joanna Red	Zaiger, USA	Simon	Simon Brothers, USA
John W	USDA, USA	Songold	ARC Infruitec, South Africa
Kelsey	Imported from Japan	Songria 10	Planasa, Spain
Laroda	USDA, USA	Songria 15	Planasa, Spain
Larry Ann	Topfruit, South Africa	Sordum	Imported from Japan
Late blue	Zaiger, USA	Souvenir	ARC Infruitec, South Africa
Mariposa	Armstrong Nursery, USA	Splash	Zaiger, USA
Mark	Ben Dor, Israel	Speckled Egg	Ben Dor, Israel
Methley	Burbank, USA	Sundew	ARC Infruitec, South Africa
Morris	Texas AM, USA	Sunkiss	ARC Infruitec, South Africa
Nubiana	USDA, USA	Sweet Treat	Zaiger, USA
October Red	Unknown	Tc Sun	Chamberlin, USA
Owen T	USDA, USA	Winner	Ben Dor, Israel
Ozark Premier	Missouri State Univ., USA	Z001	Zaiger, USA
P001	Provedo, Spain	Z002	Zaiger, USA
P002	Provedo, Spain	Z003	Zaiger, USA
P003	Provedo, Spain	Zanzi Sun	Unknown
P004	Provedo, Spain	Ziv	Ben Dor, Israel
P005	Provedo, Spain		

2.2. DNA Extraction and SSR Analysis

Young leaf samples were collected in spring and preserved in silica gel [56]. The dried leaves were ground on a TissueLysser (Qiagen, Hilden, Germany) prior to the DNA extraction. Genomic DNA was extracted following the protocol described by Hormaza [40] and using a Speedtools Plant DNA Extraction Kit (Biotools, Madrid, Spain) according to the manufacturer's instructions [13,57,58]. Quantity and quality of DNA was assessed using a microvolume spectrophotometer NanoDrop 1000 (ThermoScientific, Delaware, USA) and diluted at 10 ng/µL prior to PCR amplification [13].

A total of 13 SSR markers developed in Japanese plum, peach, and sweet cherry were used (Table 2). The DNA fragments were amplified using six sets of multiplex PCR reactions (M01 to M06). Each multiplex reaction was designed by combining the expected molecular size (pb) of the fragments amplified by each SSR primer pair and four fluorescent dyes (PET, 6-FAM, VIC, NED). Multiplex PCRs M01-M04 were performed in a final volume of 12.5 µL, and M05 and M06 in a final volume of 11.5 µL. A Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) was used for all reactions according to the manufacturer's instructions, with different concentrations for each SSR marker (Table 2) and 10 ng of genomic DNA. The temperature profile used in M01 to M04 had an initial step of 15 min at 95 °C, 35 cycles of 45 s at 95 °C, 45 s at 57 °C, and 2 min at 72 °C, and a final step of 30 min at 72 °C [31]. M05 and M06 were performed using the same conditions with modifications at the annealing temperature of 46 and 62 °C, respectively [35]. All PCR reactions were carried out using a SimplyAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were separated by capillary electrophoresis using a genetic analyzer ABI3730 (Applied Biosystems, Foster City, CA, USA). The amplified fragments were sized and scored with a size standard GeneScan 500LIZ (Applied Biosystems, Foster City, CA, USA) [57] on "Fragman" v. 1.0.9 [59], an R package [60] for fragment analysis and revised with the software PeakScanner v. 1.0 (Applied Biosystems, Foster City, CA, USA). The genetic profiles were organized in a table in csv format for the subsequent analysis.

Table 2. Multiplex (Mp) design, SSR loci, linkage group (LG), fluorescent dyes, primer concentration (PC), PCR details, and characteristics of the 13 SSR markers analyzed in this study.

Mp	Locus	LG	Dye	PC (μM)	Primer Sequence	SSR Motif	Size Range (bp)	Species
M01	CPPCT029 * [61]	G1	VIC	0.2	F: CCAAATTCCAAATCTCCTAACA R: TGATCAACTTTGAGATTTGTTGAA	$(CT)_{24}$	170–194	Peach
	pchgms2 [30]	G4	6-FAM	0.2	F: GTCAATGAGTTCAGTGTTACACTC R: AATCATAACATCATTCAGCCACTGC	$(CT)_{24}$	130–200	Peach
	CPPCT033 [61]	G7	NED	0.2	F: TCAGCAAACTAGAAACAAACC R: TTGCAATCTGGTTGATGTT	$(CT)_{16}$	151	Peach
M02	UDP96-008 * [26]	G3	PET	0.3	F: TTGTACACACCCTCAGCCTG R: TGCTGAGGTTCAGGTGAGTG	$(CA)_{23}$	140–160	Sweet cherry
	UDP98-412 * [26]	G6	NED	0.15	F: AGGGAAAGTTTCTGCTGCAC R: GCTGAAGACGACGATGATGA	$(AG)_{28}$	100–140	Peach
	UDP98-409 * [26]	G8	6-FAM	0.3	F: GCTGATGGGTTTTATGGTTTTC R: CGGACTCTTATCCTCTATCAACA	$(AG)_{19}$	125–165	Peach
	UDP98-406 * [26]	G2	VIC	0.2	F: TCGGAAACTGGTAGTATGAACAGA R: ATGGGTCGTATGCACAGTCA	$(AG)_{15}$	30–100	Peach
M03	BPPCT-007 [31]	G3	6-FAM	0.2	F: TCATTGCTCGTCATCAGC R: CAGATTTCTGAAGTTAGCGGTA	$(AG)_{22}(CG)_2(AG)_4$	143–151	Peach
	UDP96-005 [26]	G1	VIC	0.3	F: GTAACGCTCGCTACCACAAA R: CCTGCATATCACCACCCAG	$(AC)_{16}TG(CT)_2CA(CT)_{11}$	100–250	Peach
M04	BPPCT-039 [31]	G3	PET	0.3	F: ATTACGTACCCTAAAGCTTCTGC R: GATGTCATGAAGATTGGAGAGG	$(GA)_{20}$	148–158	Peach
	BPPCT-025 [31]	G6	VIC	0.3	F: TCCTGCGTAGAAGAAGGTAGC R: CGACATAAAGTCCAAATGGC	$(GA)_{29}$	178–202	Peach
M05	CPSCT026 [35]	G7	6-FAM	0.3	F: TCTCACACGCTTTCGTCAAC R: AAAAAGCCAAAAGGGGTTGT	$(CT)_{16}$	177–213	Japanese plum
M06	CPSCT005 [35]	G4	NED	0.3	F: CTGCAAGCACTGCGGATCTC R: CCCATATTCCCAACCCATTA	$(CT)_{15}$	171–191	Japanese plum

* SSR excluded from subsequent analyses due to poor amplification.

2.3. Genetic Diversity Analysis and Genetic Relationships among Accessions

The analysis of genetic diversity and genetic relationships were performed using R software v. 3.6.0 (R Development Core and Team, 2020). For the genetic diversity and population structure analysis, the data of alleles generated by the SSR markers were converted to an object of the class *genind* using the "df2genind" function of the "adegenet" package v. 2.1.2 [62].

Number of alleles per locus (N_A), private alleles (P_A), Polymorphism Information Content (*PIC*), allelic richness (A_R), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and the F-statistics (F_{IS} and F_{ST}) were determined on the whole population and on each predetermined group using the packages: "adegenet" v. 2.1.2 [62]; "hierfstat" v. 0.5-7 [63]; "pegas" v. 0.13 [64]; and "PopGenReport" v. 3.0.4 [65]. The correlation matrix of the pairwise F_{ST} values was plotted with the package "corrplot" v. 0.90 [66].

A R script was developed to detect synonymies and homonymies in the data. Synonymies were identified by comparison of the allele data using the "duplicated" function to detect identical genetic profiles considered as synonymies. All accession names were also compared by the "duplicated" function to detect homonymies.

The genetic relationships among accessions were determined using an Unweighted Pair Group Method with Arithmetic averages (UPGMA) cluster analysis according to Nei and Li [67]. The "poppr" package v. 2.8.5 was used to generate an UPGMA dendrogram with a "bootstrap" supported by 1000 replicates [68]. The genetic structure was also analyzed using the "adegenet" package v. 2.1.2. [61] by a Discriminant Analysis of Principal Components (DAPC). The optimal number of groups (*K*) in the whole population was inferred using the "find.clusters" function according to the lowest Bayesian Information Criterion (BIC) value. A cross-validation function, "xvalDapc" [61], was used to determine the correct number of Principal Components (PCs) to be retained. An Analysis of Molecular Variance (AMOVA) was conducted using the "poppr" package v. 2.8.5 to calculate the variance components among the inferred groups and among the accessions [68].

3. Results

3.1. SSR Genotyping

Eight of the 13 SSR primers pairs (62%) showed good amplification and were selected to evaluate the genetic diversity and population structure. The remaining five (CPPCT-029, UDP96-008, UDP98-406, UDP98-409, and UDP98-412) were excluded from the analysis due to null or poor amplification (Table 2). A total of 104 alleles were amplified using eight SSR primers across 161 Japanese plum-type accessions (155 commercial cultivars and selections, and six reference cultivars). The number of alleles per locus (N_A) ranged from nine (CPPCT033) to 16 (BPPCT007), with an average value of 13 and an allele size range of 93–208 pb. Polymorphism Information Content (*PIC*) values ranged between 0.56 (CPPCT033) and 0.84 (CPSCT005), with an average of 0.75 per locus. The lowest observed heterozygosity (*Ho*) was 0.45 for BPPCT039 and the highest was 0.85 for pchgms2 with a mean of 0.65 for all accessions. The values of expected heterozygosity (*He*) ranged from 0.51 (CPPCT033) to 0.80 (CPSCT005), with an average of 0.68. The *F* statistics showed moderate population differentiations for each locus. F_{IS} varied from −0.13 (pchgms2) to 0.22 (BPPCT025) with a mean of 0.05, whereas F_{ST} ranged between 0.06 (CPSCT005 and UDP96005) to 0.30 for BPPCT039 with an average of 0.12 (Table 3).

Table 3. Number of alleles and allele size range in base pairs amplified by eight polymorphic SSR in Japanese plum-type accessions. Number of alleles (N_A), Polymorphism Information Content (*PIC*), Observed heterozygosity (*Ho*), Expected heterozygosity (*He*), Inbreeding coefficient (F_{IS}), and Wright's Fixation index (F_{ST}).

Locus	N_A	Allele Size (bp)	*PIC*	*Ho*	*He*	F_{IS}	F_{ST}
pchgms2	11	130–170	0.77	0.85	0.75	−0.13	0.07
CPPCT033	9	129–147	0.56	0.47	0.51	0.07	0.15
BPPCT007	16	117–155	0.83	0.82	0.74	−0.11	0.13
BPPCT039	13	121–167	0.73	0.45	0.55	0.18	0.30
BPPCT025	13	140–194	0.75	0.52	0.67	0.22	0.13
CPSCT026	15	156–208	0.83	0.66	0.77	0.14	0.11
CPSCT005	13	165–193	0.84	0.76	0.80	0.05	0.06
UDP96005	14	93–153	0.67	0.68	0.68	0.00	0.06
Mean	13	-	0.75	0.65	0.68	0.05	0.12

3.2. Genetic Relationships among Accessions

The UPGMA dendrogram grouped the accessions into two major clusters supported by a strong bootstrap value (100) (Figure 1), allowing the identification of 159 genotypes and two pairs of synonymies ("Red Beaut" and "606", "Fortune" and "Green Sun"). The clustering of the accessions by their SSR profile was consistent with the available parentage information (Supplementary Materials, Table S1), but weak correspondence with the program breeding or geographical origin was found. According to the dendrogram, "Black Satin" and the accession "S030" clustered separately, forming the smallest cluster (A). Cluster B was the largest cluster, comprising 152 accessions distributed in seven subclusters. The subcluster B1 comprised nine accessions, some of them derived from the same pedigree as "Methley", "Morris", and "AU Amber", and the remaining accessions shared a common and known South African origin, with the exception of "Speckled Egg". The subcluster B2 comprised a set of Californian cultivars of "Eldorado" (cultivar released by Luther Burbank), "Friar", "Angeleno", "Black Diamond", "Royal Diamond", and 19 other accessions, including "Alpha" (*Prunus maritima*). The subcluster B3 comprised 20 accessions, most of which were commercial cultivars and early selections from South Africa, such as "Sunkiss", "Honey Sweet", "Honey Down", and "Honey Star". The subcluster B4 comprised 22 accessions, including some commercial cultivars: "African Rose", "Black Beaut", "Crimson Glo", "Earliqueen", "Golden Kiss", and "Souvenir". The subcluster B5 was formed by two reference genotypes ["Abundance" (*P. salicina*) and "Simon" (*P. simonii*)] and 15 other accessions, including "Burmosa" and its descendants, "Red Beaut" and "606". The subcluster B6 comprised eight accessions, including the reference genotypes of *P. salicina* "Kelsey" and "Formosa", in addition to the traditional cultivars "Golden Japan" and "Songold". The subcluster B7 encompassed 49 accessions and the reference genotype "Mariposa" (*P. salicina*). Finally, the cluster C comprised seven accessions, including two accessions from USA ("October Red" and "Sweet Treat"), four accessions from South Africa ("African Pride", "Ruby Star", "S018", and "S026"), and the rootstock cultivar "GF81".

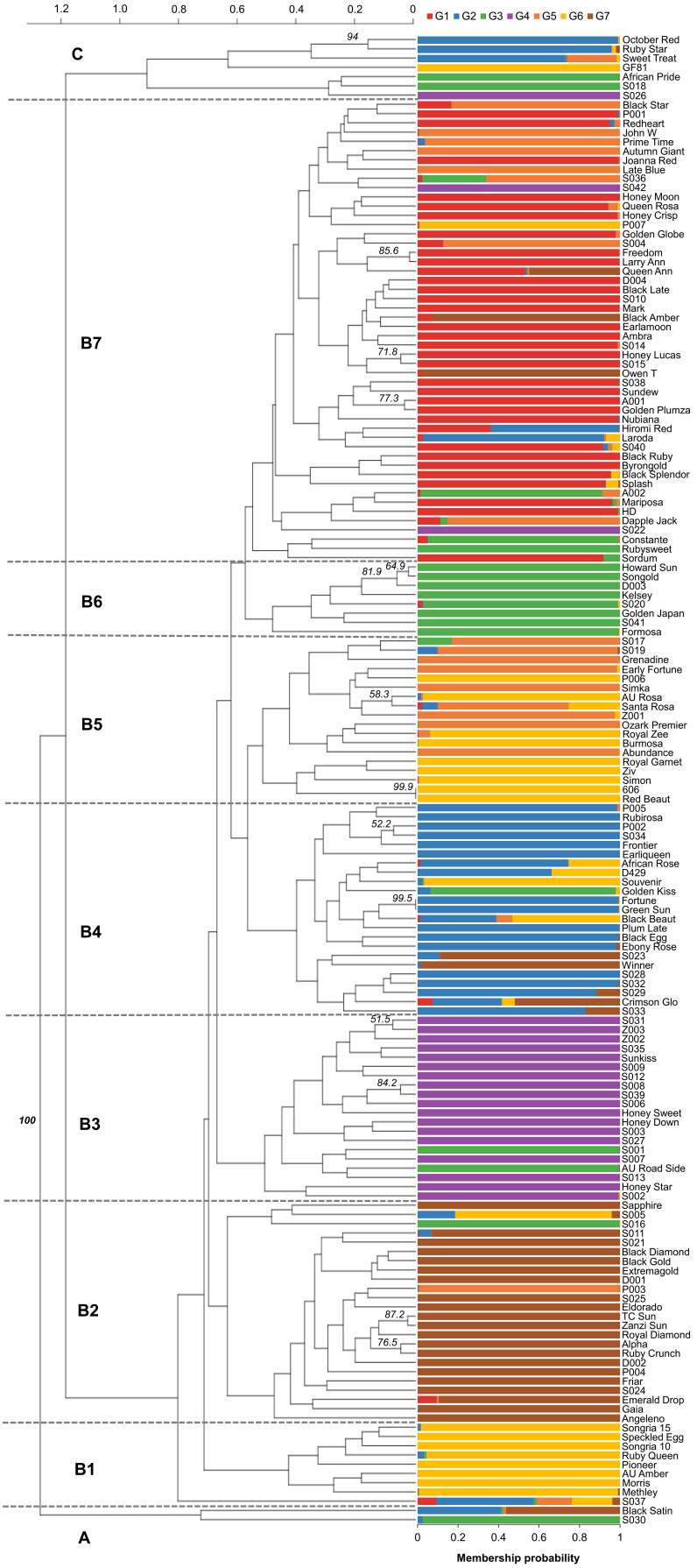

Figure 1. Genetic relationships and genetic structure from 161 Japanese plum-type accessions by

DAPC. The genetic relationships are represented by a UPGMA dendrogram created from 1000 bootstrap replications. Bootstrap values >50% are placed on the branches. The stacked bar charts represent different assigned groups with the following color codes: G1 = red, G2 = blue, G3 = green, G4 = purple, G5 = orange, G6 = yellow, and G7 = brown. The *x*-axis provides the probability of each accession belonging to the assigned group.

3.3. Analysis Genetic Structure

The genetic structure analyzed by DAPC showed a $K = 7$ value as the optimal clustering, according to the lowest BIC value. The optimal number of PCs to be retained for the subsequent analysis was 10 (Supplementary Materials, Figure S1). This scenario showed groups 1 to 3 and 5 to 7 (G1–G3 to G5–G7) overlapped, and group 4 (G4) clearly differentiated from them across the first two linear discriminant functions (LD1 and LD2) (Figure 2). The reports of the allele frequencies (loadings) in the dataset allowed determination of the contribution of alleles to the distribution of accessions in the DAPC scatterplot (Supplementary Materials, Figure S2).

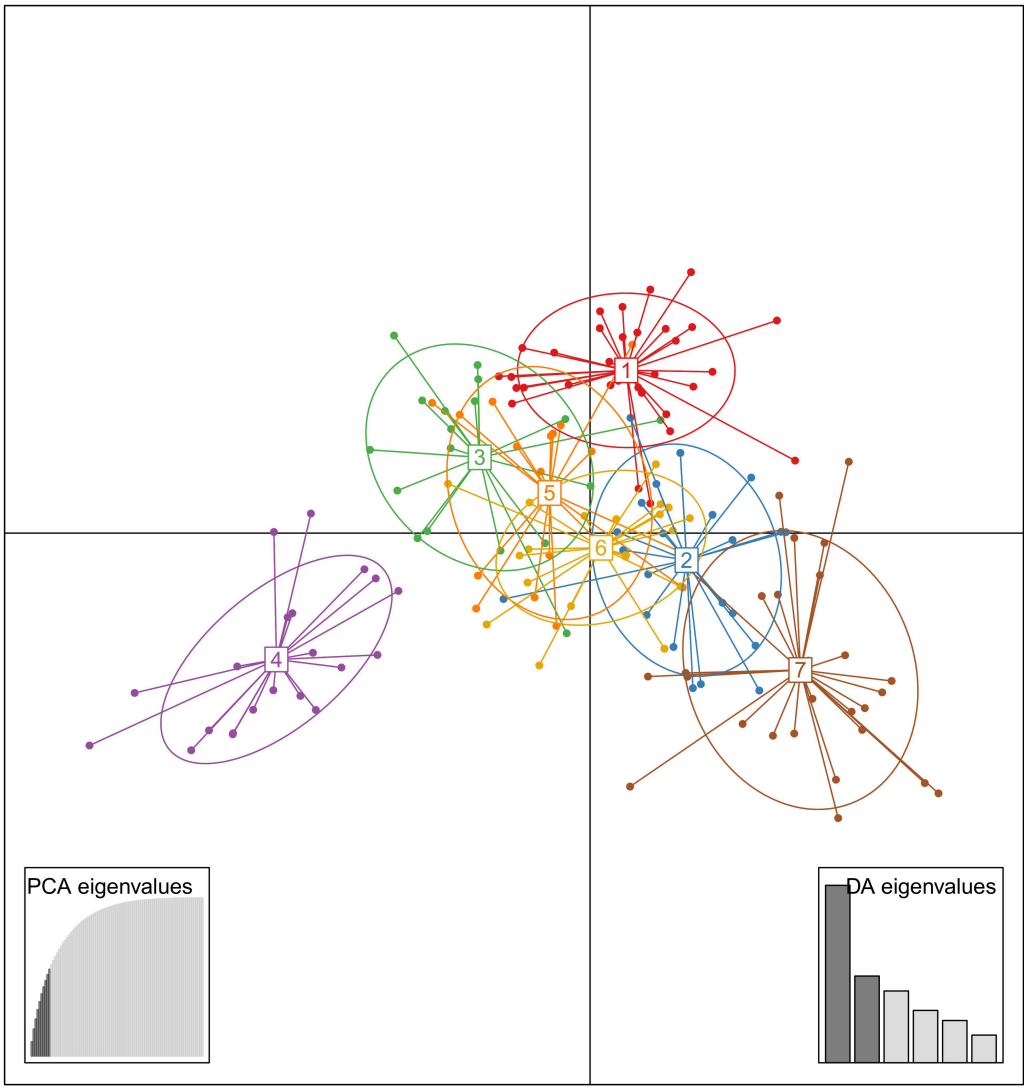

Figure 2. Scatterplot of DAPC of population structure of 161 Japanese plum-type accessions, showing the first two principal linear discriminants of the DAPC according to the optimal *K* value ($K = 7$). Each colored circle represents a group: G1 = red, G2 = blue, G3 = green, G4 = purple, G5 = orange, G6 = yellow, and G7 = brown. Each dot represents an accession. The insets represent the eigenvalues of the Principal Component Analysis (PCA) and Discriminant Analysis (DA).

The DAPC analysis allowed allocation of most of the accessions to their original group according to a membership probability up to 0.9, indicating clear-cut groups. However, some accessions showed lower membership probabilities, ranging from 0.3 to 0.7, which indicate some admixtures in the structured population (Figure 1). Group G1 comprised a set of 32 accessions (19.9%), including the *P. salicina* reference "Mariposa" and other commercial cultivars of "Black Splendor", "Queen Rosa", and "Queen Ann". Group G2 comprised 23 accessions (14.3%), mostly cultivars from California ("Hiromi Red", "Earliqueen", "Frontier", and "Green Sun", among others) and South Africa ("African Rose", "Ruby Star", and some advanced selections). Group G3 (n = 18, 11.2%) included the two *P. salicina* genotype-references "Kelsey" and "Formosa". Group G4 comprised 21 accessions (13%), most of modern cultivars ("Honey Down", "Honey Star", "Honey Sweet", and "Sunkiss"), and some advanced selections from South Africa. A group of 18 accessions (11.2%) formed the group G5, including "Abundance" (*P. salicina*) and traditional cultivars of "John W", "Santa Rosa" and "Simka". The group G6 comprised 22 accessions (13.7%), including the genotype-reference "Simon" (*P. simonii*) and some accessions with *P. cerasifera* in their pedigree ("Methley", "Morris", and the rootstock "GF81′). Finally, group G7 was formed by 27 accessions (16.8%) and encompassed a high diversity of origins of the traditional cultivars "Angeleno", "Black Diamond", "Eldorado", "Friar", "TC Sun", and "Zanzi Sun".

3.4. Genetic Diversity among Groups

Significance variance differences ($p < 0.01$) were found within the accessions and the AMOVA showed that 81.8% of the total variance observed in the $K = 7$ scenario was also due to differences within accessions, 14.2% was due to differences among groups, and the remaining 4.0% was due to differences among accessions within groups (Table 4).

Table 4. Analysis of molecular variance (AMOVA) for 161 Japanese plum-type accessions clustered in seven groups.

Source of Variation	df	Sum of Square	Mean Sum of Square	% of the Variance	Phi
Among groups	6	281	46.9	14.2	0.182
Among accessions within groups	154	876	5.7	4.0	0.046
Within accessions	161	834	5.2	81.8 *	0.142
Total	321	1991	6.2	100.0	

* Significant values at $p < 0.01$ significance level.

The statistics of genetic diversity were calculated and summarized per group ($K = 7$) (Table 5). The number of alleles (N_A) per locus ranged from 6.25 (G4) to 7.63 (G2). The total number of alleles varied from 50 (G4) to 61 (G2). The allelic richness (A_R) ranged from 6.01 (G4) to 7.25 (G3). The highest number of private alleles (P_A), those present in only one group, was 10 (G2), and only one was observed in G5. Among the groups, the lowest *H*o was determined in G1 (0.59), and the highest was observed in G3 and G7 (0.69). The lowest *H*e was recorded in G1 (0.63), and the highest in G3 (0.73). All groups had Inbreeding Coefficient values (F_{IS}) close to zero, ranging from −0.01 (G7) to 0.15 (G6), showing no excess of homo- or hetero-zygotes.

To validate the genetic differentiation among the seven groups, the F_{ST} values based on Nei's genetic distance among groups were determined (Figure 3). The overall pairwise F_{ST} values of 0.14 suggested a moderate differentiation between groups and varied from 0.11 (between G2 and G6) to 0.19 (between G4 and G7). Most of the groups with paired G4 exhibited higher F_{ST} values than the other pairs. All of these comparisons had non-zero lower and upper 99% confidence intervals (Supplementary Materials, Table S2).

Table 5. Statistics of genetic variation for 161 Japanese plum-type accessions clustered in seven groups. Number of accessions (n), Number of alleles (N_A), Number of private alleles (P_A), Allelic richness (A_R), Observed heterozygosity (*Ho*), Expected heterozygosity (*He*), Inbreeding coefficient (F_{IS}).

Group	n	$N_{A\ PER\ LOCUS}$	$N_{A\ TOTAL}$	A_R	P_A	*Ho*	*He*	F_{IS}
G1	32	7.38	59	6.06	3	0.59	0.63	0.04
G2	23	7.63	61	7.04	10	0.64	0.67	0.06
G3	18	7.25	58	7.25	2	0.69	0.73	0.05
G4	21	6.25	50	6.01	3	0.67	0.68	0.00
G5	18	7.13	57	7.13	1	0.66	0.66	0.00
G6	22	6.50	52	6.23	3	0.61	0.71	0.15
G7	27	6.75	54	6.17	4	0.69	0.69	−0.01

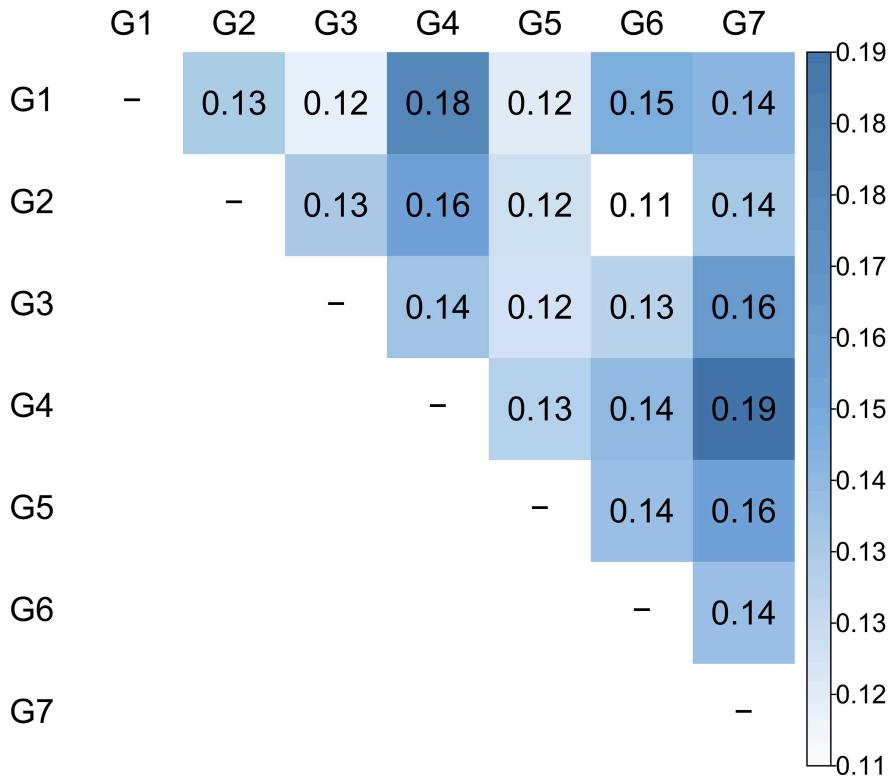

Figure 3. Weighted pairwise F_{ST} values estimated from the seven inferred groups ($K = 7$).

4. Discussion

The analysis of the genetic relationships and the genetic diversity in the germplasm analyzed, including 155 accessions and six Japanese plum-type reference-genotypes, by SSR markers, showed the correct amplification in eight of the 13 SSR markers used in this study, which were previously developed in Japanese plum [35], peach [26,30,31,61], and sweet cherry [26]. Although extrapolation of the results generated by this approach is complex due to the differences in the number of accessions and SSR markers used in the different studies [69], this approach has proven to be highly useful for cultivar identification because SSR are multi-allelic, codominant markers and most of them are transferable within *Prunus* species [31].

A total of 104 alleles were amplified by the set of SSR markers, emphasizing their high degree of polymorphism. Similar results were found in a previous study analyzing 47 accessions of Japanese plum using eight SSR markers ($N_{A\ TOTAL}$ = 104, average of $N_{A\ PER\ LOCUS}$ = 13) [51]. The *PIC* values for all loci found in this study were higher than 0.5, and therefore they were considered highly informative [70].

The observed heterozygosity found in this study was higher than that determined in previous reports in apricot (*Ho* = 0.51, 48 accessions, 31 SSR markers [40]), sweet cherry (*Ho* = 0.49, 76 accessions, 24 SSR markers [47]), and peach (*Ho* = 0.47, 50 accessions, 26 SSR markers [33]; *Ho* = 0.45, 28 accessions, 10 SSR markers [30]). This higher heterozygosity can be explained by the high number of accessions used in this study, and also by the high degree of introgression of the analyzed accessions, which mostly derived from interspecific crosses between the original species of *P. salicina* with up to 15 other *Prunus* species [12].

Genotypes were considered to be duplicated (synonymies) when they were paired on all alleles of the whole set of SSR markers. Two pairs of duplicates were found based on the SSR profile. "Red Beaut" showed 100% of similarity with "606" as expected, because "606" is a selection of cv. "Red Beaut" [15]. The other pair of duplicates was "Fortune" with "Green Sun", although both cultivars are well known and have different phenotypic characteristics [9]. Further research with samples of the same cultivars from other collections or additional SSR markers would be needed to distinguish them.

The UPGMA dendrogram arrangement indicated a stronger correlation with the parentage background (progenitors) of the accessions than their geographical or breeding program origin. The reference genotypes were allocated across the dendrogram, showing an introgression degree correlation. Two clusters (A and C) displayed a highly degree of admixture. The other cluster (B) was larger and divided into seven subclusters (B1 to B7). In subcluster B1, "Methley", "Morris", and "AU Amber" were allocated together, confirming their parentage with *P. cerasifera* [9]. In subcluster B2, several cultivars were closely related according to their pedigree, such as "Black Diamond" ("Angeleno" × OP), "Angeleno" ("Eldorado" × "Queen Ann") [55], and "Eldorado" (hybrid *P. salicina* × *P. simonii*, [71]). The presence of "Royal Diamond" in this subcluster indicates its possible parentage with "Angeleno", as has been previously suggested [9]. The closeness between "Alpha", a cultivar selected from wild trees of *P. maritima* Kerr. [72], and "Ruby Crunch" suggests possible common ancestry. The subcluster B3 was formed mostly by cultivars and selections from South Africa, where plum breeding represents a slightly different gene pool by the use of local cultivars as parents [9]. In B4, several cultivars with common genetic background were grouped: "African Rose", "Souvenir", and "Golden Kiss" from South Africa [13,73]; "Crimson Glo" and "Fortune" and their ancestors "Laroda" and "Queen Ann" [9]; "Rubirosa" and "Black Beaut" [74]. The subcluster B5 contained "Red Beaut" and its selection "606" [15] and comprised the reference genotypes "Mariposa" (*P. salicina*) and "Simon" (*P. simonii*), and their descendants "Santa Rosa" and its mutant "AU Rosa" [75]. "Red Beaut" and "Santa Rosa" were also grouped in the same subcluster in a previous report [76]. The closeness observed between "Burmosa" and "Red Beaut", in B5, and "Formosa", in B6, may be related to the use of "Formosa" as the parent of "Burmosa", which is the parent of "Red Beaut" [9]. Subcluster B6 comprises "Kelsey" (*P. salicina*) and its descendent "Songold", and "Howard Sun" and "Golden Japan", both cultivars with yellow flesh fruits, like "Songold" [6]. Subcluster B7 comprises a group of cultivars with common genetic background: "Laroda", "Black Amber", "Black Splendor" ("Black Amber" × OP), and "Queen Rosa", descendants of "Santa Rosa" [9]; and "Mariposa", "Rubysweet" ("Mariposa" × "Methley") [8], and "Byrongold", which are closely related to "Rubysweet" due to both having *P. cerasifera* in their parentage [9,49].

According to the DAPC clustering, the 161 accessions were distributed in seven groups, in which the main source of the total genetic variation was attributed to variance within accessions. The percentage variation among groups was low, resulting in high similarity among these groups. Six groups were clustered together and difficult to differentiate, which may be due to the use of the same cultivars as the parents in different breeding

programs, which could lead to a gene flow across the groups. Most genetic variation within groups rather than among groups has been also found in apricot accessions [41]. In almond, *He* values higher than *Ho* values, consistent with the results reported herein, have been attributed to the human selection and the exhausting breeding activity [37]. The highest values of P_A were found in G2, indicating that the cultivars in this group may have potential for use for breeding purposes to avoid bottleneck effects, and to be conserved in germplasm banks to maintain diversity [77–79]. In this study, moderate genetic diversity (*He*) was found in all groups and F_{IS} values ranged close to zero, indicating no excess of homo- or –hetero-zygotes. Similarly, F_{ST} values indicated a moderate degree of genetic differentiation [80], supporting the genetic structure obtained herein. The distribution of all accessions across the inferred seven groups corresponded with the genetic relationships observed in the UPGMA dendrogram, revealing a high correlation with the parentage background.

Further research is required to determine the optimal number of SSR markers needed for the analysis of genetic diversity and genetic structure. Although the addition of a new marker should not significantly affect the structure inferred by a sufficiently informative set of SSR [81], the optimal number of markers required to consistently infer the genetic structure in this and other fruit tree species remains unknown.

5. Conclusions

The SSR markers used herein were highly informative and revealed high genetic diversity within accessions. The entire population was structured in seven groups and confirmed the genetic relationships observed in the UPGMA dendrogram. Although a higher number of accessions were analyzed herein, the genetic diversity was similar to that of previous studies [46,51,53–55]. This may be due to the high number of modern cultivars and advanced selections of breeding programs analyzed, which reveal a bottleneck effect caused by the breeding system practices. The establishment of genetic relationships in Japanese plum-type accessions is highly complex due to their interspecific origin, but can be supported by the knowledge of the parentage lines in commercial cultivars. However, the genealogy of some of the ancestors widely used in most breeding programs is not available [5]. The use of chloroplast markers (cpDNA), and the application of next-generation sequencing technologies (NGS) and high-density SNP-based genotyping [19,76], may lead to additional insight into the degree of diversity among Japanese plum hybrids and the reconstruction of the genealogy of each cultivar.

The conservation of the native *Prunus* germplasm used in early plum breeding may help to maintain and improve the genetic diversity in Japanese plum-type cultivars. Unfortunately, only a few selections of this material are currently available for breeders [8]. The knowledge of the genetic diversity among Japanese plum-type accessions can enable more informed decisions by breeders for the selection of parents, to maintain biodiversity through germplasm conservation, and to find genotype–phenotype association patterns to be applied by producers and genetic research.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091748/s1, Figure S1: Clustering and DAPC Cross-validation. (a) Inference of the optimal number of clusters in the 161 Japanese plum-type accessions and (b) DAPC cross-validation for the optimal number of Principal Components (PCs) retained for the analysis in the seven predefined groups. Figure S2: Loading plots for the alleles contributions to the (a) Linear Discriminant Function 1 (LD1) and (b) Linear Discriminant Function 2 (LD2) of the DAPC when $K = 7$. Each plot computes the most informative and contributing alleles to the discriminant analysis. Table S1. Genealogical information of the analyzed accessions in which it is available. Table S2. Lower limit (below the diagonal) and upper limit (above the diagonal) of the 99% confidence interval based on 1000 bootstrap replicates.

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