Outcrossing Rate and Genetic Variability in Mexican Race Avocado

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ABSTRACT. The blooming behavior of the avocado *Persea americana* Mill. is a sophisticated mechanism that prevents effective self-pollination, enables close pollination, and encourages cross-pollination. However, there is no information on outcrossing rate among Mexican race avocado genotypes (*P. americana* var. *drymifolia* Schltdl. & Cham.). Therefore, the objective of this study was to assess the outcrossing rate and genetic variability in progenies of Mexican race avocado genotypes by simple sequence repeat (SSR) and intersimple sequence repeat (ISSR) markers. SSR marker analysis showed a considerable genetic differentiation among avocado families [total expected heterozygosity (H_e) = 0.540], whereas the total heterozygosity value observed (H_o = 0.098) showed the presence of genetic structure per family. The total Nei's unbiased average heterozygosity (nH_e) value found with ISSR markers was 0.482. The results of the analysis of molecular variance (AMOVA) combining both type of markers showed that genetic variation within avocado families was 58.6%, and among families was 41.6% ($P < 0.0001$). The outcrossing population rate in *P. americana* var. *drymifolia* was 0.774 ± 0.091 (sd), and the 'Criollo 3' and 'Plátano Temprano' families showed the lowest (-0.083 ± 0.031) and highest (0.814 ± 0.060) outcrossing rates, respectively. Variability in outcrossing rate depends on many factors, including edaphoclimatic, agronomic, and genetic, and needs to be considered to define strategies for the conservation and genetic improvement of outstanding native genotypes. SSR and ISSR markers are useful for estimating genetic variability within and among families of avocado, as well as for determining the outcrossing rates among closely related individuals and with a rather small sample size.

Avocado is one of the most economically important sub-tropical/tropical fruit crops in the world (Bost et al., 2013) and is culturally valuable in Mexico. It is member of the Lauraceae family and there are three different ecological races or botanical varieties widely recognized by horticulturists: Mexican (*P. americana* var. *drymifolia*), Guatemalan (*P. americana* var. *guatemalensis* L.O. Williams.), and West Indian (*P. americana* var. *americana* Mill.), which are originally from highland Mexico, highland Guatemala, and lowland (coastal) Guatemala to Costa Rica, respectively (Galindo-Tovar et al., 2008).

As a diploid species, *P. americana* has 24 chromosomes ($2n = 24$) and a genome size of ≈ 907 Mbp (López-Gómez et al., 2007). It presents a blooming mechanism called synchronous protogynous dichogamy. Its flowers are perfect, and the male and female parts are functional at different times of the day. The avocado genotypes can be classified as blooming types A or B. Type A genotypes open in the morning as functional female flowers, and then reopen the next day in the afternoon as functional male flowers; type B genotypes open in the afternoon as functional female flowers, and the next morning as functional male flowers. It is thought that this mechanism promotes outcrossing between avocado trees of opposite blooming types (Borrone et al., 2008).

Molecular markers based on DNA are identifiable DNA sequences found at specific locations on the chromosomes and transmitted by the Mendelian laws of inheritance from one generation to the next one. SSRs, also known as microsatellites, are tandemly arranged repeats of mono, di-, tri-, tetra-, and pentanucleotides with different lengths of repeat motifs (Bhat et al., 2010). SSR markers are characterized by their hyper-variability, reproducibility, codominant nature, locus specificity, and random genome-wide distribution (Jiang, 2013). ISSRs are segments of DNA flanked at both ends by such microsatellite sequences. Based on a single primer having microsatellite sequence complementary to microsatellite regions in the genome, random DNA segments in the genome can be amplified by polymerase chain reaction (PCR; segments within an amplifiable size range) and used as markers. These markers are assumed dominant and detect variation in the size of the genomic region between the two adjacent microsatellite sequences used as the primers binding sites (Ng and Tan, 2015; Singh and Singh, 2015). To minimize human and stochastic errors, only clearly distinctive bands with strong intensities and within a standardized size range should be scored (Ng and Tan, 2015).

Molecular markers in avocado have been used to assess genetic diversity among and within racial groups (Boza et al., 2018; Cuiris-Pérez et al., 2009; Gross-German and Viruel, 2013), in phylogenetic studies (Ashworth and Clegg, 2003; Reyes-Alemán et al., 2013), and in construct linkage maps (Borrone et al., 2009); to identify quantitative trait loci (Sharon et al., 1998), and in outcrossing studies (Alcaraz and Hormaza, 2011; Borrone et al., 2008; Chen et al., 2007; Garner et al., 2008; Kobayashi et al., 2000; Schnell et al., 2009; Violi et al., 2009). Outcrossing rate studies based on molecular markers

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have been done in different plant species such as *Brassica carinata* A. Braun (Teklewold et al., 2013), *Foeniculum vulgare* Mill. (Salami et al., 2017), *Olea europaea* L. (Souza et al., 2012), *Passiflora alata* Curtis (Ferreira et al., 2010), *Pinus densiflora* Sieb. and Zucc. (Lian et al., 2001), *Sorghum bicolor* (L.) Moench (Adugna, 2012), *Spondias tuberosa* Arruda (Fernandes and Cândido de Souza, 2013), *Theobroma cacao* L. (Efombagn et al., 2009), *T. grandiflorum* (Willd. ex Spreng.) Schum (Alves et al., 2003), and *Warburgia ugandensis* Sprague (Muchugi et al., 2008) to propose effective schemes for germplasm conservation and/or to establish strategies of selection and improvement.

In avocado, most outcrossing studies have been done in cultivars such as Hass, Bacon, Zutano, and others (Kobayashi et al., 2000; Schnell et al., 2009) to detect potential donors of pollen and to calculate the correlation between outcrossing and yield (Kobayashi et al., 2000), to determine the incidence and effectiveness of self- and cross-pollinated avocado fruit (Davenport et al., 2006), and to assess the relation among outcrossing rate with yield and alternate bearing (Garner et al., 2008). In addition, the outcrossing rate has been used to assess the proportion of progeny resulting from parents with complementary blooming (Borrone et al., 2008; Violi et al., 2009) and to determine whether an outcrossed progeny is more tolerant to *Phytophthora* root rot than a self-pollination progeny (Violi et al., 2009). However, the outcrossing rate results presented in all studies show variation according to location, tree distribution within orchards, and genotypes used as potential pollen donors.

In past decades, native Mexican avocado populations have been threatened due to the destruction of their ecosystems—a result of the opening of new areas for agricultural and livestock production; factors such as overgrazing, forest fires, urban area expansion, and logging (Torres-Gurrola et al., 2009); the presence of soil diseases and drought (Barrientos-Priego and López-López, 2002); and their replacement by improved cultivars or high productivity hybrids (Rincón-Hernández et al., 2011). Therefore, it is important to understand the biology of pollination to develop effective strategies to conserve the diversity of these genetic resources for future crop improvement.

It is assumed that avocado blooming behavior is a sophisticated mechanism that prevents effective self-pollination (within a flower), enables close pollination (between neighboring flowers within a tree), and encourages cross-pollination (between different cultivars), according to Ish-Am (2005). There is no information on outcrossing rate among Mexican race avocado genotypes. Therefore, the objective of this study was to assess the outcrossing rate and genetic variability in progenies of Mexican race avocado genotypes by SSR and ISSR markers, which will enable the establishment of new strategies for the conservation and genetic improvement of outstanding native genotypes.

Materials and Methods

Plant material and DNA extraction

This research was carried out at the Agronomy School of Universidad Autónoma de Nuevo León in Mexico during Summer 2017. Progenies of 10 Mexican race avocado genotypes from the state of Nuevo León with contrasting morphological traits were used. Genotypes ‘Bola’, ‘Criollo 3’, ‘Criollo 6’, ‘Leonor’, ‘María Elena’, ‘Plátano’, ‘Plátano Delgado’,

‘Plátano Temprano’, and ‘Todo el Año’ were in an orchard at the municipality of Aramberri (lat. 24°06’28.8”N, long. 99°49’42.4”W; elevation, 1080 m). These genotypes are local germplasm resources that have been adapted and selected empirically by the farmers. ‘Silvestre’ genotype (single tree) was growing in a forest environment at the municipality of General Zaragoza (lat. 23°57’18.573”N, long. 99°46’17.27”W; elevation, 1432 m). Each genotype is a seed parent (mother tree) and its progeny is a half-sib family. Twenty mature fruit were collected from each genotype (one tree per genotype) and kept separately. Seeds from each fruit were extracted and washed. The seeds were raised under greenhouse conditions in individual pots containing a mix of peatmoss, perlite, and vermiculite in proportions 3:1:1 as a substrate. After 3 months of establishment, 12 healthy seedlings were selected randomly by genotype for genomic DNA extraction. DNA was extracted from leaves using the extraction buffer described by Cheng et al. (1997), during which the cetyltrimethylammonium bromide concentration was increased to 650 μ M. For DNA precipitation and washing steps, washing buffer solutions 1 and 2 indicated by Hoisington et al. (1994) were used. DNA was also isolated from mother trees. The integrity, quality, and quantity of DNA were assessed in 0.8% agarose gel and by spectrophotometry (Cheng et al., 1997). The DNA was diluted to a working concentration of 10 ng· μ L⁻¹ in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0).

SSR markers

The PCR reactions were carried out in a 15- μ L volume containing molecular biology-grade water (Sigma-Aldrich, St. Louis, MO) 1× PCR buffer (Promega, Madison, WI), 2 mM MgCl₂, 0.4 mM deoxyribonucleotide triphosphate (dNTP) mix (Promega), 0.01% polyethylene glycol sorbitan monolaurate (Tween® 20, Sigma-Aldrich), 0.4 μ M each primer, 25 ng DNA template, and 0.5 U GoTaq® Flexi DNA Polymerase (Promega). Four primer pairs developed by Gross-German and Viruel (2013) and one primer pair developed by Ashworth et al. (2004) were used (Table 1). DNA amplifications were conducted in a thermal cycler (Applied Biosystems Veriti PN 4375786; Thermo Fisher Scientific, Waltham, MA). The thermal program started with a denaturing step at 94 °C for 1 min followed by 35 cycles of 30 s at 94 °C, 30 s at the specific annealing temperature for each primer, and 1 min at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were separated by electrophoresis in 4% agarose gel at 120 V for 3.5 h, stained with SYBR Gold nucleic acid gel stain (Invitrogen, Eugene, OR), and visualized using an ultraviolet light transilluminator. The band sizes were estimated using a 25-bp DNA ladder (HyperLadder; Bioline, Boston, MA) and scored by their molecular weight.

ISSR markers

PCR was carried out in a 25- μ L volume containing molecular biology-grade water, 1× PCR buffer (Promega), 3 mM MgCl₂, 0.25 mM dNTP mix (Promega), 0.2 μ M primer, 50 ng DNA template, and 1 U GoTaq® Flexi DNA Polymerase (Promega). Four ISSR primers were used because of their high percentage of polymorphism (>80%) reported previously by Reyes-Alemán et al. (2013) (Table 2). DNA amplifications were conducted in a thermal cycler (Applied Biosystems Veriti PN 4375786). The thermal program started with a denaturing step at 94 °C for 2 min followed by 45 cycles of 1 min at 94 °C, 1 min at the specific annealing temperature for each primer, and

Table 1. Simple sequence repeat description and genetic variation parameters per locus of Mexican race avocados.

Marker	Primer sequences 5' → 3'	Annealing temp (°C)	Na	Ne	Ho	He	PIC	Size range (bp)
LMAV01	F-CAGGTATGCTTGGATTCTG R-TGAGCGTACAAGCACTAAAA	55	6	2.85	0.00	0.65	0.59	175–255
LMAV02	F-TGGTTTAAGGAAGGGTGTTA R-TACAGCCTCCATTTTGT	55	5	1.50	0.17	0.34	0.32	200–260
LMAV13	F-AGGCACTGTGTTTTGT R-TCATCACGGA CT CACAAATA	60	3	2.92	0.13	0.66	0.58	240–275
LMAV24	F-CCCTTTCCAAGTTTCCTAAC R-GTGCAGAGGTAAGTCACCAT	60	2	1.96	0.19	0.49	0.37	250–270
AVD.001	F-GTTTCCAAGCGACTCACGAG R-GATTCCATGCTGAATTGCCG	60	4	2.27	0.00	0.56	0.47	275–340

Na = number of alleles per locus; Ne = number of effective alleles; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphism information content.

Table 2. Intersimple sequence repeat (ISSR) description and summary per primer of Mexican race avocados.

Marker	Primer sequences 5' → 3'	Annealing temp (°C)	No. of bands	No. of polymorphic bands	Amplification (%)	nHe	PIC
ISSR1	(AC) ₈ YG	53	14	14	47	0.50	0.37
ISSR2	(GACA) ₄	53	8	8	42	0.49	0.37
ISSR4	(TCC) ₅ RY	53	5	5	63	0.47	0.36
ISSR5	(GA) ₈ YC	48	6	4	63	0.47	0.36

nHe = Nei's unbiased average heterozygosity; PIC = polymorphic information content.

2 min at 72 °C; and a final extension step of 7 min at 72 °C. PCR products were separated by electrophoresis in 3% agarose gel at 120 V for 3 h, stained with SYBR Gold nucleic acid gel stain (Invitrogen), and visualized in ultraviolet light. The band sizes were estimated using a 100-bp DNA ladder (Axygen Biosciences, Union City, CA) and scored into a binary character matrix (1 for presence and 0 for absence).

Data analyses

GENETIC VARIATION. With SSR data, the number of alleles per locus (Na), number of effective alleles (Ne), Ho, He, and polymorphism information content (PIC) were calculated. For the ISSR data description, the number of total bands (Nb), number of polymorphic bands (Np), PIC, percentage of amplification, and nHe were calculated. Along with the genotypic data of each kind of marker, a hierarchical genetic variation among families, including individuals within families, was estimated using nonparametric AMOVA, with 10,000 permutations using the distance method (Balzarini et al., 2010). These analyses were performed using InfoGen version 2016 (Balzarini and Di Rienzo, 2016).

OUTCROSSING RATE PARAMETERS. For population level, the data of both types of markers were analyzed using the multi-locus mixed mating program, version 3.2 (Ritland, 2002), which assumes that progenies are the product of either outcrossing or self-fertilization. The estimated parameters were multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), full siblings rate ($t_m - t_s$), fixation index for maternal genotypes (F_m), correlation of outcrossed paternity (r_p), probable number of pollinators [$1/r_p$], and correlation of selfing among loci (r_s). These parameters were estimated using maximum likelihood procedures and the Newton-Raphson method. Standard deviation for each parameter was calculated based on 500 bootstraps. For family level, only t_m , t_s , and $t_m - t_s$ were estimated using codominant data.

Fig. 1. Example of electrophoretic profiles of amplified genomic DNA fragments of 'Leonor' avocado family. (A) Profile obtained with the simple sequence repeat primers LMAV02. (B) Profile obtained with the intersimple sequence repeat primer ISSR2. L = ladder; 1–12 = seedlings; M = mother tree.

Results and Discussion

PERFORMANCE OF SSR MARKERS. Five polymorphic SSR loci amplified a total of 20 alleles (Fig. 1A), with an average of four alleles per locus, varying from two (LMAV24) to six (LMAV01), and with a size range of 175 to 340 bp (Table 1). The allele frequency mean was 0.192 and frequencies varied from 0.008 to 0.480. The average Ne (number of effective alleles) value was 2.3. Ho was 0.098 and ranged from 0.00 (LMAV01, AVD.001) to 0.19 (LMAV24). He was 0.54 and ranged from 0.34 to 0.66 for loci LMAV02 and LMAV13, respectively. The average PIC value was 0.47, with the greatest value found in LMAV01 (PIC = 0.59) and the least in LMAV02 (PIC = 0.32). These PIC values are like those

Table 3. Heterozygosity estimates, multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), and full siblings rate ($t_m - t_s$) per Mexican race avocado family.

Family	SSR		ISSR	t_m	t_s	$t_m - t_s$
	Ho	He	nHe			
Bola	0.083	0.492	0.488	0.420 (0.143) ^z	0.079 (0.027)	0.341 (0.140)
Criollo 3	0.067	0.212	0.478	-0.083 (0.031)	-0.021 (0.008)	-0.062 (0.030)
Criollo 6	0.017	0.167	0.496	0.086 (0.082)	0.018 (0.017)	0.068 (0.080)
Leonor	0.233	0.177	0.504	0.687 (0.067)	0.297 (0.048)	0.390 (0.047)
María Elena	0.083	0.334	0.498	0.109 (0.091)	0.059 (0.056)	0.050 (0.072)
Plátano	0.167	0.291	0.495	0.436 (0.152)	0.149 (0.052)	0.287 (0.143)
Plátano Delgado	0.050	0.340	0.511	0.170 (0.110)	0.082 (0.053)	0.088 (0.096)
Plátano Temprano	0.267	0.349	0.494	0.814 (0.060)	0.362 (0.042)	0.452 (0.043)
Silvestre	0.000	0.278	0.505	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)
Todo el Año	0.017	0.341	0.488	0.085 (0.081)	0.016 (0.015)	0.069 (0.080)
Total	0.098	0.540	0.482	0.774 (0.091)	0.431 (0.063)	0.342 (0.113)

^zSD based on 500 bootstraps.

SSR = simple sequence repeat; ISSR = intersimple sequence repeat; Ho = observed heterozygosity; He = expected heterozygosity; nHe = Nei's unbiased average heterozygosity.

Table 4. Analysis of molecular variance for 10 families of *Persea americana* var. *drymifolia* based on simple sequence repeat and intersimple sequence repeat markers with 10,000 permutations using the distance method.

Source of variation	Df	SS	MS	Variance component	Variation (% total)	P value
Among populations	9	378.294	42.033	3.29	41.40	<0.0001
Within populations	104	483.592	4.650	4.65	58.60	<0.0001
Total	113	861.886	7.627			

SS = sum of squares; MS = mean squares.

reported by Efombagn et al. (2009), which ranged from 0.19 to 0.82. The number of alleles per locus was expected to be low as a result of the closely related individuals in the populations. The results obtained for Na, He, and PIC indicate that these kinds of markers are useful for determining genetic variation within and among populations of closely related individuals (Efombagn et al., 2009), as well as for calculating the degree of inbreeding within and among populations (Boza et al., 2018; Gross-German and Viruel, 2013).

PERFORMANCE OF ISSR MARKERS. A total of 33 clear and reproducible bands were amplified with the four ISSR primers used (Fig. 1B), with an average of 8.25 bands per primer and a size range of 500 to 3000 bp. Thirty-one amplified bands (93%) were polymorphic, and three of four primers were 100% polymorphic. The Nb and amplification per primer ranged from 5 (ISSR4) to 14 (ISSR1), and from 42% (ISSR2) to 63% (ISSR4 and ISSR5), respectively. The PIC and nHe values were very similar for all primers, with averages of PIC = 0.365 and nHe = 0.482 (Table 2). The average values of Nb, the percentage of polymorphic bands, and the average PIC value per ISSR primer are like those reported by Fasih et al. (2013) in populations of *Festuca arundinacea* Schreb. They obtained values of Nb that ranged from 4 to 13, percentages of polymorphic bands from 50% to 100%, and an average PIC value of 0.32. These results confirm the usefulness of the ISSR markers in estimating genetic variation among relatively close individuals exhibiting low levels of polymorphism (Reyes-Alemán et al., 2013), and they contrast with the values obtained when it comes to distinguishing genotypes with diverse origins (average Nb = 22, 100% of polymorphic bands, and average PIC = 0.92) (Cuiris-Pérez et al., 2009).

HO, HE, AND AMOVA FOR AVOCADO FAMILIES. Considering that heterozygosity is a measure of genetic diversity (Galindo-Tovar et al., 2011), with SSR markers, the total He value (0.540) showed a considerable genetic differentiation among avocado families, whereas the total Ho value (0.098) showed the presence of genetic structure per family (Table 3). The 'Silvestre' family presented the lowest value of Ho (0.000) and the 'Plátano Temprano' family, the highest value Ho (0.267). On the other hand, He values ranged from 0.167 to 0.492 for families 'Criollo 6' and 'Bola', respectively. Ho values close to zero within avocado families could be the result of a high inbreeding rate or low rate of cross-pollination as result of a lack of pollen donors or adverse environmental conditions that interfered during the pollination stage. The nHe values found with ISSR markers were very similar for all families. Total nHe was 0.482 and ranged from 0.478 to 0.511 for families 'Criollo 3' and 'Plátano Delgado', respectively. These re-

sults showed a low genetic variability within avocado families and also demonstrated that ISSR markers are an efficient tool to identify genotypes, even among closely related individuals and a rather small sample size (Thangjam, 2014; Tuisima et al., 2016). According to Nei (1978), the number of individuals for estimating genetic distance can be very small (≤ 10) if the number of loci is more than 50, or if the genetic distance is large, and the average heterozygosity of the two species compared is low. Even a single individual may be enough for obtaining the correct topology of a dendrogram if the differences are large. Using dominant markers such as ISSRs, the minimum number of markers required and number of individuals to be sample depend on many factors, including level of neutral genetic diversity, population differentiation, migration, unequal sample sizes, and the analyses to be performed. For AMOVAs, as few as 30 markers and sampling between 5 to 10 individuals yield reliable results (Nelson and Anderson, 2013). AMOVA results for each kind of marker were similar and highly significant ($P < 0.0001$). Genetic variation among individuals within families of avocado genotypes was 69.17% and among families was 30.83% with SSR, whereas with ISSR it was 63.37% and 36.63%, respectively. AMOVA results combining both types of markers showed that genetic variation within families was 58.6% and among families was 41.6% ($P < 0.0001$). In this case, codominant data were transformed manually to dominant (Table 4).

OUTCROSSING POPULATION RATE PARAMETERS. The results with both types of markers showed a t_m of 0.774 ± 0.091 , indicating that *P. americana* var. *drymifolia* is predominantly outcrossed with a small amount of selfing (22.6%). The t_s was 0.431 ± 0.063 , and $t_m - t_s$ was 0.342 ± 0.113 , indicating full

siblings occurring among families. The correlation of outcrossed population paternity indicates the probability that two siblings are outcrossed full-sibs (Ritland, 2002). In our study, the population value was $r_p = 0.525 \pm 0.125$, showing the presence of full siblings or nonrandom mating, which means the number expected of pollen donors was low. The ratio $1/r_p$ estimates the probable number of effective pollen donors; in this case, the number of potential male parents involved in each progeny was 1.9, suggesting that, on average, two pollinators contributed to the crosses. Another way to estimate the outcrossing rate is by means of the fixation index for maternal genotypes. Here, F_m was equal to 0.166 ± 0.063 , indicating the occurrence of inbreeding in the maternal generation and supporting that *P. americana* var. *drymifolia* is a moderate to high outcrossing species. Correlation of selfing among loci approximates the fraction of selfing rate (as opposed to full-siblings rate) (Ritland, 2002). In our study, its value was very low ($r_s = 0.022 \pm 0.127$), and because this value is positive, it implies that any two selfed progeny samples from the same parent are not the result of two independent selfing events (Muchugi et al., 2008). Selfing occurrence results in complete correlation of selfing among loci, whereas full siblings result in a looser correlation (Ritland, 2002).

OUTCROSSING FAMILY RATE PARAMETERS. ‘Criollo 3’ and ‘Plátano Temprano’ families showed the lowest and highest values of t_m , t_s , and $t_m - t_s$ respectively (Table 3). The negative values for outcrossing estimates in the ‘Criollo 3’ family can be explained by a possible overrepresentation of homozygous individuals within this family. The ‘Silvestre’ family showed values of zero for the three estimates. These results were expected because the mother tree grows in a forest environment, isolated from any pollen donors. ‘Todo el Año’ is characterized by producing flowers throughout the year. Its low outcrossing values are explained because of forwardness in its blooming period in comparison with the others genotypes, which implies an absence of pollen donors and, consequently, low allelic abundance in its progeny. ‘Criollo 6’, according to the description given by the growers, presents early onset of fruit production in comparison with the rest of the genotypes, which explains the low outcrossing rate in its progeny. The moderate to high values for the outcrossing estimates in the families ‘Bola’, ‘Plátano’, ‘Leonor’, and ‘Plátano Temprano’ indicate the existence of outcrossing within the families; their positive values of $t_m - t_s$ indicate a moderate level of full-siblings rate. These results are expected because of their overlapping blooming periods and proximity, and because other unsampled trees surround them.

Traditionally, it has been accepted that natural hybridization in avocado is controlled by flower type A (open as female during the morning and reopen as male the following afternoon) and type B (first open as female in the afternoon and reopen as male the next morning) restricting or even preventing self-pollination (Boza et al., 2018). However, several studies have revealed that cross-pollination is not always maintained, and that self-pollination can be favored by several factors. In Florida, Davenport et al. (1994) indicated a self-pollination rate of 90% to 95% for avocado flowers. Moreover, Borrone et al. (2008), detected an avocado outcrossing rate average of 85%, with 96% for ‘Tonnage’ vs. 74% for ‘Simmonds’, and the identity of the potential pollen donor varied, demonstrating that, under certain conditions, high levels of outcrossing can occur. Outcrossing rates values of ‘Hass’ ranging from 6.6% to

100% for seven California ‘Hass’ orchards, with an average of 31.4%, were reported by Kobayashi et al. (2000). They conclude that both climatic region and proximity to a pollen source are important factors controlling the mating process. Davenport et al. (2006) found that 68% of fruit in southern California was self-pollinated; in addition, they concluded that the proportion of self-pollination vs. cross-pollination might be influenced by cool weather conditions to which the trees are exposed. Garner et al. (2008) found a range of outcrossing rates for individual trees of 0% to 68% (average, $11\% \pm 5\%$). They mentioned that numerous factors could limit outcrossing rates, including the degree of blooming overlap between ‘Hass’ and pollen donors, the pollinizer effectiveness, the distance of ‘Hass’ trees to the nearest pollen donor, and the weather conditions, with cool day- and nighttime air temperatures that cause the outcrossing mechanism to break down, resulting in close pollination among ‘Hass’ trees. Alcaraz and Hormaza (2011), using a single SSR locus (AVAG21), found that the outcrossing rate of ‘Hass’ by ‘Fuerte’ at commercial harvest ranged from 32% to 68% in 2005 and from 31% to 74% in 2006. They reported that a high proportion of self-fertilized fruit was derived from flowers fertilized during the last weeks of the ‘Hass’ blooming season when ‘Fuerte’ presented either few or no flowers, and a decrease in the percentage of fruit derived from outcrossing with increasing distance to the pollinators. The results of the outcrossing rates in *P. americana* reported in these studies coincide with those found in our research, allowing us to conclude that 1) the outcrossing rates in *P. americana* var. *drymifolia* are very variable and depend on many factors, including as edaphoclimatic, agronomic and genetic; 2) this variability needs to be considered to define strategies for the conservation and genetic improvement of outstanding native genotypes; and 3) SSR and ISSR markers are useful for estimating genetic variability within and among families of avocado, as well as for determining the outcrossing rates among closely related individuals and with a rather small sample size.

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