Morphological Characterization of Southern Jalisco, Mexico, Pomegranate Genotypes Using AFLP Markers

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Abstract: Pomegranate is gaining popularity because of its proved nutraceutical properties and is highly adaptable to different climates. In southern Jalisco, Mexico, 18 genotypes were characterized on the basis of fruit characteristics (21 traits) and AFLP (Amplified Fragment length polymorphism) markers. The first three components of a principal components analysis (PCA) explained 71.5% of the variation. The most important variables were related to fruit size and weight. Fruit weight, equatorial diameter, polar diameter, and rind and membrane weight were the variables that most contributed to principal component one (PC1) 46.4%. The variables juice per fruit, edible proportion, proportion of rind and membrane, and seed length contributed most to principal component two (PC2) 15.2%, while juice pH, weight of one seed, and aril width contributed the most to principal component three (PC3) 9.9%. With the six combinations of AFLP primers, 315 fragments were obtained (an average of 52.5 fragments per primer); of these, 229 were polymorphic (72.7% polymorphism). Grouping by both morphological traits and AFLP markers separated all the evaluated individuals so that there were no repeated genotypes. In both analyses, the grouping did not obey geographic origin of the genotypes (r = −0.35), suggesting that both techniques were useful and complementary in the characterization of pomegranate genotypes. The commercial cultivars Wonderful and Apatseo had low levels of similarity to genotypes from southern Jalisco. The level of polymorphism found and compared with the results obtained by other authors suggests that the pomegranate genotypes evaluated are highly polymorphic. We found broad genetic diversity that can be used in breeding programs.

Keywords: fruit characteristics; genetic resources; molecular markers; Punica granatum

1. Introduction

Pomegranate (Punica granatum L) is native to Iran [1], but because it can be grown in arid regions in high-saline soils and can adapt to different soil types, its production has spread to tropical and subtropical regions around the world [2]. In recent years, pomegranate industrialization has increased considerably in United States markets. There are more than 500 sub-products made from different parts of the plant [2], including beverages [3], food products (jams and jellies), dietary supplements [4], packaged ready-to-eat fresh or prepared fruit [5], capsules, softgels, tablets, seed oil, juice, tea, lipstic, lip balms, eye and face cream, masques, shampoo, conditioner, deodorant, body scrub, lotion, toothpaste and soap [6], and food preservers [7]. The high demand is related mainly to
its antioxidant properties that benefit health [8–12]. Pomegranate chemical composition includes many active biological substances such as flavonols, flavanols, anthocyanins, proanthocyanidins, ellagitannins, and gallotannins [13]. It can be used as a source of natural fiber and antioxidants to develop functional foods [14]. A wide range of in vitro and in vivo studies have pointed out that the whole pomegranate fruit, including seeds, juice, pericarp, and seed oil, possess strong anti-proliferative, anti-inflammatory, anti-tumorigenic, pro-apoptotic, antiestrogenic, antioxidant [13], and antibacterial [4] effects. Worldwide, pomegranate is considered a superfood [15].

Pomegranate production increased by 65% between 2010 and 2015 in Mexico [16]. This trend is expected to continue in the short and/or medium term because of the information released on its nutraceutical properties and the introduction of new pomegranate products. In Jalisco, the crop has shown the same trend; between 2010 and 2016, the area cultivated with pomegranate increased from 10 to 120 ha. This accelerated increase in pomegranate cultivation in Mexico requires information on the genotypes being established in this region to select the most appropriate cultivar for its environmental conditions and market demand.

Examination of genetic variation in pomegranate germplasm is key to accelerating plant improvement [17]. The IPGRI (International Plant Genetic Resources Institute) states that there are more than 500 varieties of pomegranate worldwide, but only 50 of them dominate the world market. This made it necessary to explore genotypes that exist in different parts of the world because they are important sources of genes that can be used in breeding programs [18]. Although genotypical diversity of the pomegranate species is broad, studies on selection and characterization of the plant material are recent and scarce. Most works in evaluation of plant genetic resources are based on morphological traits. In the case of pomegranate, genotypes from different parts of the world have been characterized mainly by using fruit characteristics [19–21]. However, the use of molecular markers has been generalized in the study of collections of germplasm. In the case of pomegranate, different types of molecular markers have been used to evaluate diversity of the collections. The use of RAPD (Random Amplified Polymorphic DNA) has been reported in Tunisia [20,22] and in India [23,24], while AFLP markers have been used in Iraq [25], Tunisia [26], and China [27], and pcr-RFLP (Restriction fragment length polymorphism) was used in Spain [28] and SSR (Simple-sequence repeats) in India [17] and Tunisia [29]. Molecular markers have also been used together with morphological characteristics [20,30–32]. However, we found no studies of this kind on pomegranate genotypes in Mexico.

When semi-arbitrary primers are used, AFLP markers have the advantage that information of the genome of the species to be studied is not necessary. Moreover, a large quantity of fragments is generated by combining primers that are useful for evaluating plant genetic resources. The objective of this study was to characterize 18 pomegranate genotypes from southern Jalisco using fruit characteristics and AFLP molecular markers. The hypothesis was that there is a broad diversity in the pomegranate genotypes from southern Jalisco, Mexico.

2. Materials and Methods

2.1. Plant Material

Sixteen pomegranate genotypes were used for the study, and two commercial cultivars Wonderful and Apaseo were included. The genotype Wonderful is a cultivar widely cultivated internationally, while Apaseo was obtained by selection in Apaseo, Guanajuato, Mexico. Samples of these genotypes (fruits for morphological trait analysis and young leaves for AFLP analysis) were taken (August–September 2019) from commercial plantations and small orchards in southern Jalisco (Table 1). Four representative trees of each genotype were sampled (3 fruits per tree) at random to establish a completely randomized experimental design with 12 replications and to measure physical and biochemical characteristics of the fruit. The evaluated variables were fruit weight (FW, g); polar diameter including the calix (PD, cm); equatorial diameter (DE, cm); juice per fruit (JP, mL); aril
weight per fruit (ArW, g); seed weight (fibrous part, SeW, mg); rind thickness (RT, mm); rind and membrane weight (RMW, g); pH; total soluble solids in juice (TSS, °Brix); aril length, width, and thickness (AL, AW, and AT, respectively, cm); number of seeds per fruit (SN); weight of one seed (WOS, g); seed width (SW, cm); and seed length (SL, cm). TSS were measured with a Reichert® AR200 refractometer (Reichert Analytical Instruments, New York, NY, USA), and pH was obtained with a Spectrum® D-54 potentiometer (Spectrum Technologies, Aurora, IL, USA). Weight was measured with an electronic balance Velab® VE 1000 (0.01 g precision) (Velab, Mexico). DE, PD, AL, AW, AT, SL, and SW were measured with a Foy 142070 vernier. The proportion of juice (JP, %), edible portion (EP, %), proportion of seed (PRS, %), and proportion of rind and membranes (PRRM, %) were calculated with the following equations: JP = (JU × 100/FW), EP = (ArW × 100/FW), PRS = (SeW × 100/FW), and PRRM = (RMW × 100/FW). An analysis of variance was performed, and means were compared with the Tukey test (p ≤ 0.05). In addition, the data were standardized and subjected to principal components analysis (PCA). Data were analyzed using Statistical Analysis System software, version 9.13 [33].

| Genotypes | Taste          | Geographical Coordinates | Altitude |
|-----------|----------------|-------------------------|----------|
| Zapote D  | sweet          | 20°07′36″ N 103°32′04″ W | 1354     |
| Zapote A  | sour           | 20°07′36″ N 103°32′04″ W | 1354     |
| Verde teca | sour           | 19°34′17″ N 103°30′36″ W | 1781     |
| Cuco D    | sweet          | 19°34′17″ N 103°30′36″ W | 1781     |
| Chichona 1| sour           | 19°34′17″ N 103°30′36″ W | 1781     |
| Cuco criolla | sour      | 19°34′17″ N 103°30′36″ W | 1781     |
| Tectona chapeada | sour | 19°34′17″ N 103°30′36″ W | 1781     |
| Verde tecata delgada | sour | 19°34′17″ N 103°30′36″ W | 1781     |
| Chichona 2| sweet          | 19°41′20″ N 103°30′26″ W | 1563     |
| El tío     | sweet-sour     | 19°43′08″ N 103°28′11″ W | 1511     |
| El tío criolla | sour        | 19°43′08″ N 103°28′11″ W | 1511     |
| Guizar     | sweet-sour     | 19°43′08″ N 103°28′11″ W | 1511     |
| Negra      | sour           | 19°37′21″ N 103°24′42″ W | 1298     |
| Toro D     | sweet          | 19°41′51″ N 103°29′33″ W | 1527     |
| Toro A     | sour           | 19°41′51″ N 103°29′33″ W | 1527     |
| Toro R     | sweet-sour     | 19°41′51″ N 103°29′33″ W | 1527     |
| Wonderful  | sweet-sour     | 19°41′20″ N 103°30′26″ W | 1563     |
| Apaseo     | sweet          | 19°41′20″ N 103°30′26″ W | 1563     |

Data of morphological traits were standardized using default options of the STAND module. Duplicated measurements for each specimen were averaged and used to design a data matrix of pairwise similarities between genotypes by calculating the Jaccard coefficient (J). Principal component analysis (PCA) was used to depict non-hierarchical relationships among the specimens. Eigenvalues and eigenvectors were calculated by the EIGEN module using a correlation matrix as input (calculated using standardized morphological data), and 2D plot was used to generate the two-dimensional PCO plot. All the calculations, modules and procedures mentioned in this paragraph were carried out with the NTSYs 2.11 software (New York, NY, USA) [34].

2.2. DNA Isolation

Very young and visibly healthy leaves from each genotype were collected (Table 1), labeled, packed in ice, and stored at –80 °C at the Plant Biotechnology laboratory from CIATEJ A.C. in Jalisco, Mexico. DNA was extracted using the Saghai–Maroof protocol with some modifications: two grams of tissue were ground to powder in a mortar with liquid nitrogen. Nine milliliters of buffer (100 mM Tris (pH 7.7), 700 mM NaCl, 50 mM EDTA (ethylenediaminetetraacetate; pH 8.0), 1% CTAB (mixed alkyltrimethyl-ammmonium bromide), 140 mM β-mercaptoethanol) were added and incubated for 60 min in a 65 °C oven. We added 4.5 mL chloroform/octanol (24:1) and centrifuged the mixture at 1500 × g
for 10 min at room temperature. The supernatant was transferred to a new tube, and the chloroform/octanol step was repeated; 30 μL of RNase (10 mg/mL) was added, and the mixture was incubated at 37 °C for 45 min. An equal volume of isopropanol was added and mixed for incubation at −20 °C for 15 min. Precipitated DNA was centrifuged for 10 min at 1500×g at room temperature. The supernatant was poured off, and the pellet was left to dry out. The DNA was rinsed with 10 μL NaCl and 800 μL 100% ethanol, then dissolved in 100 μL of distilled water and stored at 4 °C [35].

2.3. AFLP Analysis

AFLP methodology was based on the Vos protocol: 1 μg of genomic DNA and one pair of restriction endonucleases (MseI + EcoRI) were used. DNA was digested 4 h under conditions recommended by the supplier. The fragments produced were ligated to adaptors previously annealed; they were then pre-amplified using primers with one selective base for each restriction enzyme. Finally, pre-amplified products were selectively amplified using three selective base primers for each restriction enzyme [36]. They were then analyzed in an 6% acrylamide solution with 1× TBE buffer on a Bio-Rad sequencing gel apparatus (Bio-Rad, Hercules, CA, USA) at 100 W for 3 h. Fragments were detected by silver nitrate staining using the Bassam and Caetano-Anolles protocol [37].

AFLP were scored on the basis of the presence (coded as 1) or absence (coded as 0) of polymorphic fragments for each primer. These scores were used to calculate a genetic similarity matrix using the Jaccard (J) coefficient. Cluster analysis was performed on both morphological and molecular similarity matrices using the unweighted pair group method using arithmetic means (UPGMA) algorithm, from which dendrograms depicting similarity among varieties were drawn and plotted with NTSYS-pc software version 2.0 (Exeter software, East Setauket, NY, USA). The cophenetic correlation was calculated to find the degree of association between the original distance matrix and the tree matrix in both morphological and AFLP analysis. Comparison of the two methods was performed for the accessions for which morphological and AFLP data were available, correlating the two dataset means with the Mantel test using NTSYS pc software [34].

3. Results and Discussion

3.1. Principal Components Analysis

3.1.1. Morphological Analysis

In terms of fruit characteristics, we found that the analysis of variance revealed significant differences (p < 0.05) for all the evaluated variables (Figure 1), indicating broad variation. In a previous study [21] over three cycles, we evaluated the characteristics of 11 of the 18 pomegranate genotypes included in this study and found that there were statistical differences in the evaluated traits among the cycles and species. They concluded that the evaluated traits were greatly affected by production environment. Pomegranate production in southern Jalisco began in home gardens. The main use in this region was to make the traditional drink ponche with pomegranate juice and alcohol, as well as, in a lesser measure, to eat fresh. Because of the high demand for this crop worldwide, the cultivated area in pomegranate has increased considerably. For this reason, it is important to characterize the genotypes that exist in the region to plan new plantations for specific production purposes.
Figure 1. Mean value of the most important fruit traits (according to the PCA). (A) Polar diameter, (B) Equatorial diameter, (C) Fruit weight, (D) Juice pH, (E) Rind and membranes weight, (F) Rind and membrane proportion, (G) Editable proportion, (H) Juice proportion, (I) Seed length, (J) Aril width of 18 pomegranate genotypes from southern Jalisco. VT (Verde tecatona), CH1 (Chichona1), CC (Cuco criolla), TCH (Tecatona chapeada), VTD (Verde tecata delgada), ET (El tío), ETC (El tío criolla), GUI (Guizar), TD (Toro D), TA (Toro A), TR (Toro R), ZD (Zapote D), ZA (Zapote A), CD (Cuco D), CH2 (Chichona 2), NE (Negra), WO (wonderful), APA (Apaseo).
The basic statistics (mean, standard deviation) of the 21 fruit traits of the different pomegranate genotypes are shown in Table 2. According to the PCA of the 21 fruit traits, the first three components explained 71.5% of the variation of the analyzed pomegranate genotypes (Table 2). A study in Spain evaluated the genetic diversity of a collection of pomegranate genotypes [19]. They used fruit, seed, flower, and leaf characteristics and found that the first three components explained 53.75% of the variation. On the basis of this analysis, they concluded that fruit characteristics were the traits that had more power of discrimination for the characterization of the pomegranate genotypes evaluated. Another study evaluated pomegranate accessions in Turkey. On the basis of morphological and biochemical characteristics of the fruit, they reported that the first three components explained 50% of the variation [38]. Our study was based on fruit traits, and the contribution of the first three components was greater than that reported by these authors and very similar to that reported in a collection of 20 cultivars from the European Union. They included total soluble solids, titratable acidity, organic acids, sugars, antioxidant capacity, and total content of phenols, finding that the first three components explained 78.17% of the total variation [14]. Still, another study reported that the first three PC with fruit characteristics explained 80% of the variation. However, they evaluated only 11 of the 18 genotypes we used in our study and did not include commercial cultivars [21].

Table 2. Descriptive statistics and eigenvector coefficients of quantitative fruit characters evaluated in *Punica granatum* genotypes from south Jalisco.

| Characters                        | Mean   | Std Dev  | Min  | Max  | PC1      | PC2     | PC3     |
|----------------------------------|--------|----------|------|------|----------|---------|---------|
| Polar diameter (PD, cm)          | 8.92   | 1.42     | 5.9  | 11.18| 0.9402   | 0.0767  | 0.0368  |
| Equatorial diameter (DE, cm)     | 8.14   | 1.25     | 6.13 | 10.48| 0.9112   | 0.194   | 0.1072  |
| Fruit weight (FW, g)             | 287.33 | 101.28   | 128.4| 472.4| 0.9705   | 0.0411  | 0.0209  |
| Rind thickness (RT, mm)          | 0.4154 | 0.0917   | 0.278| 0.625| 0.7675   | -0.0832 | 0.067   |
| Number of seeds per fruit (SN)   | 579.06 | 124.51   | 397  | 811.5| 0.6579   | 0.3968  | 0.3295  |
| Rind and membrane weight (RMW, g)| 135.43 | 59.35    | 45.53| 288.52| 0.9595  | -0.1621 | 0.081   |
| Aril weight (ArW, g)             | 151.90 | 49.36    | 82.88| 252.13| 0.8377  | 0.4844  | -0.0545 |
| Juice per fruit (JU, mL)         | 110.72 | 40.58    | 53.21| 197.93| 0.8068  | 0.5159  | 0.0144  |
| Seed weight (fibrous part, SeW, mg)| 41.066| 13.32    | 29.2 | 84   | 0.647   | 0.1866  | -0.2075 |
| Juice pH                         | 2.73   | 0.1948   | 2.47 | 3.13 | -0.4512 | -0.0756 | -0.6798 |
| Total soluble solids (TSS, Brix) | 14.95  | 1.2634   | 12.7 | 17   | 0.3317  | -0.0751 | -0.0032 |
| Edible proportion (EP, %)        | 53.88  | 6.47     | 38.77| 64.48| -0.6268 | 0.6592  | -0.3612 |
| Juice proportion (JP, %)         | 38.49  | 5.35     | 29.32| 47.29| -0.2798 | 0.8077  | -0.1791 |
| Seed proportion (PRS, %)         | 15.34  | 4.092    | 9.75 | 23.01| -0.6313 | -0.0395 | -0.3014 |
| Rind and membrane proportion (PRRM, %) | 46.12 | 6.4741  | 35.52| 61.23| 0.6268  | -0.6592 | 0.3612  |
| Weight of one seed (WOS, g)      | 0.0721 | 0.0771   | 0.0534| 0.1157| 0.5502  | -0.4118 | -0.5064 |
| Aril width (AW, cm)              | 0.6215 | 0.0990   | 0.49 | 0.8961| 0.7541  | -0.234  | -0.4917 |
| Aril length (AL, cm)             | 0.9123 | 0.0785   | 0.8  | 1.096 | 0.5837  | 0.2795  | -0.3502 |
| Thickness                        | 0.5200 | 0.1090   | 0.3  | 0.7  | 0.672   | 0.1753  | -0.4335 |
| Seed width (fibrous part, SW, g) | 0.3150 | 0.0435   | 0.245| 0.415 | 0.3483  | -0.4136 | -0.4296 |
| Seed length (SL, cm)             | 0.6764 | 0.0526   | 0.5616| 0.7425| -0.1406 | 0.6102  | 0.2444  |

PC1 explained 46.4% of the variation, and the variables that most contributed to this component were those related to fruit size and weight (FW, DE, PD, and RMW) (Table 2). The evaluated genotypes varied greatly in terms of these characteristics: FW ranged from 128.4 to 472.4 g (average 287.33 g) (Figure 1 and Table 2). Average fruit weight was close to the values of 325–414 g reported in pomegranate of Spain [19]. Moreover, a study of 100 pomegranate genotypes from the Saveh part of Markazi province in Iran reported values of 106.60–496.91 [9], while another study in Spain reported fruits ranging from...
In Turkey, other authors showed a wide range of the variable FW with 69.9–795.3 g [38]. In our study, DE and PD were 6.13 to 10.48 cm (average 8.14 cm) and 5.9 to 11.18 cm (average 8.92 cm), respectively (Tables 2 and 3), lower than those reported in Turkey [38], Morocco [39], and Spain [40]. The variable RMW varied from 45.53 to 288.52 (mean 135.43) (Table 3). The genotype with the lowest FW was Zapote D with 128.4 g, followed by Toro D with 141.25 g. The heaviest genotype was Wonderful with 472.4 g, followed by Tío criolla, Chichona 1, and Apaseo (442.78, 434.6, and 384.11 g, respectively) (Figure 1). The other variables that contributed most to PC1 (PD, DE, and RMW) were highly correlated with FW, and similar behavior was observed: those individuals that had lower weight (Zapote D and Toro D) also had lower value of the variables DE, PD, RMW, and vice versa. Fruit size is one of the most important characteristics for the international market of table fruits.

Table 3. Primer combinations, fragments, and polymorphism found in 18 pomegranate genotypes using AFL markers.

| Primer Combination | Total Fragments | Polymorphic Fragments | Polymorphism % |
|--------------------|-----------------|-----------------------|----------------|
| Mse + CTA/Eco + AGG | 48              | 32                    | 66             |
| Mse + CAT/Eco + ACC | 44              | 22                    | 50             |
| Mse + CAG/Eco + ACC | 27              | 17                    | 63             |
| Mse + CAG/Eco + ACT | 67              | 50                    | 74             |
| Mse + CAT/Eco + AGG | 92              | 78                    | 84             |
| Mse + CTA/Eco + ACC | 37              | 30                    | 81             |
| Total              | 315             | 229                   | 72.7           |

In the case of PC2, which explained 15.2% of the variation, the variables that most contributed to this component were those related to fruit proportions: JP, EP, PRMM, and seed length (Table 2). As in the case of PC1, these variables had widely ranging averages. In the case of the variable JP, the statistically lowest value was 29.32%, found in the genotype Wonderful, while the statistically highest value was found in the genotype Verde tecata delgada with 47.29% (Figure 1). These results contrast with commercial pomegranate genotypes from Oman. They reported small differences for this variable (average values of 57.33–67.33%) [41]. These authors also related JP to aril hardness, which was not included in our study. Other authors also reported low variability in JP of new genotypes in Granada, Spain (50.25–64.17%). Like for other fruits, JP is an important characteristic for pomegranate [42] since, currently, pomegranate juice is gaining much popularity, not only as a food, but also as a natural dye, and is becoming important in the health and cosmetics industries [41].

In the case of the variable EP, the extreme values were 38.77 (Wonderful) and 64.48 (Zapote D) (Figure 1), similar to those obtained in six pomegranate cultivars from Morocco (53.4–61.2%) [39] and in seven pomegranate accessions grown in southeastern Spain (52.7–65.9) [40]. These percentages indicate that the fruits have an acceptable edible proportion. Lower values indicate that they also have a large proportion of rind and membranes, which are attributes that are not favorable if the fruit arils are to be consumed or if it will be used for juice. However, if antioxidants are to be extracted from the rind, they are high potential genotypes because of the amount of rind and the great antioxidant capacity that has been reported for this portion of the fruit, 23.4 times more than in juice [43]. Our results differed from findings of a study carried out in Spain, which reported an EP of 58–75% in pomegranate genotypes [2], while other authors report results similar to ours [39,40].

For PRRM, the lowest value of 35.52 was found in the genotype Zapote D, while the highest, 61.23, was found in the genotype Wonderful. For the variable SL, the extreme values were 0.5617 in Wonderful and 0.720 to 0.7424 mm (statistically equal) in Chichona 1, Verde tecatona, Toro D, and Guizar. It is notable that, although the genotypes from southern Jalisco have smaller average sizes than the cultivar Wonderful, they have a larger juice and edible proportion and a lower proportion of rind and membranes (Figure 1).
In general, the genotypes collected in southern Jalisco had smaller fruit sizes and weights than the cultivars Wonderful and Apaseo. The genotypes Chichona 1 and El tío criolla had average values similar to those of the commercial materials, but they are sour genotypes (Table 1) used to make pomegranate *ponche*. They cannot be consumed fresh, but they have a large proportion of juice (37.12% to 45%, respectively) compared with Wonderful and Apaseo, which are 29.32% and 32.49% juice, respectively (Figure 1).

PC3, with 9.9% of the variation, was represented by the variables pH, WOS, and AW (Table 2). Average pH was 2.77, and the extreme values found were 2.47 and 2.5 (statistically equal) for Toro R and Toro A, respectively, and 3.13 for the genotype Toro D (Figure 1), indicating that they are very sour, compared with 3.13–6.66 reported in another study [14]. However, similar values were found in pomegranate accessions of Turkey; the study reported a pH range of 2.6–3.9 and mentioned that these levels of acidity were higher than those reported in other studies because many of the evaluated accessions are used in regional cuisine of Turkey (salads, sauces) and less as a beverage [38], which is the case of the genotypes of southern Jalisco. In our case, 14 of the 18 genotypes are sour or sweet-sour and have been selected in the region for use in preparing the traditional drink *ponche*. Martinez-Nicolas [19] did not find significant difference in pH among the different populations analyzed, but pH was one of the important variables for defining diversity on the basis of a PCA, like that used in our study.

Regarding the variable weight of one seed (WOS), for which only the fibrous part was considered, the values obtained were highly variable (0.0534–0.116 g, average 0.095, data not shown). In five new pomegranate cultivars, values of 0.030–0.058 g of the fibrous portion of the seed [42] were found. This characteristic, together with other variables such as TSS and juice acidity, determine how the genotypes are consumed: fresh or as juice. Most of the pomegranate genotypes from southern Jalisco have a larger fibrous part of the seed than that found in other studies, and in addition, because they are sour and sweet-sour fruits, they are ideal for processing, while the variable AW had extreme values of 0.49 for the genotype Toro D and 0.89 for the cultivar Wonderful (average 0.62) (Figure 2).

The projection of the 18 genotypes characterized on the basis of the first three components is shown in Figure 2a,b. Because of the high variability found among the genotypes evaluated, their distribution on the plane generated for PC1 and PC2 does not have a clear tendency to group by collection region or flavor (sweet, sour, sweet-sour). However, those that had intermediate values of fruit size (Verde tecatona, Verde tecata delgada, El tío, Toro A, Cuco D, Cuco criolla, Toro R, Tecatona chapeada, Chichona 2 and Guizar) appeared in the center of the graph. Genotypes with on average smaller fruits (Zapote D and Toro D) were positioned on one extreme of the graph, while those with larger fruits (Wonderful, Apaseo, and Chichona 1) were placed on the opposite extreme of the graph.

The dendrogram of the grouping generated with the 21 morphological characteristics evaluated are presented in Figure 3. As in the case of PCA, there was no clear formation of groups relative to the collection area or juice flavor. On the basis of this analysis, we observed the clear formation of three main groups; this grouping is associated mainly with the variables of fruit size. Group 1 includes the genotypes Zapote D, Toro D, Zapote A, and Negra, which had the lowest values of FW, PD, and DE. In group 2, we found those individuals with medium-size fruit: Verde tecatona, Verde tecata delgada, El tío, Toro A, Cuco D, Cuco criolla, Toro R, Tecatona chapeada, Chichona 2, and Guizar. The genotypes with the heaviest and largest fruit were placed in group 3: Chichona 1, El tío criolla, and the cultivars Apaseo and Wonderful. Only the genotypes Zapote A and Negra, which were morphologically similar in terms of the characteristics mentioned in the PCA, were observed to be closely associated in the dendrogram, and they shared the characteristic of having sour fruits.
Figure 2. PCA distribution of 18 pomegranate genotypes: (a) PC1 vs. PC2, (b) PC1 vs. PC3. Based on fruit traits.
Figure 3. UPGMA dendrogram (based on Euclidean distance) of 18 pomegranate genotypes from southern Jalisco, obtained with fruit traits. I, II and III: the three main groups formed from the analysis.

3.1.2. AFLP Analysis

Level of Polymorphism

From the six combinations of primers, a total of 315 fragments were obtained. Of these, 229 were polymorphic (72.7%) (Table 3). With RAPD markers, other authors evaluated 24 pomegranate genotypes using 16 RAPD decamer primers; they reported 178 fragments
(11.1 per primer), of which 102 (57.3%) were polymorphic [20]. In contrast, another study evaluated pomegranate progenies produced by controlled crosses; using 26 RAPD primers, they found 325 fragments (12.5 fragments per primer), of which only 70 (21.18%) were polymorphic [32].

In general, the level of polymorphism was high, reflecting the existing level of diversity among the pomegranate genotypes characterized. The combination Mse + CAT/E + AGG was that which generated the largest number (92) of fragments as well as the largest percentage of polymorphisms (84%), followed by the combination Mse + CAG/E + ACT (67 fragments, 74% polymorphic). The combination that generated the fewest fragments was Mse + CAG/Eco + ACC (27 fragments, 63% polymorphic). The number of fragments was small for some of the combinations. Vos et al. [36] mention that 50–100 fragments can be obtained using AFLP, but in our case, two combinations, Mse CAG/Eco ACC and Mse + CTA/Eco + ACC, produced 27 and 37 fragments, respectively. This contrasts with other authors who evaluated genetic diversity of 85 pomegranate genotypes from six different populations in China. They tested eight combinations of AFLP primers and found 158 fragments on average per combination, with 73.26% polymorphism, which is similar to our findings [27]. Moreover, Jbir et al. [26] tested six combinations of AFLP primers in 34 pomegranate genotypes of Tunisia; they found 345 fragments (57.6 fragments per combination on average) with 94.7% polymorphism. In general, the level of polymorphism found in our study, compared with the results obtained by other authors, suggests that the pomegranate genotypes evaluated were highly heterozygous, due to their allogamic reproductive systems. For this reason, we found broad genetic diversity that can be used in breeding programs.

The similarity matrix generated from the AFLP fragments had average similarity values of 0.71. The least similarity found was between the genotypes El tío criolla and Wonderful (0.51% similarity), followed by the genotypes El tío criolla and Cuco D (0.52% similarity). On the other hand, the genotypes El tío with Toro A and El tío with Toro D were those that had the highest values of similarity (0.90%). Wonderful was the genotype that had the lowest levels of similarity to the rest of the genotypes evaluated (average similarity 0.60). This means that the genotype Wonderful shared fewer fragments with the rest of the characterized genotypes. The ranges of similarity (0.51–0.9) were smaller than those reported for pomegranate genotypes in other countries. In Iran, the range was 0.26–0.89 [20], and in Tunisia, it was 0.1–0.86 (average similarity 0.48) [26]. The difference could be attributed to the smaller number of individuals we analyzed—16 of the 18 genotypes we evaluated were from a single region (southern Jalisco), and a greater range of similarity (0.08–0.79) was reported in wild pomegranate genotypes [24].

The dendrogram of groupings generated from AFLP fragments is presented in Figure 4. As in the case of morphological traits, no clear relationship of the groups to the collection region or fruit flavor (sweet, sour, sweet-sour) was observed. Cophenetic correlation produced an r = 0.88, indicating good representation of the dendrogram based on the AFLP data. At 67% similarity, the formation of two groups was observed. Group 1 was formed by the genotypes Toro A, El tío, Toro D, Guizar, Zapote D, Verde tecata delgada, Cuco criolla, Negra, Verde tecatona chapeada, Chichona 2, Toro R, and Zapote A. In this group, we observed that Toro A was very similar to El tío and Cuco criolla to Negra, with 90% similarity. This may suggest that these genotypes have very close genetic origins, although morphologically they are not so similar. Group 2 included Verde tecatona, Cuco D, and Chichona 1. The genotypes Apaseo, El tío criolla, and Wonderful were placed outside these groups and, although morphologically they have common characteristics for variables related to fruit weight and size, in this case, there was no association trend.
outside these groups and, although morphologically they have common characteristics for variables related to fruit weight and size, in this case, there was no association trend.

Figure 4. UPGMA dendrogram (Jaccard’s coefficient) of 18 pomegranate genotypes from southern Jalisco using AFLP markers ($r = 0.88$). I and II: the two main groups formed from the analysis.

The genotypes that were closely associated by morphological traits, such as Zapote A and Negra, were not associated in the same way in the dendrogram generated by AFLP. Although both genotypes were placed in the same group (0.81% similarity), they were not closely related. This suggests that the morphological similarity observed in these genotypes was due to environmental effects (climate and soil condition in the year of study). In this study, on the basis of the AFLP analysis, there were no duplicated genotypes. That is, we did not observe genotypes with identical band patterns that would suggest the existence of duplicates with different names among the characterized genotypes.
3.2. Correlation between Morphology and AFLP

Both analyses showed a clear separation of the cultivars Wonderful and Apaseo from the rest of the characterized individuals. However, the correlation obtained on the basis of the Mantel test resulted in values that indicated little correlation \((r = -0.35)\). Morphological characteristics and AFLP markers provided information for our study of pomegranate genotypes that was different but complementary for a more adequate valuation of the existing genetic material. As in our case, Jbir et al. [26] found little relationship between the formed groups and geographic origin or genotype denomination of Tunisian pomegranate. The low correlation between morphological data and molecular markers in pomegranate has been reported previously [20], wherein the authors characterized pomegranate genotypes using fruit characteristics and RAPD markers \((r = -0.36)\). Likewise, another study reported little correlation between fruit morphological traits and RAPD \((r = 0.03)\) [32]. These authors attributed the low correlation to the fact that they measured only fruit characteristics, which do not cover the entire pomegranate genome. The molecular markers covered more diverse regions of the genome, beyond the morphological traits studied, and hence the association was scant. Both RAPD and AFLP markers use arbitrary primers that are not necessarily associated with regions or genes of the genome of the analyzed individuals. For this reason, they can be considered exploratory techniques that can provide us with an idea of the genetic diversity of the individuals, as well as low correlation relative to coding regions (specific genes or fragments related to a specific trait) of individuals is to be expected.

Most of the genotypes we characterized have sour fruit due to their traditional use in making the traditional pomegranate drink (ponche). However, given the growing importance of pomegranate cultivation in southern Jalisco and throughout Mexico, it is necessary to select local genotypes with characteristics demanded by the market for fresh consumption and/or industrialization, without ruling out introduction of new germplasm to achieve these goals.

4. Conclusions

Although pomegranate molecular characterization studies have been performed using different types of molecular markers, this is the first study to characterize pomegranate accessions of Mexico with AFLP markers. The study shows that molecular markers are highly useful, not only to determine the status of the collected accessions, but also as a tool for future programs of selection and improvement of genotypes to evaluate the inclusion of new genotypes with characteristics of interest.

With both morphological traits and AFLP markers, a broad variability of pomegranates was found. This diversity offers an opportunity to select outstanding genotypes for fresh consumption, industrialization, or breeding. The characteristics related to FW and fruit size, DE and PD, were the most important variables for classification of the pomegranate genotypes evaluated in this study. The level of polymorphism found (70.7%) was high. Mse + CAT/Eco + AGG was the combination that yielded the highest number of fragments and polymorphism in the pomegranate genotypes evaluated. Although there is little correlation between morphological characteristics and the AFLP markers used in this study, the information obtained with the two methods was useful and complementary in classifying the pomegranate genotypes from southern Jalisco. Therefore, the hypothesis is accepted.

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