

GENETIC DIFFERENTIATION OF CULTIVATED CITRUS FRUITS (*Citrus* spp.) IN COLOMBIA USING SSR MOLECULAR MARKERS

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ABSTRACT

Citrus is the second most important fruit crop in Colombia after bananas, with production taking place in 26 of the country's 32 departments. Oranges, sour limes, and mandarins are the most economically important crops in terms of area planted and production. Microsatellites were used to assess the genetic differentiation of oranges, mandarins, tangelos, grapefruits, and acid limes from the Colombian Agricultural Research Corporation AGROSAVIA germplasm bank and collection of micro-grafted plants at Palmira Research Center in Colombia. A total of 121 samples from eight citrus groups were analyzed with 30 fluoromarked simple sequence repeats (SSR) microsatellites. The mean expected heterozygosity and mean observed heterozygosity were 0.58 and 0.57, respectively, and the coefficient of genetic differentiation was 0.558, confirming very high genetic differentiation among the citrus groups evaluated. Microsatellites mCrCIR01B02, AMB5, Ci01C09, mCrCIR08B08, and Ci01C07 were the most informative, presenting a high number of alleles and polymorphic loci percentages of more than 45 %; in addition, they allowed the identification of unique alleles, which can be used to establish the genetic fingerprint of citrus. Genetic differentiation was achieved for seven out of the eight groups evaluated. The SSRs used failed to differentiate the orange groups, possibly due to genetic origin; for this group, other molecular markers are recommended.

Keywords: Germplasm, genetic identity, characterization, planting material, fluorolabeled markers.

INTRODUCTION

Citriculture is one of the most important agricultural and economic activities in the world since these are the most productive fruit trees. Citrus fruits, due to their agronomic and commercial use, are grouped into four large varietal groups: 1) oranges; 2) mandarins; 3) grapefruit; and 4) limes and lemons. Sweet oranges lead world production with 56 %, followed by mandarins with 32 %, limes and lemons with 12 %, and grapefruits or pomelos with 7 % (FAO, 2020). Citrus is grown in more than 130 countries with tropical, subtropical, and borderline subtropical/temperate

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climate zones (FAO, 2020). In Colombia, citrus production in 2021 was 1 450 071 Mg with a harvested area of 87 638 ha and an average yield of 15 Mg ha⁻¹ (MADR, 2021), demonstrating the economic importance of citrus genetic resources in the fruit industry, as they are produced commercially in 21 of the 32 departments in the country.

Citrus growers rely on both registered and unregistered nurseries with the Colombian Agricultural Institute (ICA) for the supply of planting material. Unfortunately, many of these nurseries do not have mother orchards to supply budwood and collect it from growers' farms, which poses a risk to the genetic traceability of the plants because of the possibility of mixing genotypes and the transmission of diseases through grafting. The citrus chain prioritized 15 cultivars, including oranges, mandarins, tangelos, grapefruits, and acid limes, as a bet of economic interest to develop the country's citrus industry.

In 2019, the ICA, through resolution 12816, established the requirements for the registration of nurseries and basic orchards, producers, and marketers of sexual and asexual seed (plant propagation material) of citrus. Nurseries are requested to ensure the genetic fidelity of the materials by using molecular markers as an alternative to the conventional method of morphological identification, which makes it difficult to distinguish between cultivars since some are differentiated by fruit traits and mother plants do not reach this phenological stage in nurseries.

In citrus, the use of molecular markers is useful for different purposes, such as the identification of hybrids and cultivars, characterization and classification of germplasm (García-Lor *et al.*, 2015), relationships between canopies and rootstocks (Rohini *et al.*, 2020), and obtaining genetic fingerprints, which allow detecting polymorphisms in DNA sequences and certifying the authenticity of the analyzed materials (Abdelaali *et al.*, 2018; Chungada *et al.*, 2021), since DNA profiles are not influenced by the environment.

One of the most widely used molecular markers are SSRs (simple repeated sequences). These markers are short DNA sequences consisting of motifs of 1 to 6 nucleotides repeated in tandem, of Mendelian inheritance. With a reliability higher than 95 %, they are co-dominant and highly informative. The number of repeats is variable and the degree of polymorphism increases with the total length of the microsatellite (Oliveira *et al.*, 2002).

There are several methods used to determine the alleles generated by SSRs, one of which is the use of primers with fluorescent marking at the 5' end, which reduces genotyping errors and allows the use of several fluorochromes for the marking of the primers, allowing the detection by fluorescence emission of several markers in the same PCR product. This method consists of amplifying several SSR loci simultaneously, which requires the optimization of PCR conditions and selecting primers that, with similar hybridization temperatures, do not interact with each other, and with alleles in different size ranges (Abdelaali *et al.*, 2018).

The objective of this study was to carry out genetic differentiation of oranges, mandarins, tangelos, grapefruits, and acid limes from AGROSAVIA germplasm bank and the collection of micro-grafted plants that are commercially grown, using

fluorolabeled SSR markers. This research aims to contribute to the identification of citrus that will give rise to the mother plants of commercial varieties for nurseries registered with the competent authority within the citrus certification program in Colombia. It will also provide valuable information on the distribution of genetic variability and relationships within and among various citrus species.

It was hypothesized that citrus cultivated in Colombia is highly diverse and polymorphic in the 30 SSR loci analyzed. No studies have been conducted in Colombia to evaluate the genetic differentiation of citrus grown in different regions of the country using codominant molecular markers such as SSRs.

MATERIALS AND METHODS

Plant material collection

A total of 121 plants from 17 citrus accessions were selected from the germplasm bank and the collection of micro-grafted plants preserved in a protected environment in an aphid-proof net house located at the Colombian Agricultural Research Corporation AGROSAVIA, Palmira Research Center, Colombia. Each accession was represented by seven replicates; in addition, Eureka lemon and Rangpur lime accessions were included as comparison genotypes (Table 1).

Table 1. Citrus accessions used for the genetic differentiation study.

Number	Scientific name	Common name	Origin*
1	<i>Citrus reticulata</i> Blanco × <i>C. paradisi</i> Macf.	Minneola tangelo	Meta (Colombia)
2	<i>C. reticulata</i> Blanco × <i>C. paradisi</i> Macf.	Orlando tangelo	Corsica (France)
3	<i>C. clementina</i>	Clemenules mandarin	IVIA 22-19 (Spain)
4	<i>C. reticulata</i> Blanco	ICA Bolo mandarin	Palmira (Colombia)
5	<i>C. reticulata</i> Blanco	Oneco mandarin	California (USA)
6	<i>C. reticulata</i> Blanco	Arrayana mandarin	Quindío (Colombia)
7	<i>C. sinensis</i> (L.) Osbeck.	Campbell Valencia orange	California (USA)
8	<i>C. sinensis</i> (L.) Osbeck.	Frost Valencia orange	California (USA)
9	<i>C. sinensis</i> (L.) Osbeck.	García Valencia orange	Valle del Cauca (Colombia)
10	<i>C. sinensis</i> (L.) Osbeck.	Olinda Valencia orange	California (USA)
11	<i>C. sinensis</i> (L.) Osbeck.	Salustiana orange	California (USA)
12	<i>C. sinensis</i> (L.) Osbeck.	Sweetie orange	Quindío (Colombia)
13	<i>C. sinensis</i> (L.) Osbeck.	Frost Washington orange	Valle del Cauca (Colombia)
14	<i>C. sinensis</i> (L.) Osbeck.	Valle Washington orange	Valle del Cauca (Colombia)
15	<i>C. aurantifolia</i> (Christm.) Swingle	Pajarito/Castillo acid lime	Quindío (Colombia)
16	<i>C. × latifolia</i> Tanaka ex Q. Jiménez	Tahití acid lime	Corsica (France)
17	<i>C. paradisi</i> Macf.	Star Ruby Grapefruit	California (USA)
18	<i>C. limon</i> (L.) Burm. F.	Eureka lemon	California (USA)
19	<i>C. limonia</i> Osbeck	Rangpur lime	California (USA)

*Source: SNBGVAA (National System of Plant Germplasm Banks for Food and Agriculture), Palmira Research Center.

DNA extraction

Citrus DNA was extracted from young leaves using the Doyle (1991) protocol. A 100 mg sample of tissue macerated with liquid nitrogen was resuspended with extraction buffer (100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0, 3 % CTAB (cetyltrimethylammonium bromide), 0.02 % B-Mercaptoethanol, and 1 % polyvinylpyrrolidone). The supernatant was washed with chloroform and precipitated with absolute ethanol and 5 M ammonium acetate. The pellet was eluted in deionized water treated with DEPC (diethylpyrocarbonate) and RNase (10 mg mL⁻¹). DNA was evaluated in terms of concentration (ng µL⁻¹) and quality (absorption spectra 260–280 and 260–230) by spectrophotometry using a NanoDrop™ 1000 (ThermoFisher, USA).

PCR conditions

Thirty SSR microsatellite markers (Table 2) were selected from studies conducted in different citrus populations (Oliveira *et al.*, 2002; Novelli *et al.*, 2006; Froelicher *et al.*, 2008). The markers were fluorescently labeled at the 5' end of the sense primer (Alpha DNA) and grouped into panels or multiplexes according to fluorochrome and amplification size (Table 2). The dye kit for SSR microsatellite labeling was DS-33 (Applied Biosystems, USA). The fluorochrome was selected based on the formation of panels where the amplification size and intensity of the bands at the time of visualization of the PCR products in 3 % agarose gels prior to labeling were taken into account. The markers with lower intensity were labeled with FAM and the rest with NED, VIC, and PET.

Table 2. SSR microsatellite markers used for citrus genetic differentiation analysis.

Primer name	Primer sequence F 5'-3'	Primer sequence R 5'-3'	Size (pb)	Melting temperature	Fluorophore
Ci01C07	TTG CTA GCT GCT TTA ACT TT	GTC ACT CAC TCT CGC TCT TG	236–278	55	FAM
mCrCIR01E02	TGA ATG GTA CGG GAA ATG C	CAG GGT CGG TGG AGA GGA T	153–175	53	VIC
mCrCIR01F04a	AAG CAT TTA GGG AGG GTC ACT	TGC TGC TGC TGT TGT TGT TCT	184–224	55	NED
mCrCIR08B08	GTG AAA GAG AGC AAG AAA AAT	AGC CAA AAA GAG AAG AAA TG	116–137	50	PET
Ci02A04	AAA GAA GTT AAA GAA AAA ATG	AGC GGT ATC GTA ATT CTC	156–165	55	FAM
mCrCIR01B02	AGC CAA AAA GAG AAG AAA TG	TTA GCA ATA TCA ACA TCA T	194–210	55	PET
Ci07C09	TAA GAT AAA AAC AGC ACA	GTG GCT GTT GAG GGG TTG	241–263	55	VIC
Ci07B09	TGC TGC TGC TGT TGT TGT TCT	AAA GAA GTT AAA GAA AAA ATG	186–196	50	NED

Table 2. Continue...

Primer name	Primer sequence F 5'-3'	Primer sequence R 5'-3'	Size (pb)	Melting temperature	Fluorophore
Ci01D11	TCG CTT TCT TAT TTC ACA CTC ACC	AGG ACA GAT GAC CCA GAT GAC A	197–210	55	FAM
Ci01G11	AAC ACC AAG AAG GAA GAG	TCG CTT TCT TAT TTC ACA CTC ACC	102–111	55	VIC
mCrCIR07H06	TTA TTT ATC TGT TTT CGC CTA	AGA TAC TTC ATT TGA TTG GC	180–194	56	PET
Ci01C09	GAC AGA ATG GGA GAG GAG A	TTG TCC CTT CCC TTT GTA	263–294	50	VIC
Ci02B07	CAG CTC AAC ATG AAA GG	TTG GAG AAC AGG ATG G	151–174	50	NED
Ci07C07	TAT CCA GTT TGT AAA TGA G	TGA TAT TTG ATT AGT TTG G	220–240	50	PET
mCrCIR06B07	CGG AAC AAC TAA AAC AAT	TGG GCT TGT AGA CAG TTA	91–109	50	FAM
Ci01D12	ATT TAT TTT CTT TCC CTT GTC TTA	TTT TCT TCT TCA TCT CTT TAC TGC	153–165	46	FAM
Ci02B10	TTT CAC AGC CAT CAC A	AAC ACC AAG AAG GAA GAG	180–200	50	PET
Ci07E06	AAT AAA CGC CCA CCT GAG AC	CAG TTG TTA AAA GGG AAG AAT GAA	223–244	55	NED
Ci08A10	GAGACTTA CTTGAATGAA	ATCTCGTGTGA AAATAA	152–165	50	VIC
Ci01H05	AAA ACA ACC AAA AGG ACA AGA TT	TTC AAA CTA AAC AAA CCA ACT CG	97–107	55	NED
Ci02F07	GCA GCG TTT GTT TTC T	TGC TGG TTT TCA GAT ACT T	169–200	55	PET
Ci06A05b	TCT CTG GTT GGT TTT TGT GA	ATG ATG AAA AGC AAG GGG	172–228	50	FAM
mCrCIR06A03	GGG TTG CGA CGA TGA GC	TCT CGG TTT GGC AGT TCG	231–237	55	VIC
Ci02F03	TAT CGA CAA CTC TTT CTC AT	TAA AGC GCA TGG ATA CT	158–171	55	PET
CCSM13	CTA GAG CCG AAT TCA CC	AAC AGC TAC CAA GAC ACC	160–177	53	NED
AMB5	CCC TGC ACA AAA ACT CAC AC	TGG GGG TGT TGA ATG GTA AT	108–143	56	FAM
AMB10	TAC TGT GGG GAA GGG ATC TG	GAC TCC TTC CAG CAC TTT GC	160–177	59	VIC
AMB8	TGA ACA TAT TTG CCC TTG GA	TTG TTT TGT GTG CTT GTG AGG	151–163	55	PET
CCSM6	ATC TGT GTG AGG ACT GAA	CCT CTA TTA ATG TGC CTG	204–248	51	FAM
CCSM147	AGA CTC ACG TAA CCT ACT TC	GCT ATG TTA TGA TAC GTC TG	109–133	54	VIC

pb: base pairs; F: forward; R: reverse.

For a final volume of 20 μ L, 60 ng of DNA, 1.2 mM Buffer $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 0.8 mM dNTP's, 0.48 mM for each primer (sense and antisense), 0.2 X bovine serum albumin (BSA), 10 % trehalose, and 1 U Taq polymerase (recombinant) (Thermo Scientific, USA) were added. The amplification process was performed on an SureCycler 8800 thermal cycler (Agilent, USA) with the following thermal profile: 94 °C for 5 min, one cycle (94 °C for 45 s, 48–61 °C (2 °C above aligning temperature) for 45 s, 72 °C for 1 min), 35 cycles (94 °C for 30 s, 48–59 °C for 30 s, 72 °C for 1 min), 72 °C for 10 min, and 20 °C for 5 min.

After PCR, the amplified products were visualized on 1.6 % agarose gels, which were run in 0.5 X TBE buffer at 90 V and 400 mA for 60 min, stained with GelRed TM at a concentration of 13.3 X added to the sample, and visualized using an Enduro GDS gel photodocumenter (Labnet International, USA). The detection of fluorescent molecular markers was performed using an ABI 3730 xl automated sequencer (Thermo Scientific, USA) with a conformational analysis polymer.

Data analysis

The sequenced alleles were read using the bioinformatics program GeneMapper (Applied Biosystems, USA), which identifies the amplified microsatellite fragments as fluorescent peaks. The size of the alleles was estimated in base pairs, and homozygotes or heterozygotes were identified, as appropriate. The genetic parameters of number of alleles (NA), observed heterozygosity (Ho), expected heterozygosity (He), and private alleles per population were estimated with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006). The polymorphic information content (PIC) of each SSR microsatellite was calculated with PowerMarker version 3.25 (Liu and Muse, 2005). The coefficients of genetic differentiation (Fst), inbreeding coefficient within populations (Fis), and total inbreeding coefficient (Fit) were determined with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006).

To determine the kinship relationships between the populations evaluated, the Nei genetic distance (Nei, 1973) was estimated. The clustering analysis was performed using the UPGMA method with program TFPGA version 1.3 software. Genetic distances were visualized in three dimensions with principal coordinate analysis (PCoA) and calculated with GenAlex version 6.5.0.1 (Peakall and Smouse, 2006). Genetic variability within and between populations was determined by analysis of molecular variance (AMOVA) using GenAlex version 6.5.0.1 (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

Population genetic diversity

A total of 7260 allelic data were obtained from the evaluation of 121 individuals, representing 19 citrus populations with 30 SSR markers, of which eight primer pairs

were excluded for having more than 30 % null data (mCrCIR01F04a, mCrCIR07H06, Ci01D12, Ci08A10, mCrCIR06A03, CCSM13, AMB10, and AMB8). Analyses were performed with the remaining 22 markers, where 118 alleles (NA) were identified, with an average of five alleles per locus (Table 3). These results contrast with those obtained by Abdelaali *et al.* (2018), who evaluated seven SSR markers in a population of 22 citrus accessions, identifying a total of 44 alleles and six alleles per locus. The mean observed heterozygosity (Ho) and mean expected heterozygosity (He) ranged from 0.07 to 0.93 and from 0.20 to 0.79, with means of 0.57 and 0.58, respectively. This indicates a high genetic diversity among the populations under study. The markers mCrCIR01B02, Ci01C09, AMB5, mCrCIR01E02, Ci02B07, Ci02F07, Ci06A05b, CCSM6, and Ci01C07 were the most informative, with Ho and He values higher than 0.67, which also presented the highest number of alleles. These results agree with those obtained by García-Lor *et al.* (2013), who studied the genetic organization of

Table 3. Genetic diversity parameters of 22 SSR microsatellite markers used in 122 cultivated citrus individuals.

Number	Microsatellite	NA	He	Ho	PIC	Fis	Fit	Fst
1	Ci01C07	7.00	0.79	0.91	0.76	-0.92	-0.05	0.45
2	mCrCIR01E02	6.00	0.73	0.79	0.69	-0.96	0.31	0.65
3	mCrCIR08B08	6.00	0.48	0.36	0.45	-0.97	0.03	0.51
4	Ci02A04	3.00	0.33	0.02	0.30	-0.67	0.54	0.72
5	Ci07B09	3.00	0.60	0.62	0.52	-0.93	0.52	0.75
6	Ci07C09	5.00	0.62	0.79	0.54	-0.97	0.10	0.54
7	mCrCIR01B02	7.00	0.68	0.73	0.63	-0.88	0.35	0.66
8	Ci01D11	4.00	0.46	0.05	0.39	-1.00	0.85	0.93
9	Ci01G11	2.00	0.20	0.19	0.18	-1.00	0.34	0.67
10	Ci01C09	6.00	0.71	0.89	0.67	-0.92	-0.14	0.41
11	Ci02B07	5.00	0.73	0.88	0.69	-1.00	-0.05	0.47
12	Ci07C07	3.00	0.46	0.19	0.41	-0.50	0.56	0.71
13	mCrCIR06B07	5.00	0.62	0.33	0.58	-0.84	0.44	0.70
14	Ci02B10	6.00	0.60	0.66	0.55	-0.96	0.09	0.53
15	Ci07E06	4.00	0.53	0.66	0.47	-1.00	-0.08	0.46
16	Ci01H05	5.00	0.26	0.07	0.24	-1.00	0.44	0.72
17	Ci02F07	5.00	0.74	0.71	0.70	-0.88	0.21	0.58
18	Ci06A05b	8.00	0.75	0.77	0.71	-0.97	0.26	0.63
19	Ci02F03	5.00	0.46	0.13	0.43	-0.94	0.55	0.77
20	AMB5	10.00	0.72	0.88	0.69	-0.98	-0.01	0.49
21	CCSM147	5.00	0.62	0.94	0.55	-0.93	-0.40	0.28
22	CCSM6	8.00	0.75	0.93	0.71	-0.98	-0.04	0.47
Average		5.36	0.58	0.57	0.54	-0.92	0.22	0.60
D.E		1.85	0.16	0.32	0.16	0.03	0.06	0.03

NA: number of alleles; He: expected mean heterozygosity; Ho: observed mean heterozygosity; PIC: percentage of polymorphic loci; Fis: inbreeding coefficient; Fit: total inbreeding coefficient; Fst: coefficient of genetic differentiation; D.E: standard deviation ($p \leq 0.05$), 10 000 permutations.

C. reticulata with 50 SSR markers, which included genotypes of *C. maxima*, *C. medica*, subgenus *Papeda*, *Fortunella* spp., *Microcitrus* spp., *Eremocitrus*, and *Poncirus trifoliata*, and obtained an average H_e of 0.71 with a range between 0.39 and 0.86, where Ci01C07, Ci02F07, Ci02B07, and mCrCIR01E02 presented H_e values higher than 0.57.

Polymorphic information content (PIC) ranged from 0.18 for marker Ci01G11 to 0.76 for marker Ci01C07 (Table 3). The average value for all markers was 0.54, indicating a medium level of discrimination. Similar values were reported by Chungada *et al.* (2021), who obtained an average PIC of 0.59 when evaluating in Nagpur (India) the genetic diversity of six citrus species with 14 SSRs. These results also coincide with those reported by Froelicher *et al.* (2008), who evaluated *C. reticulata* germplasm and obtained PIC values above 0.52. Conversely, Kumari *et al.* (2022) reported a PIC of 0.41 in 70 *C. aurantifolia* genotypes from the Indian breeding program.

In general, the F_{st} value for each of the SSR markers was high, with intervals between 0.276 and 0.927. The average value was 0.595 ($DS = 0.032$), which indicates very high genetic differentiation among the evaluated populations, according to Wright (1978). The average F_{is} value for the 22 markers was -0.918 (Table 3). The values ranged from -0.502 for marker Ci07C07 to -0.978 for marker Ci07C07. Negative F_{is} values were obtained for all 22 markers, indicating an excess of heterozygotes and that factors favoring variability are present in the populations studied.

In five of the 22 loci evaluated, we detected a total absence of homozygotes (Table 3). The total fixation index (F_{it}) obtained was 0.220 ± 0.064 , with no significant differences between markers ($p = 0.041$); the values ranged from -0.006 to 0.854. These results contrast with those reported by García-Lor *et al.* (2013), who obtained an average F_{it} of 0.41.

Genetic distance

The dendrogram for the 19 populations constructed using the UPGMA method with a bootstrap of 10 000 replicates showed a high percentage of loci (95.45 %) supporting the formation of the nodes, which indicates a high reliability of the SSR markers used in the genetic differentiation analysis. Seven groups were formed in the dendrogram. Group I included the orange populations (*C. sinensis*), Olinda Valencia, Valle Washington, García Valencia, Campbell Valencia, Salustiana, Frost Valencia, Sweet Orange, and Frost Washington, which were not genetically differentiated (Figure 1).

Citrus sinensis shows a high diversity in morphological, physiological, and agronomic aspects, which may be due to spontaneous somatic mutations (Barkley *et al.*, 2006), mainly affecting fruit characteristics; however, very little genetic variation has been detected using molecular markers such as random amplified polymorphic fragments (RAPD) (Malik *et al.*, 2012; Tripolitsiotis *et al.*, 2013), inter simple sequence repeats (ISSR) (Tripolitsiotis *et al.*, 2013), DNA amplification fingerprinting (DAF) (Luro *et al.*, 2012), even with SSR microsatellite markers (Novelli *et al.*, 2006; Ruiz *et al.*, 2018).

This low genetic differentiation in the orange group is possibly due to the fact that they share ancestral hybrids that maintain low variability due to reproduction by apomixis

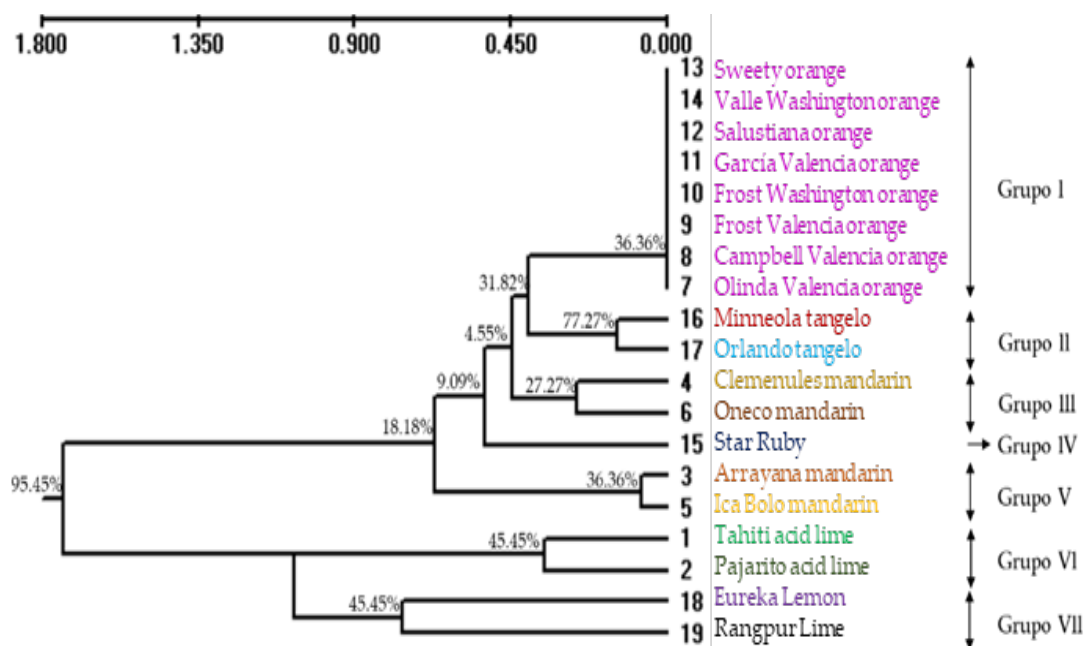


Figure 1. Nei genetic distances between the evaluated citrus populations, exemplified by a dendrogram constructed by using the UPGMA method, with a bootstrap of 10 000 permutations and the TFPGA program.

and that commercial variety multiplication is vegetative through the use of grafting (Shahnazari *et al.*, 2022). Some authors have reported the use of molecular markers for genetic differentiation in *C. sinensis*. Juibary *et al.* (2021) reported the use of SCoT markers for the analysis of the structure and genetic fingerprint of 25 sweet orange cultivars from Iran, managing to identify some unique loci that can differentiate these cultivars. Similarly, Sagawa *et al.* (2018) reported the use of DArT seq markers as some efficient markers to identify genetic diversity at the species level in *Citrus*.

Group II consisted of Orlando and Minneola tangelos (*C. reticulata* Blanco × *C. paradisi* Macf.). These populations had a dissimilarity index of 0.144. The mandarins Oneco and Clemenules were located in group III with a dissimilarity of 0.261, and they were clearly differentiated from Ica Bolo and Arrayana, which were associated in group V. *Citrus reticulata* exhibited high genetic variability, as determined by morphological characters and molecular markers (Froelicher *et al.*, 2008; Tripolitsiotis *et al.*, 2013; Suárez-Contreras *et al.*, 2020). Star Ruby grapefruit (*C. paradisi* Macf.) was placed in group IV.

When comparing the interspecific genetic distances of the genotypes evaluated, Star Ruby was found to be genetically distant from Tahiti acid lime (*C. × latifolia* Tanaka ex Q. Jimenez) (1.943) and Pajarito acid lime (*C. aurantifolia* (Christm.) Swingle) (1.894), but showed a closer genetic relationship with the orange group with a dissimilarity index

of 0.430. The SSR analysis revealed a genetic affinity among the orange, mandarin, tangelos, and grapefruit group (Figure 1), possibly explained by their phylogenetic and common ancestral relationships. *C. reticulata* has been considered as one of the main ancestral groups of cultivated citrus, along with *C. medica*, *C. maxima*, and *C. micrantha* (Nicolosi *et al.*, 2000; Wu *et al.*, 2018). *C. reticulata* includes gene introgression into other species, such as tangors (*C. reticulata* × *C. sinensis*) and tangelo; moreover, mandarin has been proposed to be a parent of *C. sinensis*. Roose *et al.* (2009) suggested that *C. sinensis* has 75 % *C. reticulata* and 25 % *C. maxima*, indicating backcross 1 (BC1) (*C. maxima* × *C. reticulata*) × *C. reticulata*.

This same hypothesis was also postulated in the orange genome sequencing work developed by Xu *et al.* (2013). However, this theory differs from the hypothesis of Nicolosi *et al.* (2000), who proposed that orange originated from a direct hybridization between *C. maxima* and *C. reticulata*. García-Lor (2013) contradicts these hypotheses and propose that the two orange parents are interspecific hybrids. Their results show that *C. sinensis* has three-allele genes that appear to be solely inherited from the *C. maxima* gene cluster and eight-allele genes from *C. reticulata*. Meanwhile, Star Ruby grapefruit has shared alleles from the *C. maxima*, *C. reticulata*, and *C. sinensis* gene pools (Luro *et al.*, 2012).

Group VI included acid limes (Tahiti and Pajarito), which showed a dissimilarity of 0.351 and were genetically distant from the rest of the genotypes; this could be partly explained by the fact that they do not share common ancestors. According to molecular data, *C. aurantifolia* is a hybrid between *C. medica* and *C. micrantha* (Nicolosi *et al.*, 2000; Ollitrault *et al.*, 2012), while *C. latifolia* is a triploid hybrid between *C. aurantifolia* and *C. limon* (Rouiss *et al.*, 2017), hence the close genetic relationship between these two species.

Group VII included Eureka lime (hybrid between *C. aurantium* × *C. medica*) and Rangpur lime (hybrid between *C. reticulata* × *C. medica*) with a dissimilarity index of 0.759, which showed closer genetic relationships with acid limes and a high genetic differentiation with the rest of the citrus populations. These results agree with those obtained by Nicolosi *et al.* (2000) and Kumar *et al.* (2022), who found close phylogenetic relationships between *C. medica*, *C. aurantifolia*, and *C. limon*.

The PCoA principal coordinate analysis in the first three coordinates explained 62.44 % of the total variability of the 19 citrus populations (Figure 2). The first coordinate explained 38.03 % of the genetic variability of the sour lime populations (Tahiti and Pajarito) and the oranges of the rest of the populations. The second coordinate explained 24.42 % and separated the Oneco, Ica Bolo, Arrayana, and Clemenules mandarin populations. Tangelos (Orlando and Minneola) were more closely related to the mandarin group, and grapefruit was located close to oranges, which can be explained by their phylogenetic relationships and common ancestors.

In general, citrus genotypes exhibited a distinctive genetic background; the greatest dispersion was observed among the acid limes in comparison to the rest of the populations because they do not share a common origin. The orange cultivars showed

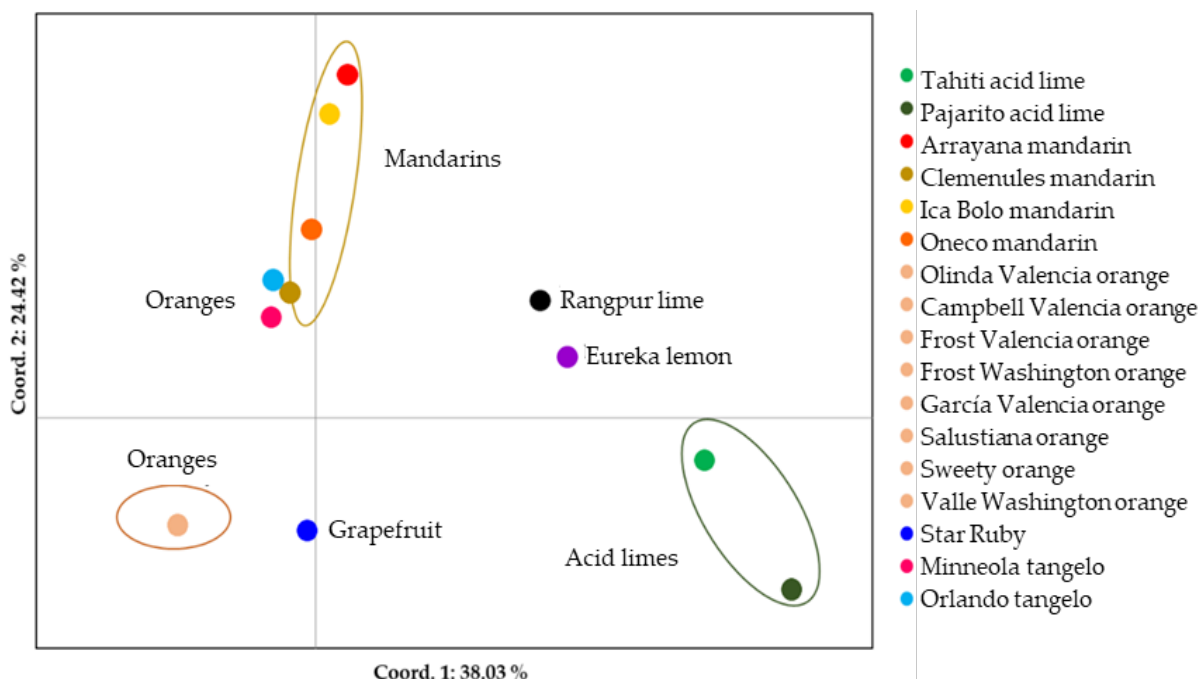


Figure 2. Two-dimensional principal coordinate analysis (PCoA) of genetic variation (*Nei* genetic distance) among citrus genotypes.

a high intragroup affinity, suggesting that they have a very close genetic basis. In contrast, the mandarin group was more variable. The reference species Eureka lemon and Rangpur lime showed closer relationships with the acid limes.

Genetic differentiation (*F_{st}*)

According to the Wright (1978) scale, acid limes presented *F_{st}* values above 0.442 when compared with oranges, mandarins, tangelos, and grapefruit, which indicates a very high genetic differentiation between these populations. The mandarin cultivars Arrayana and Ica Bolo showed intermediate genetic differentiation (*F_{st}* = 0.133), while Oneco and Clemenules had very high genetic differentiation (*F_{st}* = 0.253) (Figure 3). The Orlando and Minneola tangelos also showed high genetic differentiation (*F_{st}* = 0.185); however, the orange cultivars of the seven groups evaluated could not be genetically differentiated with the use of these microsatellites, so some other molecular markers could be used, such as single nucleotide polymorphisms (SNP) and insertion or deletion markers (InDel), which have been used by other authors for the genetic differentiation of orange germplasm (Ferrer *et al.*, 2021). For other citrus groups, these results confirm the discriminatory power of SSR microsatellites and agree with García-Lor *et al.* (2013), who were able to demonstrate a high degree of genetic differentiation between the different citrus taxa evaluated.

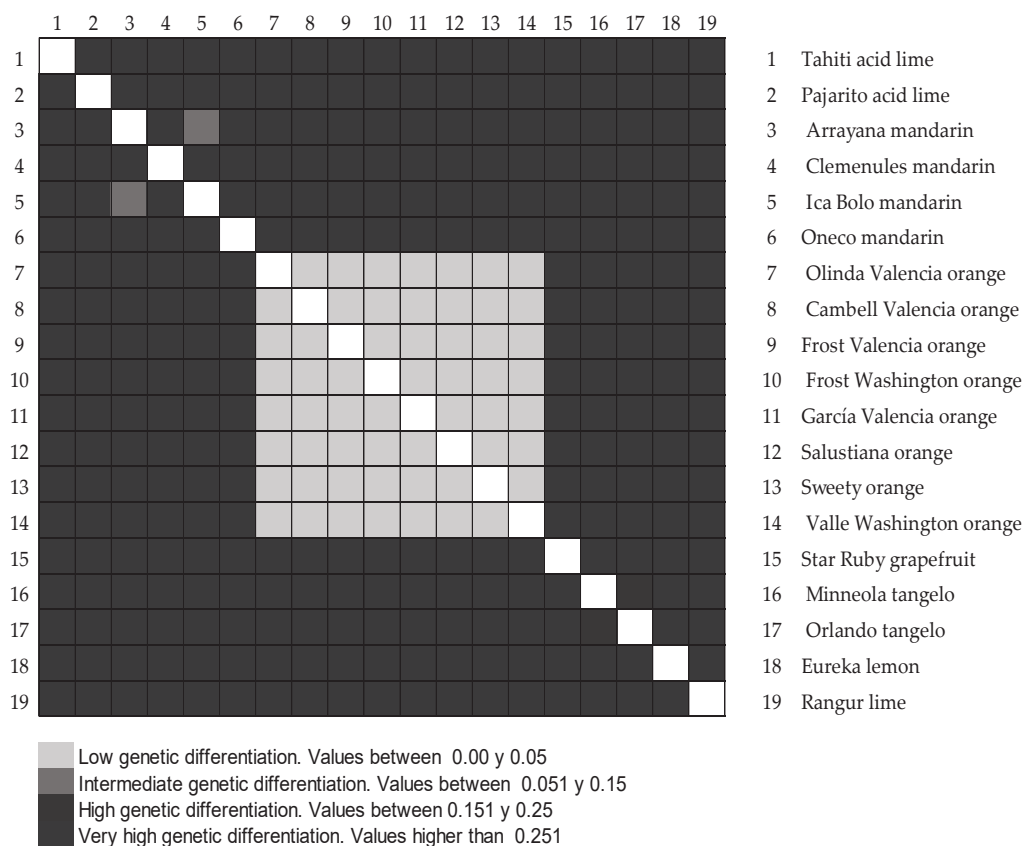


Figure 3. Heat map from Fst values among the evaluated populations. The darker the color tone, the greater the genetic differentiation between populations.

Analysis of molecular variance

The results of the AMOVA revealed that the greatest percentage of variation corresponded to differences between populations (89 %), followed by the variation between individuals within populations, which corresponded to 11 %, with significant ($p = 0.041$) and non-significant values (Table 4). The variation within populations did not show significant differences ($p = 1.0$), due to the asexual reproduction method used to obtain the plants. The 17 citrus varieties micrografted were obtained from the germplasm bank plant collection using shoot-tip grafting *in vitro*, and thus are considered clones.

Single alleles

The number of single alleles was estimated for Tahiti acid lime, Pajarito acid lime, Arrayana mandarin, Clemenules mandarin, Oneco mandarin, Star Ruby, Eureka lemon, and Rangpur lime cultivars (Table 5).

Table 4. Analysis of molecular variance (AMOVA) for the 19 citrus populations.

FV	G.L	SM	CM	S ² (%)	Fixation indexes
Between populations	18	543.26	46.586	89	Fst: 0.595 **
Between individuals within the population	104	84.208	0.745	11	Fis: -0.918 ns
Total	122	627.47	6.870		

FV: Sources of variation; G.L.: Degrees of freedom; SM: Sum of squares; S²: Total variance; CM: Mean square; *P-value based on 10 000 permutations, ** $p < 0.0001$; ns: not significant.

Table 5. Single alleles obtained in citrus cultivars by SSR markers.

Cultivar	Markers	Single alleles
Tahití acid lime	mCrCIR01B02, Ci02B10, AMB5	3
Pajarito acid lime	mCrCIR01B02, Ci07E06, Ci02B10, AMB5, CCSM6	5
Arrayana mandarin	AMB5	1
Clemenules mandarin	Ci01C09	1
Oneco mandarin	mCrCIR08B08	1
Ica Bolo mandarin	AMB5	1
Star Ruby grapefruit	mCrCIR08B08, Ci02A04, Ci07C09, mCrCIR01B02, Ci02B10, Ci01C09	6
Minneola tangelo	Ci01C07, Ci01C09	1
Orlando tangelo	Ci01C07	1
Eureka lemon	Ci01C07, Ci01H05, AMB5	3
Rangpur lime	Ci07C09, mCrCIR01B02, Ci01D11, Ci01C09, Ci01H05, Ci02F03, AMB5, CCSM6	7

The mCrCIR01B02, AMB5, Ci01C09, mCrCIR08B08, and Ci01C07 markers identified single alleles in sour lime, mandarin, grapefruit, Eureka lemon, and Rangpur lime. These SSR microsatellites were very informative, presenting a high number of alleles and a polymorphic information content above 45 % (Table 5), which can be suggested as genetic fingerprinting patterns (Chungada *et al.*, 2021).

CONCLUSIONS

There is a high genetic diversity among citrus (*Citrus* spp.) cultivated in Colombia. The fluoromarked SSR microsatellite markers used in this study allowed genetic differentiation of the groups of mandarins, tangelos, grapefruits, and acid limes within and between species. Oranges exhibit little genetic differentiation. These markers can

be used in studies of genetic diversity and variability, as well as to establish barcodes as genetic fingerprints of citrus in germplasm banks and populations with special agricultural characteristics, which can be used for genetic improvement programs or the implementation of strategies for the conservation of genetic resources.

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REFERENCES

- Abdelaali SB, Debbabi OS, Abdelaali NB, Hajlaoui MR, Mars M. 2018. Fingerprinting of on-farm conserved local Tunisian orange cultivars (*Citrus sinensis* (L.) Osbeck) using microsatellite markers. *Acta Biologica Cracoviensia Series Botanica* 60 (1): 83–93. <https://doi.org/10.24425/118045>
- Barkley NA, Roose ML, Krueger RR, Federici CT. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theoretical and Applied Genetics* 112 (8): 1519–1531. <https://doi.org/10.1007/s00122-006-0255-9>
- Chungada AS, Gahukar SJ, Akhare AA, Behere GT, Solanke AU, Patil SR. 2021. DNA fingerprinting in citrus for species identification. *The Pharma Innovation Journal* 10 (5): 306–309.
- Doyle J. 1991. DNA protocols for plants. In Hewitt GM, Johnston AWB, Young JPW. (eds.), *Molecular Techniques in Taxonomy*. NATO ASI Series 57. Springer: Berlin, Germany, pp: 283–293. https://doi.org/10.1007/978-3-642-83962-7_18
- FAO (Food and Agriculture Organization). 2020. Datos sobre cítricos fruta fresca y procesados, boletín estadístico 2020. United Nations Food and Agriculture Organization. Rome, Italy. <https://www.fao.org/3/cb6492en/cb6492en.pdf> (Retrieved: March 2022).
- Ferrer V, Costantino G, Paoli M, Paymal N, Quinton C, Ollitrault P, Tomi F, Luro F. 2021. Intercultivar diversity of sour orange (*Citrus aurantium* L.) based on genetic markers, phenotypic characteristics, aromatic compounds and sensorial analysis. *Agronomy* 11 (6): 1084. <https://doi.org/10.3390/agronomy11061084>
- Froelicher Y, Dambier D, Bassene JB, Costantino G, Lotfy S, Didout C, Beaumont V, Brottier P, Risterucci AM, Luro F, Ollitrault P. 2008. Characterization of microsatellite markers in mandarin orange (*Citrus reticulata* Blanco). *Molecular Ecology Resources* 8 (1): 119–122. <https://doi.org/10.1111/j.1471-8286.2007.01893.x>
- García-Lor A, Curk F, Snoussi-Trifa H, Morillon R, Ancillo G, Luro F, Navarro L, Ollitrault P. 2013. A nuclear phylogenetic analysis: SNPs, indels and SSRs deliver new insights into the relationships in the ‘true citrus fruit trees’ group (Citrinae, Rutaceae) and the origin of cultivated species. *Annals of Botany* 111 (1): 1–19. <https://doi.org/10.1093/aob/mcs227>

- Juibary PL, Seyedmehdi FS, Sheidai M, Noormohammdi Z, Koohdar F. 2021. Genetic structure analysis and genetic finger printing of sweet orange cultivars (*Citrus sinensis* (L.) Osbeck) by using SCoT molecular markers. *Genetic Resources and Crop Evolution* 68 (4): 1645–1654. <https://doi.org/10.1007/s10722-020-01092-2>
- Kumar JPT, Thirugnanavel A, Upadhyay DY, Kamde SA, Jalamkar PR, Murkute AA. 2022. Genetic diversity and population structure of sweet orange [*Citrus sinensis* (L.) Osbeck] germplasm of India revealed by SSR and InDel markers. *bioRxiv*. <https://doi.org/10.1101/2022.01.11.475964>
- Liu J, Muse SV. 2005. Power Marker: Integrated analysis environment for genetic marker data. *Bioinformatics* 21 (9): 2128–2129. <https://doi.org/10.1093/bioinformatics/bti282>
- Luro F, Venturini N, Costantino G, Paolini J, Ollitrault P, Costa J. 2012. Genetic and chemical diversity of citron (*Citrus medica* L.) based on nuclear and cytoplasmic markers and leaf essential oil composition. *Phytochemistry* 77: 186–196. <https://doi.org/10.1016/j.phytochem.2011.12.013>
- Kumari S, Sharma A, Bakshi P, Salgotra R, Sharma M, Gupta V, Rai GK. 2022. Characterization of acid lime genotypes using SSR markers. *Indian Journal of Horticulture* 79 (4): 394–401. <https://doi.org/10.5958/0974-0112.2022.00055.x>
- MADR (Ministerio de Agricultura y Desarrollo Rural de Colombia). 2021. Cadena de cítricos: indicadores e instrumentos. Primer semestre 2021. Ministerio de Agricultura y Desarrollo Rural. Bogotá, Colombia. <https://sioc.minagricultura.gov.co/Citricos/Documentos/2021-03-31%20Cifras%20Sectoriales.pdf> (Retrieved: March 2022).
- Malik SK, Rohini MR, Kumar S, Choudhary R, Pal D, Chaudhury R. 2012. Assessment of genetic diversity in Sweet Orange [*Citrus sinensis* (L.) Osbeck] cultivars of India using morphological and RAPD Markers. *Agricultural Research* 1 (4): 317–324. <https://doi.org/10.1007/s40003-012-0045-3>
- Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* 70 (12): 3321–3323. <https://doi.org/10.1073/pnas.70.12.3321>
- Nicolosi E, Deng ZN, Gentile A, la Malfa S, Continella G, Tribulato E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics* 100 (8): 1155–1166. <https://doi.org/10.1007/s001220051419>
- Novelli MV, Cristofani M, Souza AA, Machado MA. 2006. Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* L. Osbeck). *Genetics and Molecular Biology* 29 (1): 90–96. <https://doi.org/10.1590/S1415-47572006000100018>
- Oliveira AC, Garcia AN, Cristofani M, Machado MA. 2002. Identification of citrus hybrids through the combination of leaf apex morphology and SSR markers. *Euphytica* 128 (3): 397–403. <https://doi.org/10.1023/A:1021223309212>
- Ollitrault F, Terol J, Martin AA, Pina JA, Navarro L, Talón M, Ollitrault P. 2012. Development of indel markers from *Citrus clementina* (Rutaceae) BAC-end sequences and interspecific transferability in *Citrus*. *American Journal of Botany* 99 (7): 268–273. <https://doi.org/10.3732/ajb.1100569>
- Peakall R, Smouse PE. 2006. Genalex 6: genetic analysis in Excel. Population genetic programa for teaching and research. *Molecular Ecology Notes* 6 (1): 288–295. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>
- Rohini MR, Sankaran M, Rajkumar S, Prakash K, Gaikwad A, Chaudhury R, Malik SK. 2020. Morphological characterization and analysis of genetic diversity and population structure in *Citrus × jambhiri* Lush. using SSR markers. *Genetic Resources and Crop Evolution* 67 (5): 1259–1275. <https://doi.org/10.1007/s10722-020-00909-4>

- Roose ML, Federici CT, Mu L, Kwok K, Vu C. 2009. Map-based ancestry of sweet orange and other citrus variety groups. *In* Second International Citrus Biotechnology Symposium 28. Tremestieri Etneo, Italy.
- Ruiz M, Pensabene-Bellavia G, Quiñones A, García-Lor A, Morillon R, Ollitrault P, Primo-Millo E, Navarro L, Aleza P. 2018. Molecular characterization and stress tolerance evaluation of new allotetraploid somatic hybrids between Carrizo citrange and *Citrus macrophylla* W. rootstocks. *Frontiers in Plant Science* 9: 901. <https://doi.org/10.3389/fpls.2018.00901>
- Rouiss H, Bakry F, Froelicher Y, Navarro L, Aleza P, Ollitrault P. 2017. Origin of *C. latifolia* and *C. aurantiifolia* triploid limes: the preferential disomic inheritance of doubled-diploid 'Mexican' lime is consistent with an interploid hybridization hypothesis. *Annals of Botany* 121 (3): 571–585. <https://doi.org/10.1093/aob/mcx179>
- Sagawa CHD, Cristofani-Yaly M, Novelli VM, Bastianel M, Machado MA. 2018. Assessing genetic diversity of Citrus by DArT_seq™ genotyping. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* 152 (4): 593–598. <https://doi.org/10.1080/1263504.2017.1341438>
- Shahnazari N, Noormohammadi Z, Sheidai M, Koohdar F. 2022. A new insight on genetic diversity of sweet oranges: CAPs-SSR and SSR markers. *Journal of Genetic Engineering and Biotechnology* 20 (1): 105. <https://doi.org/10.1186/s43141-022-00393-6>
- Suárez-Contreras LY, Arango-Tolosa MJ, Sánchez-Pabón I. 2020. Molecular characterization of mandarins (*Citrus reticulata* Blanco) using ISSR markers. *Revista Colombiana de Ciencias Hortícolas* 14 (2): 168–177. <https://doi.org/10.17584/rcch.2020v14i2.9397>
- Tripolitsiotis C, Nikoloudakis N, Linos A, Hagidimitriou M. 2013. Molecular characterization and analysis of the Greek citrus germplasm. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 41 (2): 463–471. <https://doi.org/10.15835/nbha4129302>
- Wright S. 1978. The relation of livestock breeding to theories of evolution. *Journal of Animal Science* 46 (5): 1192–1200. <https://doi.org/10.2527/jas1978.4651192x>
- Wu GA, Terol J, Ibanez V, López-García A, Pérez-Román E, Borredá C, Domingo C, Tadeo FR, Carbonell-Caballero J, Alonso R *et al.* 2018. Genomics of the origin and evolution of *Citrus*. *Nature* 554 (7692): 311–316. <https://doi.org/10.1038/nature25447>
- Xu Q, Chen LL, Ruan X, Chen D, Zhu A, Chen C, Bertrand D, Jiao WB, Hao BH, Lyon MP *et al.* 2013. The draft genome of sweet orange (*Citrus sinensis*). *Nature Genetics* 45 (1): 59–66. <https://doi.org/10.1038/ng.2472>