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Genome size and chromosome number of *Psidium friedrichsthalianum* (O. Berg) Nied ("Cas") in six populations of Costa Rica

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Abstract. *Psidium friedrichsthalianum* (O. Berg) Nied is a species found from southern Mexico, Central America; and there are reports that it is also found in Venezuela and Ecuador. It is a common fruit component of the Costa Rican diet, and it is valued industrially for its high content of polyphenols, mainly proanthocyanidins (PACs). This crop is not completely domesticated and there are no improved varieties produced through plant breeding. Genome size or ploidy levels have not been investigated in Costa Rican populations of *Psidium friedrichsthalianum*. Information about chromosome number and genome size is paramount for plant breeding strategies. Therefore, the main objective of our study was to determine chromosome number using pollen meiocytes and genome size by flow cytometry in six populations of *P. friedrichsthalianum* in Costa Rica. We found x = 11 bivalent chromosomes in all meiocytes analysed, classifying these populations as diploid. All populations had an average nuclear DNA content of 2C = 1.960 ± 0.005 pg. No statistically significant differences in nuclear DNA content were found among populations. We conclude that the consistency in chromosome number and genome size among populations suggests a common origin among them. Our estimates of the number of chromosomes and genome size of *P. friedrichsthalianum* determined in this study will be essential for future breeding programs, hybridization practices and development of QTL (Quantitative Trait Loci).

Keywords: ploidy, fluorescent microscopy, 2C nuclear DNA, flow cytometry, Costa Rican Guava, plant breeding.

INTRODUCTION

Psidium friedrichsthalianum (O. Berg) Nied is a tropical species in the family Myrtaceae, subfamily Myrtoideae, tribe Myrteae (Lucas *et al.* 2019); commonly known as "Cas", "Sour Guava" or "Costa Rican Guava". It is a medium-sized tree with reddish branches and abundant foliage of intense green color. Flowers are perfect, possibly allogamous and pollination is performed by bees and occasionally by hummingbirds (Barahona and Rivera 1995). Fruits are fleshy globose berries, between 5 and 10 cm in diameter with a greenish to yellow exocarp and a very distinct soft and acidic pulp. In addition, it is presumed that its center of origin is in Costa Rica (Barahona and Rivera 1995; Rojas-Rodríguez and Torres-Córdoba 2013). "Cas" fruits are characterised by abundant polyphenol content, mainly proanthocyanidins (PACs); these metabolites have important antioxidant, anti-inflammatory, antimicrobial and vasodilatory properties (Cuadrado-Silva *et al.* 2017; Flores *et al.* 2013; Rojas-Garbanzo *et al.* 2019; Granados-Chinchilla *et al.* 2016; González *et al.* 2012). Vasconcelos *et al.* (2019) described the chemical composition and allelopathic properties of essential oils extracted from *P. friedrichsthalianum*, suggesting that this oil may be used as a natural weed control comparable in efficacy, to synthetic herbicides. This fruit is considered an important resource due to its photochemical properties; however, few studies have been conducted on this tropical fruit.

The germplasm of *P. friedrichsthalianum* in Costa Rica has not yet been genetically characterized, however, Srivastava (1977), reported this species as diploid (2n = 2x = 11), while Hirano (1967) reported tetraploid and hexaploid individuals in Central America samples. The diversity in chromosomal number previously reported for *P. friedrichsthalianum* may be a consequence of its ongoing domestication process. Information on chromosome numbers in the Myrtaceae is generally scarce, the fairly small chromosomes found in this taxonomic group, which rarely exceed 2 mm (Costa 2004), may curb chromosome determination. Presently, genome sizes have been reported for *Psidium acutangulum, Psidium cattleianum, Psidium guajava* L. (white cultivar), *Psidium guajava* L. (red cultivar), *Psidium guineense* and *Psidium grandifolium* (Costa and Forni-Martins 2006b, Costa *et al.* 2008; Machado-Marques *et al.* 2016; Coser *et al.* 2012; Souza *et al.*2015) (Table 1). However, the genome size or the 2C value of *P. friedrichsthalianum* have not been analyzed yet.

Estimates of the number of chromosomes and genome size for *P. friedrichsthalianum* are essential for the design of effective improvement strategies, such as hybridization practices, the development of QTL (Quantitative Trait Loci), as well as to better understand the effects of inbreeding and heterosis (Birchler 2013; Wash-

Table 1. Chromosomes number and genome size from different species of *Psidium* and *Eucalyptus* (Myrtaceae) determined in previous studies. The content of holoploid nuclear DNA (2C, pg DNA) and the content of monoploid DNA (1C, pg DNA) are also provided.

Species	2n	Ploidy level	Nuclear DNA content			Reference
			2C (pg)	1C (pg)	1C (Mbp)*	
Genus *Eucalyptus*						
Eucalyptus microcorys	22	2x	1.040	0.520	508.56	Almeida-Carvalho *et al.*(2017)
Eucalyptus botryoides	22	2x	1.350	0.675	660.15	Almeida-Carvalho *et al.*(2017)
Genus *Psidium*						
Psidium guajava	22	2x	0.950	0.475	464.55	Machado-Marques *et al.*(2016); Coser *et al.* (2012)
Psidium guajava (purple)	18	2x	0.990	0.495	484.11	Souza *et al.*(2015)
Psidium guajava ("Paluma")	22	2x	1.020	0.510	498.78	Souza *et al.*(2015)
Psidium guajava (white cultivar)	22	2x	0.507	0.253	247.43	Coser *et al.* (2012)
Psidium guajava (red cultivar)	22	2x	0.551	0.275	268.95	Coser *et al.* (2012)
Psidium grandifolium var. *cinereum*	44	4x	1.280	0.640	625.92	Costa and Forni-Martins (2009)
Psidium grandifolium var. *argenteum*	44	4x	0.820	0.410	400.98	Costa and Forni-Martins (2009)
Psidium cattleianum	44	4x	1.053	0.526	514.42	Costa and Forni-Martins (2006b)
Psidium cattleianum	44	4x	1.990	0.995	973.11	Souza *et al.*(2015)
Psidium guineense	44	4x	2.020	1.010	987.78	Souza *et al.*(2015)
Psidium guineense	44	4x	1.850	0.925	904.65	Machado-Marques *et al.*(2016)
Psidium acutangulum	44	4x	1.167	0.583	570.17	Costa and Forni-Martins (2009)

*1pg DNA = 978 Mbp (Dolezel *et al.* 2003; Bennett *et al.* 2000).

burn and Birchler 2014). Additionally, with the current development of second and third generation sequencing techniques (NGS), information on genome size or C values are essential to establish appropriate experimental conditions, to effectively prepare genomic libraries and sequencing of complete genomes (Leitch and Leitch 2008). The C-values reported here may be used as a tool for genomic analysis in this species, which should benefit genetic improvement practices in this species.

Therefore, given the absence of information on ploidy level and nuclear DNA content in populations of *Psidium friedrichsthalianum*; we aimed to determine the chromosome number via fluorescent DAPI stain and flow cytometry to determine the nuclear DNA content of this tropical fruit in six populations of Costa Rica, its likely centre of origin.

MATERIALS AND METHODS

Sample collection

We analysed individuals from six populations of *P. friedrichsthalianum* in Costa Rica. Samples were collected from local small-scale plantations from different regions in the country (Table 2). Plantations were located at different elevations ranging from sea level to over 1500 m asl (metres above sea level). Samples were always taken from reproductive trees and care was taken to collect samples from individuals that were separated by at least 10 meters to avoid collecting possible genets.

Chromosomal count using DAPI stain

Chromosome counts were performed on pollen mother cells in meiotic metaphase. At least seven flower buds were collected from each of six populations; flower buds ranged between 0.7 cm and 0.8 cm in length. Flower buds were fixed in FAA solution (96% ethanol, 5% glacial acetic acid and 40% formaldehyde) for 24 hours. As suggested by Dyer (1979), flower buds were dissected to 3/4 of their final bud size. Anthers were placed on slides and subjected to mechanical disaggregation (macerating anthers with a thin spatula) adding occasional drops of acetic acid to prevent desiccation. Macerated anthers were stained with DAPI fluorochrome (Sigma-Aldrich, Ilinois, USA) and were incubated in the dark for 5 min. We used an epifluorescence microscope (Olympus BX50, Olympus Corporation, Tokyo, Japan) to visualize and photograph stained cells. Only cells that were in a state of meiotic metaphase were photographed. For each population at least five slides were evaluated and at least one cell was recorded in a state of meiotic metaphase. Finally, to facilitate chromosome counts, image color adjustments were performed with Adobe Photoshop CS82 (Adobe Systems, San Jose, CA) and the ImageJ software (US National Institutes of Health, 2007) was used to count chromosomes.

Flow cytometry estimates of DNA content

We used flow cytometry to estimate genome size on *P. friedrichsthalianum*. We collected fruits from at least 10 individuals per population. Seeds were manually extracted, washed with tap water and dried in open air for a week to remove moisture. After one week, seeds were sown in pots with vermiculite soil and placed in a greenhouse for 12 weeks until seedling emergence. Ten seedlings per population were analyzed in a BD FACSCalibur TM (Becton Dickinson, San Jose, CA, USA) flow cytometer. After initial parameter adjustment in the flow cytometry equipment, samples were prepared following the protocol by Dolezel *et al.* (2007) with some modifications. We used 1 mg of leaf sample from *Glycine max* as a reference (2C = 2.50 pg) (Dolezel *et al.* 2007) and 5 mg of leaf tissue from *P. friedrichsthalianum*. Leaves were

Table 2. Collection sites of "Costa Rican Guava" used to determine genome size and chromosomal number. **PPT**: mean annual precipitation (mm); **Samples CMF**: number of seedlings used for flow cytometry; **Samples NM**: number of samples used to determine chromosomes count.

Population name	Geographical coordinates		Altitude (m a.s.l.)	PPT (mm)	Temp (°C)	Samples CMF	Samples NM
Cervantes	09°53′28.3″N	83°47′24.0″W	1465	2500	24	10	5
Guápiles	10°13′42.1″N	83°46′06.3″W	262	4535	27	10	5
Tacacorí	10°03′07.3″N	84°12′52.3″W	952	2100	23	10	5
Ciruelas	09°59′05.7″N	84°15′26.3″W	910	1900	23	10	5
Batán	10°04′34.4″N	83°22′37.2″W	114	3567	28	10	5
Escazú	09°55′08.3″N	84°07′42.6″W	2428	1929	24	10	5

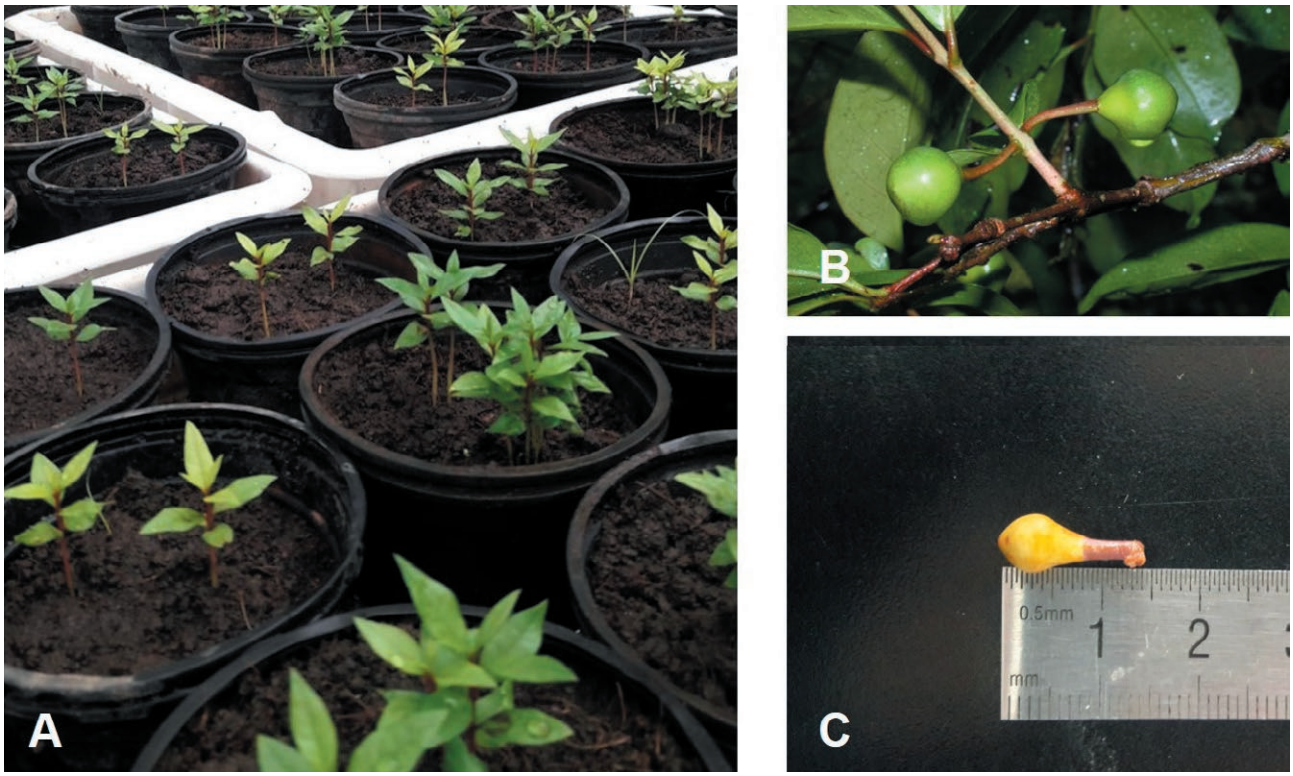

Figure 1. (a) Seedlings of *P. friedrichsthalianum* used to measure genome size by flow cytometry. (b and c) Flower buds of *P. friedrichsthalianum* used for cytogenetic observations.

placed in a petri dish on ice, then 1 ml of OTTO-I lysis buffer (0.1 M citric acid, 0.5% (vol / vol) Tween 20) supplemented with 2 mM dithiothreitol (DTT) was added to the leaf cutouts (Otto 1990). Subsequently, leaves were cut with a razor blade until homogenization. The extract was filtered through a 41µm Nylon mesh, onto a 2.0 ml microcentrifuge tube. The filtrate was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl of OTTO-I lysis buffer and incubated for 15 min at 4 ° C. After incubation and prior to analysis in a flow cytometer, 300 µl of OTTO-II buffer (0.4 M Na 2 HPO 4 · 12 H 2 O) (Otto 1990), 20 µl of propidium iodide (50 µg / ml) and 2 µl of RNase (50 µg / ml) were added to the mixture.

All measurements were based on the fluorescence of at least 5000 total events (total nuclei). We analyzed two independent replicas of each sample on different days and estimated an average nuclear DNA content. Mean fluorescence intensity (MFI), number of events per peak and variation coefficient were all calculated using the FCS Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA). Finally, the nuclear DNA content was calculated according to Dolezel *et al.* (2007) as follows:

$$A = \frac{(B \times C)}{D}$$

Where A = 2C (pg) nuclear DNA content concentration of *P. friedrichsthalianum*; B = Mean fluorescence intensity (MFI) of the G_0 / G_1 peak of *P. friedrichsthalianum*; C = 2C (pg) nuclear DNA content of the internal standard; D = MFI of the G0 / G1 peak of the internal standard. Genome size was estimated from DNA content as 1 picogram (pg) of DNA being equivalent to 978 megabase pairs (Mbp) (Bennett *et al.*2000; Dolezel *et al.* 2003). The 2C nuclear DNA content data of all individuals was compared among populations with a one-way ANOVA, followed by Tukey's test to determine individual differences ($p < 0.05$). Statistics were done using R 3.5.0 software (R Core Team 2018).

RESULTS AND DISCUSSION

We consistently found 11 bivalent chromosomes in all meiocytes from *Psidium friedrichsthalianum* (Figure 2) across all populations (Table 2). Taking into account that the basic chromosome number of the Myrtaceae

family is x = 11 (Atchison 1947; Raven 1975) we classified all Costa Rican samples of *P. friedrichsthalianum* as diploid (2n=2x=22). The diploid nature of the Costa Rican guava mirrors the results from Srivastava (1977), who similarly found a 2n = 2x = 22 diploid chromosome count in different genotypes of *Psidium friedrichsthalianum*. Costa and Forni-Martins (2006a, b, 2007) also described chromosome numbers for 50 species in the Myrtaceae, and found a predominance of 2n = 2x = 22 diploid species. Naitani and Srivastava (1965), Coser *et al.* (2012), Éder-Silva *et al.* (2007), and Souza *et al.* (2015), all found predominantly diploid species in the *Psidium* genus, such as in *Psidium chinense* and *Psidium guajava*.

Previous results reported *P. friedrichsthalianum* individuals with 2n=4x=44 and even 2n=6x=66 (Hirano 1967), suggesting that this species may have tetraploid and hexaploid members. These results clearly indicate that there may be variation in ploidy levels among populations of *P. freidrichsthalianum* in different areas. In contrast, our results show that at least in Costa Rica, cultivated populations are consistently diploid. This chromosomal uniformity may be the result of a common historical origin among populations. Alternatively, our results may also be a consequence of artificial selection by farmers who selected cytotypes with specific homogenous traits of interests such as fruit size of pulp content. Multiple cytotypes have also been found in other *Psidium* congeners, for example in *Psidium cattleyanum* the cytotypes 2n = 44, 66, 77 and 88 have been described (Costa and Forni-Martins 2006a; Costa 2009). Multiple cytotypes have been also found in populations of *Psidium guineense* and *Psidium guajava* (Srivastava 1977; Costa and Forni-Martins 2006a; Éder-Silva *et al.* 2007; Souza *et al.* 2015). Polyploidy is recognized as one of the main evolutionary forces in angiosperms (Soltis *et al.* 2015); and it is frequently associated with interspecific hybridization followed by chromosomal duplication to restore hybrid fertility (Soltis *et al.* 2009). Results from congeners suggests that *P. friedrichsthalianum* may also have the potential to create other cytotypes may represent important prospects for future breeding programs.

Our study found bivalent and univalent chromosomes in meiocytes of *P. freidrichsthalianum* (Figure 2a-2c). Chromosomes were also observed in a trivalent state (Figure 2d) and this is consistent with previous observations by Srivastava (1977) in this species. Univalent chromosomes are frequently observed in plants; these can arise through three different ways: (i) when a chromosome is not matched completely in zygotene stage; (ii) when paired bivalents separate in diplotene because robust chiasmata have not yet formed between them; (iii) due to premature disjunction of the bivalents during anaphase (Pires-Bione *et al.* 2000). The premature migration of univalent chromosomes to the poles during cell division is common in plants, giving rise to micronuclei (Pagliarini 1990; Pagliarini and Pereira 1992; Consolaro *et al.* 1996). Alternatively, univalent chromosomes may occasionally occur in plants due to environmental factors such as temperature fluctuations (Heilborn 1934; Katayama 1935). Some of our sites differ drastically in climatic conditions, however, further studies are needed to better understand the cytology of this species.

Our flow cytometry estimates were very consistent across all plant samples. Our coefficients of variation were all less than 5% (Table 3), which confirms that our suspensions had a sufficient number of stoichiometrically stained and intact nuclei. Additionally, DTT used in nuclei suspensions proved to be effective inhibiting cytosolic interfering compounds which resulted in clear histograms. DTT is commonly used in flow cytometry studies because of its broad antioxidant activity (Dolezel *et al.* 2007). In our study, DTT was very efficient because many woody species in the Myrtaceae, as is the case of *P. friedrichsthalianum*, contain abundant secondary metabolites that may interfere with DNA content staining (Loureiro *et al.*2006) (Ohri and Kumar 1986).

We determined a mean nuclear value of 2C = 1.960 ± 0.005 pg for *P. friedrichsthalianum*, equivalent to

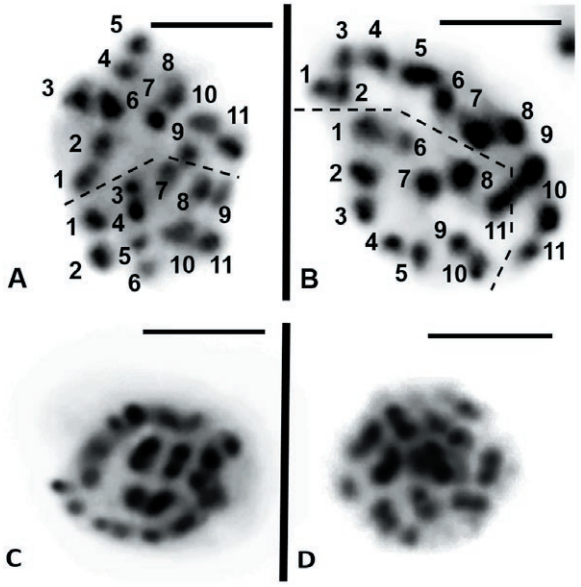

Figure 2. Bivalent chromosomes of *P. friedrichsthalianum* in meiotic metaphase, stained with DAPI, scale bar 10um. (a, b and c) Samples from the populations of Cervantes, Tacacorí and Escazú respectively, showing 11 bivalent chromosomes. (d) Image showing chromosomes in trivalent, bivalent and univalent states.

Table 3. Parameters obtained by flow cytometry to determine the genome size of *Psidium friedrichsthalianum*. **NE**: Number of events obtained; **CV**: Coefficient of variation obtained; **2C (pg)**: holoploid nuclear DNA content obtained; 1pg DNA = 978 Mbp (Dolezel *et al.* 2003; Bennett *et al.* 2000).

Species	NE	CV	1C (pg)	2C (pg)	Mbp
Psidium friedrichsthalianum	2452 ± 0.001	2.95 ± 0.007	0.980 ± 0.005	1.960 ± 0.005	1916.88
Glycine max (*standard*)	2756 ± 0.005	3.01 ± 0.005			

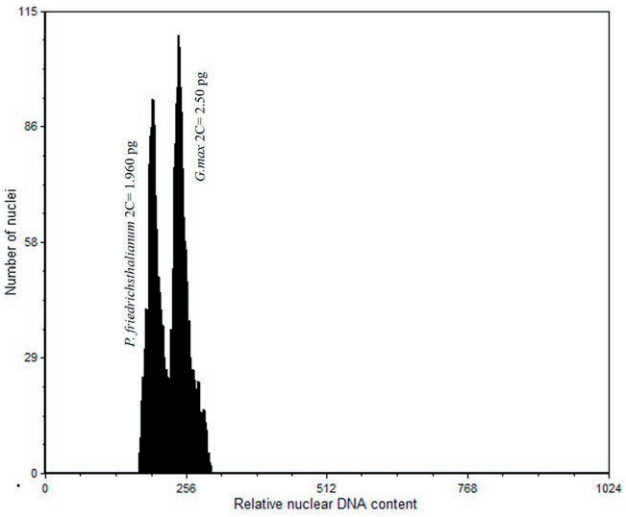

Figure 3. Relative fluorescence intensity (propidium iodide (PI)) histogram obtained after a simultaneous cytometric analysis of nuclei of reference standard (*Glycine max,* 2C=2.50 pg of DNA) and *Psidium friedrichsthalianum* (2C= 1.960 ± 0.005 pg).

1916.88 Mbp (Bennett *et al.* 2000) (Figure 3, Table 3). Nuclear DNA content did not statistically vary among all six populations (F=0.29; df=5; p = 0.917). Leitch *et al.* (1998) and Soltis *et al.* (2003) classified species with 1C ≤ 1.4 pg content as species with a very small genomes compared to other angiosperms. Therefore, given our 1C estimates (1C = 0.98 ± 0.005 pg) (Table 3) the Costa Rican guava should also be classified as a small genome species. Consistently, Machado-Marques *et al.* (2016) found that *Psidium guajava* and *Psidium guineense*, also have very small genomes as 1C = 0.475 pg and 1C = 0.925 pg, respectively (Table 1). Almeida- Carvalho *et al.* (2017) determined that 25 species of the genus *Eucalyptus* (Myrtaceae), all had 1C values between 1C=0.40 pg and 1C=0.75 pg which may indicate that the Myrtaceae family may typically contain species with smaller genomes. On the other hand, our 2C estimates (2C =1.960 ± 0.005 pg) are within the range described by Souza *et al.* (2015), who also used flow cytometry on different species of *Psidium* and found 2C values that ranged between 2C=0.99 pg and 2C=5.48 pg. However our estimates are significantly higher than those found for different varieties of *Psidium guajava*; for example, Coser *et al.* (2012) found 2C = 0.507 pg for the white varieties, and 2C = 0.551 pg or 2C = 0.950 pg for the red varieties; while Souza *et al.* (2015) found 2C = 0.990 pg and 2C = 1.020 pg in purple and "Paluma" varieties respectively (Table 1). These differences in genome size may be due to (i) natural or bred adaptations of these species to different environmental conditions (Cavallini and Natali 1990), for example, to new cultivation environments; (ii) hybridization events, or (iii) changes in repetitive DNA sequences (Martel *et al.*1997). Several authors have suggested that transposable elements (TE) may be important in the evolution of genome sizes in plants (Wang *et al.* 2016; Wendel *et al.* 2016; Zhao *et al.* 2016). For example, Almeida-Carvalho *et al.* (2017) compared the genome size of two *Eucalyptus* species, *E. botryoides* (2C=1.350 pg) and *E. microcorys* (2C=1.040 pg); and found big differences in genome size between them, although both had the same chromosome number (2n = 2x = 22) (Table 1). They argued that variations in 2C values in these *Eucalyptus* were caused by chromosome rearrangement and possibly TE elements.

Therefore, our results on ploidy level and genome size of *P. friedrichsthalianum*, contribute to the cytogenetic characterization of this economically important fruit species. This information may be used to design regional conservation strategies that preserve local genetic resources. Flow cytometry may be used to assess ploidy level in *in vitro* propagated plants (Ochatt *et al.* 2011), to screen for plants with higher ploidy levels, which may have new features of economic interest such as increased fruit size, or better juicing capabilities. Additionally, results from our study could aid the taxonomic definition of *P. friedrichsthalianum* species and the understanding of phylogenetic relationships among other members in the genus *Psidium*.

CONCLUSIONS

Populations of *Psidium friedrichsthalianum* from six different regions of Costa Rica, the likely centre of origin

of this species, have a chromosome number equal to 2n = 2x = 22, indicating that cultivated populations in Costa Rica, are all consistently diploid. Furthermore, these populations have an average 2C nuclear DNA content of 1.960 ± 0.005 pg. The uniformity found across populations in terms of chromosomal number and nuclear DNA content, suggests a common origin among them.

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GEOLOCATION DATA

Geolocation data is found on Table 2.

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