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Assessment of genetic diversity in *Psidium guajava* L. using different approaches

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ABSTRACT

Psidium guajava L. is one of the economically most relevant fruit crops in the Myrtaceae family. Genetic diversity studies are an important source of data for breeding programs, as well as taxonomic and evolutionary approaches in this species. Considering that the use of distinct methods enables more reliable information of genetic diversity, this study aimed to screen and characterize the genetic diversity among 28 *P. guajava* genotypes by morphological, karyotypical, nuclear 2*C*-value and simple sequence repeat (SSR) marker data. Karyotypic and nuclear 2*C*-value analyses showed that all genotypes have a stable and very small diploid genome ($2n = 2x = 22$; $2C = 0.95$ pg). In this regard, the species exhibits karyotypic characteristics related to ancestral angiosperm groups. Dendrogram based on morphological and SSR data evidenced diversity among *P. guajava* genotypes, with better discrimination by SSR. The data obtained for morphology, SSR molecular markers, karyotype and nuclear genome size contributed to expand the knowledge about the genome and genetic diversity of *P. guajava*. In this context, this information may aid plant breeders to structure crop improvement programs and contribute to evolutionary approaches.

1. Introduction

Psidium guajava L. belongs to the Myrtaceae family, which contains approximately 150 genera and more than 5.650 species (Govaerts et al., 2008). Guava represents an excellent source of vitamins A, B and C, as well as calcium, zinc, phosphorus and iron (Singh, 2005). Furthermore, fruits, leaves, flowers, roots, bark and stems are used in medicinal applications (Gutiérrez et al., 2008; Kamath et al., 2008). In regard of these facts, as well as the low cultivation costs, the guava crop is economically important in various tropical and subtropical countries (Rodríguez et al., 2010).

Cross-pollination is the reproductive form most frequently seen in *P. guajava* (Dasarathy, 1951; Balasubrahmanyan, 1959; Alves and Freitas, 2007), but occurrence of self-pollination has also been reported (Singh and Sehgal, 1968). Due to this reproductive mechanism and seminal propagation, some guava orchards exhibit great heterozygosity and genetic variability (De Lara et al., 2004).

Genetic diversity in the germplasm of *P. guajava* has been screened and characterized based on morphological (Molero et al., 2003; Urdaneta et al., 2007; Santos et al., 2010) and chemical features (Sharma et al., 2010); DNA molecular markers (Chen et al., 2007; Rodríguez et al., 2007; Valdés-Infante et al., 2007); karyotype characterization; and nuclear 2*C*-value measurement (Costa et al., 2008). These data have also been considered valuable for taxonomic and evolutionary approaches (Agarwal et al., 2008), sustainable management and exploration of genetic resources, bioprospection, as well as for in- and ex vitro conservation (Rodríguez et al., 2010).

Considering that data from distinct sources provide a more accurate information set to estimate genetic diversity (De Lara et al., 2004; Delgado et al., 2007; Gomes-Filho et al., 2010; Mani et al., 2011), this study aimed to assess the genetic diversity among *P. guajava* genotypes by evaluation of morphological, karyotypical, nuclear 2*C*-value and simple sequence repeat (SSR) marker data.

2. Materials and methods

2.1. Plant material

For flow cytometry approaches, 28 plants of *P. guajava* L. were used, being: six genotypes from the cultivars 'Paluma', 'Pedro Sato', 'Kumagai', 'Sassaoka', 'Rica' and 'Século XXI' (Sec XXI); 15 pre-selected genotypes obtained from open pollination orchard:

Abbreviations: bp, base pairs; C, cortibel; CV, coefficient of variation; dH_2O, distilled water; FCM, flow cytometry; pg, picograms; Sec XXI, Século XXI; SSR, simple sequence repeat; UPGMA, unweighted pair group method with arithmetic mean.

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'Cortibel (C) I', II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV and XV; four spontaneously occurring genotypes: 'São Pedro', 'Guaçuí', 'Caparaó 1' and 'Caparaó 2'; and three genotypes purchased from a plant nursery: 'Roxa', 'Cascão' and 'Campo'. These genotypes were also used in molecular markers approaches, except São Pedro, Guaçuí, Caparaó 1 and Caparaó 2, and in morphological approaches, except Kumagai, Sassaoka, Sec XXI, Rica, CXIV, CXV, Cascão and Campo.

2.2. Morphological analysis

Each genotype was evaluated for eight quantitative fruit characteristics (Table 1) in the years 2010–2011. Data were obtained from two plants per genotype by five fruits per plant. The measurements were carried out using graduated rules described by Inmetro (2010). The data collected were subjected to principal component analysis; diversity analysis using mean Euclidean distance; and finally to clustering using the unweighted pair group method with arithmetic mean (UPGMA). The statistical program Genes (Cruz, 2006) was used for these analyses.

2.3. SSR analysis

Total genomic DNA was extracted from young leaves of each accession according to protocol of Doyle and Doyle (1990), with the following changes: addition of 1% (w/v) polyvinylpyrrolidone to extraction buffer; two washings with chloroform-isoamyl alcohol (24:1); and for precipitation were used a third of ammonium acetate (7.5 M) and one volume of cold isopropanol. The samples were resuspended in 50 μl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) containing 50 μg ml^{-1} RNase A. Quantification and verification of DNA integrity were carried out with a Thermo Fisher Scientific Nanodrop 2000/2000c Spectrophotometer equipment. Twenty-six microsatellite primers, described by Risterucci et al. (2005) and Lepitre et al. (2010), were amplified by polymerase chain reaction (PCR) with a final volume of 15 μl, containing: 60 ng DNA, 1 × PCR buffer, 0.3 mM of each primer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP and 1.0 U of *Taq* DNA polymerase. The reactions were performed in a Veriti® 96-Well Thermal Cycler ABI, according to Risterucci et al. (2005). PCR products were separated by polyacrylamide (6%) and agarose (3%) gel electrophoreses and visualized through staining with ethidium bromide. The data were subjected to diversity analysis using complement of weighted index to generate a dissimilarity matrix. UPGMA was employed as clustering method, using the computational resources of the program Genes (Cruz, 2006).

2.4. Flow cytometry analysis

Before each flow cytometry (FCM) measurement, flow cytometer parameters (such as gain and channel) were determined, based on external analyses of *Solanum lycopersicum* (primary reference standard, 2*C* = 2.00; Praça-Fontes et al., 2011) and all *P. guajava* (samples) genotypes. Next, internal FCM procedures were performed.

Fragments (2 cm^2) of young leaves from the primary reference standard and sample plants were simultaneously chopped (Galbraith et al., 1983) with a razor blade, for 30 s, in 60 × 15 mm Petri dish containing 0.5 ml of OTTO-I lysis buffer (Otto, 1990; Doležel and Göhde, 1995), supplemented with 2.0 mM dithiothreitol and 50 μg ml^{-1} RNase. Subsequently, 0.5 ml of the same buffer was added, and the homogenate was filtered through 30 μm nylon filter into a 2.0 ml microcentrifuge tube, then centrifuged (ALC® microCentrifugette® 4214) at 100 × *g* for 5 min. The supernatant was poured out, and the pellet was resuspended and incubated for 10 min in 100 μl OTTO-I lysis buffer.

The nuclei suspension was stained with 1.5 ml OTTO-I:OTTO-II solution (Otto, 1990; Doležel and Göhde, 1995) in proportion 1:2. The solution was supplemented with 75 μm propidium iodide, 2.0 mM dithiothreitol and 50 μg ml^{-1} RNase (Praça-Fontes et al., 2011). After staining in the dark, for 40 min, the nuclei suspension was filtered through 20 μm nylon mesh.

All suspensions were analyzed in the same Partec PAS® flow cytometer (Partec® GmbH, Munster, Germany), equipped with a Laser source (488 nm). Nuclei-emitted propidium iodide fluorescence was collected by an RG 610 nm band-pass filter. The equipment was calibrated and aligned using microbeads and standard solutions, according to the manufacturer's recommendations (Partec®). The FlowMax® software (Partec®) was used for data analyses. Six independent repetitions were performed at three distinct days, analyzing over 10,000 nuclei each time.

Genome size of each sample was measured using peaks corresponding to the mean relative DNA contents of the 2*C* nuclei, at G_0/G_1, of both sample and primary standard plants. The genome size mean values, given in picograms (pg), were converted to base pairs (bp) considering that 1 pg of DNA corresponds to 0.978×10^9 bp (Doležel et al., 2003).

2.5. Cytogenetic analysis

P. guajava seeds were germinated in Petri dish containing distilled water (dH_2O) at 30 °C. Root-tips of 0.5–1.0 cm length were treated with solution containing 2 to 6 μm of microtubule inhibitors amiprophos-methyl or oryzalin, for a period of 1–7 h at 30 °C, or 8–24 h at 4 °C. The roots were washed with dH_2O for 15 min and subsequently fixed with fresh cold methanol:acetic acid solution 3:1 (v/v) for 24 h. The fixative was replaced three times and the roots were stored at −20 °C (Carvalho et al., 2007).

After 24 h, the roots were macerated with pectinase:dH_2O solution in the proportions 1:5 to 1:50, for 1–2 h, at 34 °C. Next, roots were washed for 20 min in dH_2O, fixed again, and stored at −20 °C. Slides were prepared by meristematic cellular dissociation, air-dried and placed on a hot-plate (50 °C) for 20 min. The slides were immediately stained with 5% Giemsa solution in phosphate buffer (pH 6.8) for 4 min, washed twice in dH_2O, and air-dried (Carvalho et al., 2007). Chromosome images were captured with a Media Cybernetics® Camera Evolution™, charge-coupled device video camera, mounted on a Nikon 80i microscope (Nikon, Japan).

3. Results

3.1. Morphological analysis

Genotype variations related to fruit characteristics are summarized in Table 1. The coefficients of variation ranged from 6.5% to 35.7%, with the highest value obtained for fruit weight and lowest for the ratio between total diameter/inner diameter. Based on all characteristics, the genotypes were clustered into four groups (Fig. 1a), adopting cut to 50% of the maximum fusion, corresponding to the maximum variation region of the dendrogram.

The UPGMA dendrogram from this data presented a good fit between the dissimilarity matrix and the graphical representation, as observed by a cophenetic correlation coefficient of 0.95.

Cluster A grouped nine Cortibel genotypes, along with Paluma, Pedro Sato, Roxa and the spontaneous genotype São Pedro; Cluster B grouped three spontaneous genotypes; Cluster C grouped three Cortibel genotypes; and cluster D grouped a single genotype, CIII. The closest genotypes were CVII and CVIII, identified with a distance of 0.14, whereas the most divergent were CIII and CVIII, with a distance of 2.59.

Table 1
Mean, standard deviation and variation coefficient from morphological data.

Genotype	Weight (g)	Length (cm)	Total diameter (mm)	Inner diameter (mm)	Total diameter/Inner diameter	Pulp weight (g)	Seed weight (g)	Number of seeds
CI	294.4 ± 110.5	9.03 ± 1.1	8.15 ± 1.3	5.09 ± 0.7	1.61 ± 0.2	66.47 ± 19.6	5.99 ± 1.1	335.75 ± 86.1
CII	199.8 ± 87.9	7.22 ± 1.3	6.88 ± 1.1	4.37 ± 0.6	1.57 ± 0.1	45.82 ± 18.3	4.39 ± 1.1	317.60 ± 62.8
CIII	89.1 ± 25.8	5.70 ± 1.0	5.22 ± 0.4	3.64 ± 0.6	1.45 ± 0.2	28.85 ± 11.8	3.42 ± 1.5	266.00 ± 129.6
CIV	183.1 ± 18.8	6.95 ± 0.4	6.93 ± 0.3	4.62 ± 0.3	1.50 ± 0.1	55.86 ± 6.3	5.53 ± 0.5	427.50 ± 17.5
CV	140.1 ± 25.3	6.47 ± 0.6	6.21 ± 0.3	3.85 ± 0.4	1.63 ± 0.2	29.85 ± 6.9	2.62 ± 0.6	224.90 ± 81.1
CVI	165.3 ± 14.5	7.24 ± 0.4	6.53 ± 0.2	4.36 ± 0.3	1.50 ± 0.1	49.75 ± 13.6	3.81 ± 0.5	312.80 ± 33.1
CVII	134.0 ± 24.5	6.90 ± 0.4	5.97 ± 0.5	4.20 ± 0.3	1.42 ± 0.1	38.82 ± 6.4	5.43 ± 0.4	427.30 ± 30.4
CVIII	193.4 ± 44.1	7.65 ± 1.0	6.83 ± 0.6	4.39 ± 0.6	1.57 ± 0.1	43.73 ± 10.9	3.88 ± 0.8	229.30 ± 49.2
CIX	168.9 ± 27.1	7.31 ± 0.4	6.75 ± 0.3	3.88 ± 0.2	1.74 ± 0.1	34.49 ± 6.1	2.95 ± 0.9	209.80 ± 82.1
CX	135.2 ± 48.0	6.52 ± 1.0	6.19 ± 0.6	4.15 ± 0.5	1.50 ± 0.1	42.10 ± 17.8	4.93 ± 2.0	323.10 ± 153.6
CXI	132.5 ± 39.0	6.56 ± 0.8	6.03 ± 0.5	4.17 ± 0.5	1.45 ± 0.1	39.37 ± 10.5	4.78 ± 0.8	436.40 ± 52.3
CXII	118.5 ± 17.4	6.19 ± 0.2	5.90 ± 0.4	4.06 ± 0.3	1.45 ± 0.1	35.88 ± 5.6	4.30 ± 0.5	369.70 ± 68.2
CXIII	138.4 ± 26.1	6.48 ± 0.4	6.18 ± 0.5	4.17 ± 0.5	1.49 ± 0.1	42.21 ± 9.0	4.96 ± 0.9	411.00 ± 104.4
Paluma	154.3 ± 23.9	7.02 ± 0.8	6.45 ± 0.3	4.38 ± 0.2	1.47 ± 0.1	47.22 ± 7.1	4.89 ± 0.6	464.40 ± 28.1
Pedro Sato	174.5 ± 51.8	7.09 ± 0.8	6.58 ± 0.7	4.47 ± 0.5	1.47 ± 0.1	51.02 ± 20.8	5.60 ± 2.0	484.80 ± 173.9
Roxa	163.8 ± 74.3	7.10 ± 0.9	6.40 ± 0.9	4.07 ± 0.5	1.57 ± 0.1	46.16 ± 18.0	6.14 ± 2.2	463.00 ± 211.2
São Pedro	122.4 ± 32.5	7.00 ± 0.7	5.70 ± 0.4	3.50 ± 0.0	1.63 ± 0.1	36.74 ± 5.9	4.07 ± 1.5	314.40 ± 102.1
Guaçuí	58.4 ± 43.5	5.10 ± 1.7	4.40 ± 0.9	2.96 ± 1.0	1.56 ± 0.3	18.95 ± 13.1	3.68 ± 1.3	428.80 ± 126.0
Caparaó 1	84.4 ± 54.0	4.36 ± 0.6	4.80 ± 0.6	2.70 ± 0.4	1.79 ± 0.1	17.82 ± 5.8	2.65 ± 0.4	171.40 ± 36.6
Caparaó 2	77.3 ± 21.5	6.16 ± 1.3	4.40 ± 0.9	2.60 ± 0.4	1.71 ± 0.3	20.68 ± 6.0	2.37 ± 0.8	250.20 ± 45.7
CV (%)	35.7	14.4	14.8	15.9	6.5	31.3	26.5	28.1

Principal component analysis evidenced that the weights for the morphological characteristics in the first autovector were highly similar, and showed total diameter with greatest weight in the last autovector (Table 2).

3.2. SSR analysis

From the 26 SSR *loci* used, 70 alleles were identified, varying from one to five per *locus*, with an average of 2.7 alleles per *locus*. Among these SSR, 24 were polymorphic, and thus used for assembly of dissimilarity matrix.

The closest genotypes were identified with 0.07 dissimilarity (CVI and CVIII) and the most highly divergent with 0.74 (Roxa and Cascão). Considering all accessions, the average dissimilarity was 0.42.

Adopting cut at 50% of maximum fusion, corresponding to the maximum variation region of the dendrogram, UPGMA analysis yielded a dendrogram exhibiting twelve groups: (a) a cluster with 11 Cortibel genotypes; (b) a cluster with two Cortibel genotypes (CXIV and CXV); (c) a cluster with Cortibel III and the genotype Rica; and (d) other nine clusters containing a single genotype (Fig. 1b).

The UPGMA analysis also showed a good fit between the dissimilarity matrix and the graphical representation, as demonstrated by a cophenetic correlation coefficient of 0.88.

3.3. 2C-value measurement and karyotype characterization

Nuclei suspensions supplied FCM histograms with G_0/G_1 peaks exhibiting CVs below 5% (Fig. 2a). Mean 2*C*-values were determined based on G_0/G_1 nuclei peak of sample and primary reference standard (*S. lycopersicum*). All *P. guajava* lines showed the same mean value, $2C = 0.95$ pg, equivalent to 0.929×10^9 bp.

The cytogenetic procedure yielded metaphases showing individualized chromosomes, flattened on the slide, without chromatin deformations and cytoplasmic background noise, and exhibiting well-defined primary constriction (Fig. 2b). These aspects were advantageous for precise scattering of the metaphases and morphometric characterization of the chromosomes. All *P. guajava* lines presented $2n = 2x = 22$ chromosomes, being five metacentric (3, 4, 8, 9, 10) and six submetacentric (1, 2, 5, 6, 7, 11).

4. Discussion

4.1. Morphological analysis

The mean values and standard deviation of morphological characteristics (Table 1) showed variation among the genotypes for all analyzed traits. Four groups were formed based on morphological data (Fig. 1a), with 65% of the genotypes clustered in group A: Paluma, Pedro Sato, nine selections of Cortibel, and the spontaneous genotype São Pedro. Regarding this clustering, the group was mainly composed by genotypes that were pre-selected for fruit production, as well as Cortibel genotypes showing a common origin. As this group exhibited a median performance for the measured traits, the spontaneous genotype São Pedro can be considered potential, since it grouped with genotypes already pre-selected for production characteristics.

Group B clustered three spontaneous genotypes (Guaçuí, Caparaó 1 and Caparaó 2), forming a group with the lowest values for the characteristics. This result corroborated to treat of wild

Table 2
Estimates of autovectors associated to the principal components (PC), obtained from the matrix correlating eight quantitative traits of *P. guajava* L.

	Weight (g)	Length (cm)	Total diameter (mm)	Inner diameter (mm)	TD/ID	Pulp weight (g)	Seed weight (g)	Number of seeds
CP1	0.3979	0.3813	0.4004	0.3999	0.2575	0.4134	0.3304	0.1723
CP2	−0.2498	−0.1909	−0.1692	0.0686	−0.351	0.0616	0.4604	0.7272
CP3	−0.0518	−0.1239	−0.2671	−0.2797	0.8578	−0.0983	0.2187	0.1978
CP4	−0.0606	0.8411	−0.2243	−0.3307	−0.0813	−0.0832	−0.2014	0.2743
CP5	0.1563	0.0909	−0.0612	−0.5	−0.2233	0.0841	0.6826	−0.4366
CP6	−0.617	0.2294	−0.3623	0.4475	0.0971	0.2517	0.1745	−0.3632
CP7	0.0712	−0.1842	−0.2294	−0.2973	−0.0288	0.8495	−0.3065	0.059
CP8	−0.6023	0.0239	0.7084	−0.3303	0.0894	0.1288	−0.0091	0.0334

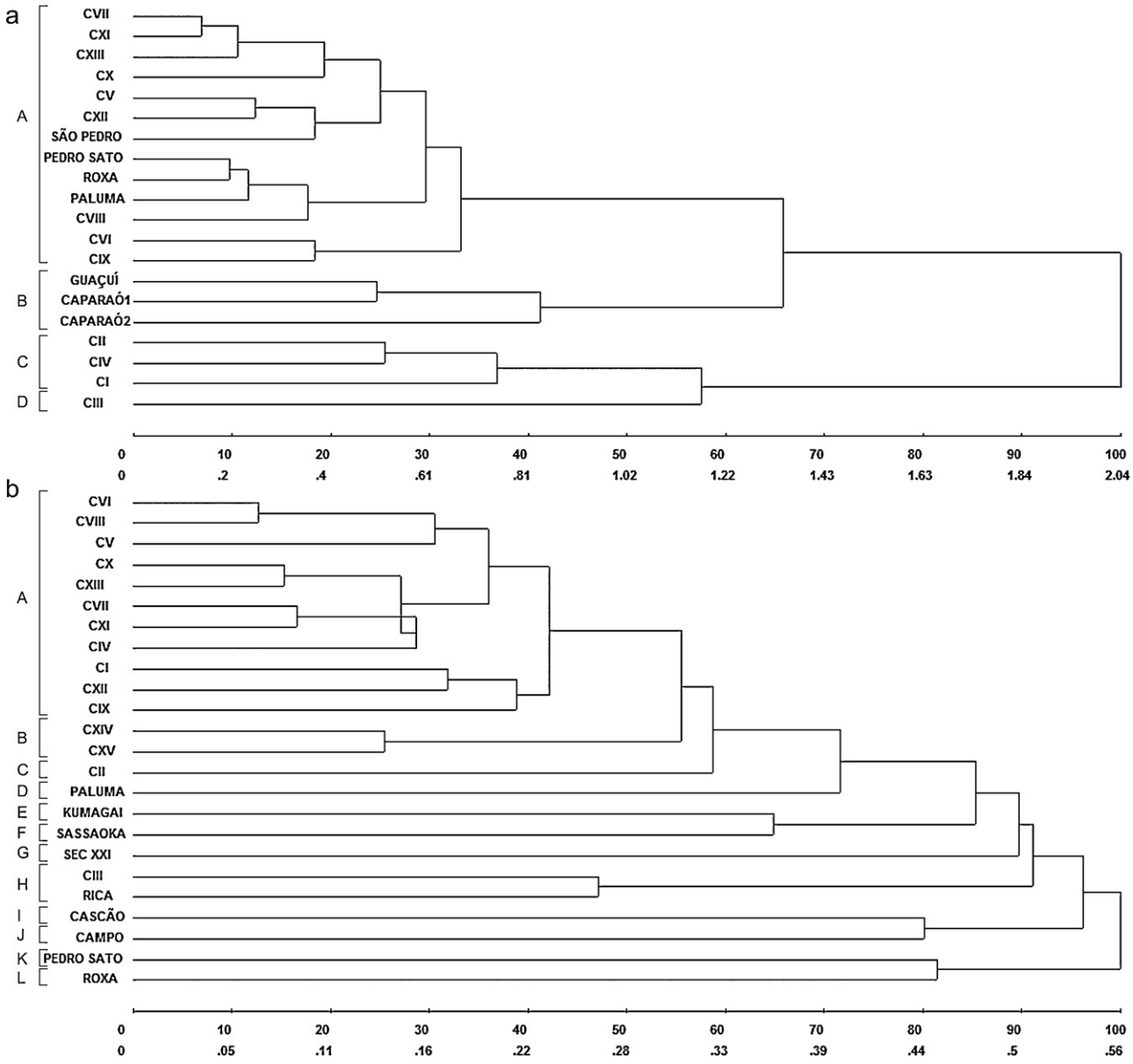

Fig. 1. UPGMA dendrogram from morphological (a) and molecular (b) data.

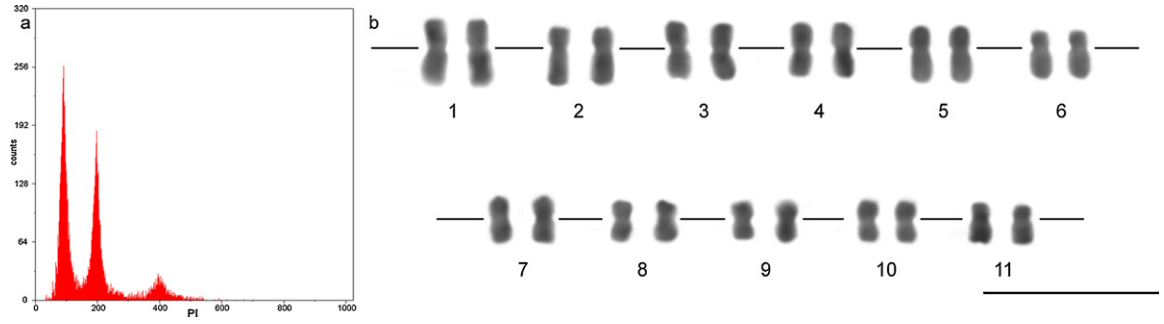

Fig. 2. (a) Representative flow cytometry histogram showing G_0/G_1 peaks obtained from simultaneous processing and analysis of nuclear suspensions stained with propidium iodide. G_0/G_1 peak of *P. guajava* 'Paluma' nuclei in the channel 95 ($2C = 0.95$ pg), and G_0/G_1 peak of *S. lycopersicum* (internal standard, channel 200, $2C = 1.96$ pg). (b) *P. guajava* 'Cortibel' karyogram assembled from chromosomes stained with 5% Giemsa. Metaphases were obtained from root tips treated with 4 μM amiprophos-methyl for 15 h, and macerated with 1:12 enzymatic solution for 2 h. From ten selected metaphases, *P. guajava* karyograms displayed $2n = 2x = 22$, consisting of five metacentric (3, 4, 8, 9, 10) and six submetacentric (1, 2, 5, 6, 7, 11) chromosomes. Bar = 5 μm.

genotypes that did not pass for any selection for production characteristics.

Cluster C grouped three Cortibel genotypes, corresponding to the highest values for production traits, evidencing the existence of variability among Cortibel genotypes, instead of a common origin, and high potential for production quality. Finally, cluster D consisted of a single genotype, CIII, which was the most divergent amongst all genotypes, with high variation for the morphological characteristics.

Principal component analysis identified total diameter as the characteristic being subjected to elimination from the analysis, with greatest weight in the last autovector. Considering this result, a new cluster was established without this characteristic, so as to verify its influence. Because the cluster had a different constitution, total diameter was considered an important characteristic for genetic diversity distinction, being therefore maintained. Since the weights for the characteristics were highly similar in the first autovector (Table 2), the most influent trait was not identified, reinforcing the importance of the all eight characteristics for the diversity analysis.

As demonstrated in this and other *P. guajava* approaches (Molero et al., 2003; Sanabria et al., 2006; Urdaneta et al., 2007; Jacobo et al., 2009; Lozano et al., 2009; Santos et al., 2010), morphological traits are valuable for genotype identification and diversity studies. Among these characteristics, the fruit quantitative has always been highlighted owing to its direct relationship with commercial and breeding qualities. Considering the inevitable environmental influence over the genotype, which may affect genetic variation for the mentioned characteristics, molecular techniques are required for making inferences at DNA level, complementing the analyses without environmental biases (Risterucci et al., 2005; Herrero et al., 2010).

4.2. SSR analysis

SSR were chosen for being extremely polymorphic, highly informative and codominant molecular markers, distinguishing between closely related individuals (Risterucci et al., 2005; Jones et al., 2009). These markers are more informative for genetic diversity studies than dominant ones, which have been previously applied for this purpose in guava (Chen et al., 2007; Delgado et al., 2007; Gomes-Filho et al., 2010; Mani et al., 2011; Liu and Yang, 2012).

Considering the low number of SSR markers applied in other approaches (Valdés-Infante et al., 2007; Herrero et al., 2010), the use of 24 polymorphic SSR was considered suitable for dissimilarity matrix assembly, allowing discrimination of the genotypes (Fig. 1b).

Most studies of genetic diversity in *P. guajava* have used dominant molecular markers, i.e. from binary data, analyzed by different coefficients (Chen et al., 2007; Delgado et al., 2007; Gomes-Filho et al., 2010; Mani et al., 2011; Liu and Yang, 2012). In comparison to codominant markers, such as SSR, dominant markers provide a larger number of monomorphic *loci*; when these are not used in statistical analyses, genetic distances between the genotypes may be overestimated. Thus, literature data for *P. guajava* from different molecular markers (i.e. dominant and codominant), as well as distinct forms of analysis, hamper comparisons between values obtained in different studies.

SSR dendrogram (Fig. 1b) showed twelve groups, with nine containing a single genotype. In cluster A, 11 Cortibel genotypes were allocated. This grouping reflects the genetic similarity observed in Cortibel, related to its origin from common parents. In cluster B, two Cortibel genotypes were clustered (CXIV and CXV), indicating their high genetic similarity, despite divergence from other Cortibel genotypes.

Cluster C was occupied by a single Cortibel genotype (CII), reflecting its singularity and genetic divergence from all other genotypes. Even though Cortibel genotypes show common parents, recombination may be responsible for the observed genetic variability and consequent divisions inside this group.

Clusters D, E, F, G and K were formed by single cultivated genotypes. This data was considered an important aspect, since these genotypes presented genetic diversity despite having been highly selected for production quality traits, so as to be registered as cultivars. Valdés-Infante et al. (2007) also detected divergence between cultivated and spontaneous genotypes when using SSR to study *P. guajava* diversity.

Cluster H gathered the genotypes CIII and Rica, representing the similarity between a Cortibel and a cultivated genotype. Clusters I, J and L also comprised single genotypes, corresponding to those collected at commercial plant nurseries, evidencing their genetic discrepancy in relation to all other materials. Groups formed by single genotypes indicate that such individuals are the most divergent, as observed in this work and by Gomes-Filho et al. (2010).

Though the resulting clusters reflect the similarity among most Cortibel plants, the genotypes CXIV, CXV, CIII and CII also indicate genetic diversity. Moreover, genetic divergence among cultivated and plant nursery genotypes could be observed, which was in accordance with their single origins.

4.3. 2C-value measurement and karyotype characterization

FCM histograms showed G_0/G_1 peaks exhibiting CVs below 5%, which are considered suitable for 2*C*-value measurements (Doležel and Bartos, 2005). Pinto et al. (2004) reported that, for most woody species, it is very difficult to obtain CV values at the level suggested by Doležel and Bartos (2005). In this sense, procedures were adopted here to yield suspensions showing high number of isolated, preserved and stoichiometrically stained nuclei, for instance the use of OTTO buffers supplemented with the anti-oxidant dithiothreitol, centrifugation of the nuclei suspensions, and supernatant discharge. These procedures supplied G_0/G_1 peaks with resolution appropriate for nuclear genome size measurement of the *P. guajava* genotypes (Fig. 2a).

P. guajava was the first fleshy-fruited Myrtaceae species in which nuclear genome size was measured. Using Feulgen microdensitometry, Bennett and Leitch (2005) found $2C = 0.7$ pg for this species. FCM was applied for 2*C*-value measurement in *P. guajava* by Costa et al. (2008). These authors used OTTO buffers and *Arabidopsis thaliana* as pseudo-internal standard, and reported that *P. guajava* 2*C*-value varied from $2C = 0.507$ pg in 'White' to $2C = 0.551$ in 'Red'.

Differently from Costa et al. (2008), *P. guajava* 2*C*-values were calculated here using *S. lycopersicum* as primary standard. This species was chosen because an ideal DNA reference standard ought to have a genome size close to that of the target species (Doležel and Bartos, 2005), thus avoiding instrumental nonlinearity errors during FCM. Moreover, revisiting the 2*C*-values of eight standards widely used in FCM and image cytometry, Praça-Fontes et al. (2011) suggested that *S. lycopersicum* is an adequate primary standard.

Following the classification of Soltis et al. (2003), *P. guajava* has a very small nuclear genome size, here reported to be $2C = 0.95$ pg. In contrast to Costa et al. (2008), no genome size variation was found at the present work for any *P. guajava* genotype.

Corroborating with FCM data, karyotype analysis showed that *P. guajava* genotypes have $2n = 2x = 22$, with relatively small chromosomes (Fig. 2b). Distinct authors have reported a predominance of $2n = 22$ chromosomes for this species (Bennett and Leitch, 2005; Costa and Forni-Martins, 2006a, 2006b, 2007). However, cytotypes showing aneuploidy and euploidy have been described in *P. guajava* (Andrade and Forni-Martins, 1998; Costa and Forni-Martins,

2006a, 2006b, 2007). As observed for nuclear 2*C*-value, no variation in chromosome number was found either for cytogenetically characterized *P. guajava* genotypes.

For the first time, morphometric characterization was carried out for *P. guajava*. According to Costa and Forni-Martins (2006a), these data lacked owing to the very small total chromosome length of this species. Considering this fact, the cytogenetic procedure was adapted to yield metaphases showing chromosomes with suitable resolution for discrimination, pairing of homologues and assembly of accurate karyograms (Fig. 2b).

4.4. Morphological, SSR, karyotypic and 2C-value data

Based on the obtained karyotype and nuclear 2*C*-values, *P. guajava* genotypes showed a diploid, stable and small-sized genome. In the evolutionary context, *P. guajava* showed karyotype characteristics related to ancestral angiosperm groups (Stebbins, 1949; Leitch et al., 1998; Costa and Forni-Martins, 2007).

Despite the similarity in genome size and karyotype, morphological and SSR data evidenced diversity among *P. guajava* genotypes, with better discrimination by SSR in relation to morphological data. Differently from morphological characteristics, molecular data are only based on the DNA molecule, without environmental influences.

In SSR and morphological dendrograms, most Cortibel genotypes were clustered, which is in accordance with their genealogy. Dendrogram analyses also evidenced that CIII was the most divergent of the Cortibel genotypes.

Relying on morphological results, Cortibel genotypes were grouped with cultivated ones, indicating adequate performance for production characteristics. This grouping was not observed in SSR analysis, which showed a genetic divergence between Cortibel and cultivated genotypes, as well as among Cortibel genotypes. The morphological similarity in association with molecular dissimilarity reinforces the relevance of these genotypes for hybridation breeding programs of *P. guajava*.

5. Conclusion

The use of distinct data from morphological, SSR molecular marker, karyotypic and genome size analyses expanded the knowledge about genetic diversity in *P. guajava*. In this context, the new data can help plant breeders to structure crop improvement programs, and contribute for evolutionary approaches.

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