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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.