

Cytogenetics and Flow Cytometry-based DNA Quantification in *Herreria salsaparilha* Martius (Herreriaceae): a Medicinal Species

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Summary Adventitious root tips developing from base of cuttings and leaf samples of *Herreria salsaparilha* were used to determine the karyotype and to estimate DNA amount, respectively. Flow cytometry methodology was used with DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide) as specific DNA fluorochromes, and leaf samples of *Pisum sativum* (9.09 pg) as an internal standard. Cytogenetic techniques determined that *H. salsaparilha* has 29 pairs of homologous chromosomes ($2n=58$), 12 acrocentric pairs (length varying from 1.75 to 10.51 μm), 16 submetacentric pairs (1.30 to 3.45 μm), and 1 metacentric pair (2.8 μm). The DAPI- and PI-based flow cytometry analysis revealed that *H. salsaparilha* has nuclear DNA content $2C=5.5$ pg, equivalent to approximately 5.31×10^9 base pairs. This karyotype asymmetric type may suggest an evolutionary process with involvement of polyploidization, being justified by the relative increased chromosome number ($2n=58$) and fusions.

Key words C-value, Flow cytometry, *Herreria salsaparilha*, Herreriaceae, Karyotype.

Species within *Herreria* genus occur predominantly in warm regions of the globe, mainly in Equatorial America; Mexico, Peru and Brazil. However, under the denomination of salsaparrilha are almost 200 species of the genus *Smilax*, some of them, being also of medicinal importance such as *Smilax salsaparilha*, native of the Meridional America (Mexico), *S. officinalis* (Houndurean salsaparrilha), *S. papyraceae* (Brazilian salsaparrilha), *S. japecanga*, *S. syringoides*, *S. syphilitica*, *S. brasiliensis* and *Salsaparrilha medica* (“salsaparrilha de Vera Cruz”). In the 17th century the Spanish used roots from *Smilax* assuming that it can be used as a medicine to control syphilis. The medicinal properties of “salsaparilhas”, mainly those belonging the genus *Smilax*, are due to the presence of steroidal saponins such as sarsaponin, parrilin and smilasaponin. Those plants were spread around the world in consequence of their depurative and diuretic properties (Teske and Trentini 1995).

Herreria salsaparilha Martius is a monocotyledon popularly known as “salsaparrilha”, “mandioquinha”, “japecanga”, “salsa do mato”, “salsa gerdas”, “salsa caroba” and “cipó salsa”. Since the colonial period the roots have been used in popular medicine as depurative and anti-syphilitic (Peckolt 1936, Rodrigues and Carvalho 2001). In Brazil, it is found in the states of Minas Gerais, Rio de Janeiro, São Paulo, Mato Grosso do Sul, Mato Grosso, Goiás and Distrito Federal. Species of the genus *Herreria* occur also in Paraguay, Uruguay, Argentina and Chile (Lopes and Andreatta

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2003).

Historically, the genus *Herreria* has already been considered as belonging to several families of Smilacaceae and Liliaceae (Lopes and Andreato 2003). However, recent reviews of the genus have concluded that it should be classified as *Herreriaceae* (Ordem Asparagales) (Lopes and Andreato 2003), which is in agreement with the classification proposed by Dahlgren and Clifford (1982). In addition, the genus *Clara*, established by Kunth in 1948 (as reviewed by Lopes and Andreato 2003) was considered to be closely related to *Herreria* Ruiz *et* Pav. Over the years, it was considered to be a valid genus or a synonym of *Herreria*, depending on the author (Lopes and Andreato 2003).

There are only a few studies on *H. salsaparilha* cytology. Satô (1942), after having studied the karyotype in Liliaceae species and correlated families, found $2n=54$ and described the karyotype, but his studies were not conclusive.

Cytogenetics studies provide important information concerning the plant genomic organization, such as number and morphology of chromosomes. This kind of information can help solving problems of taxonomic identification and/or classification (Levin 2002), as is important for evolutionary studies, as it may identify which chromosome alterations and mechanisms were involved in the speciation process (Bennett and Leitch 2005). Flow cytometry is a convenient and rapid method that has been used extensively for estimation of nuclear genome size in plants (Dolezel *et al.* 1998), and as auxiliary tool in plant taxonomy (Ohri 1998). Genome size is a character with taxonomic significance as well as other characters and in some cases even more, because variation in genome size may reflect many mutations steps (Greilhuber 1998).

In the present study cytogenetics and flow cytometry techniques were used to determine the karyotype and quantify nuclear DNA content for *Herreria salsaparilha*, aiming to contribute to further taxonomic studies of the species.

Materials and methods

Plant material

Plants of *Herreria salsaparilha* were obtained from cuttings derived from donor plants collected at the township of Teixeiras/MG (649 m altitude; 20°45'20"S; 42°52'40"W). The climate in the region of Teixeiras is classified as tropical of altitude (Cwb according to Köppen), with warm summers and temperature below 22°C in the warmest month (Köppen 1948). Cuttings (average 15 cm height) containing two axillary buds were placed vertically in a sand rooting bed, and kept under 50% shaded green house and watered on a daily basis. Rooting occurred without adding any growth regulator. Rooted cuttings were transplanted to polyethylene containers (6 l capacity) filled up with a 3 : 1 : 1 mixture (soil : manure : sand, respectively). The plants were maintained under the same 50% light reduction, being periodically watered and controlled for pests and diseases.

The specimens vouchers are deposited in the VIC Herbarium (Universidade Federal de Viçosa, Viçosa, MG, Brasil) under the number 25.825. The identification of the species was confirmed by a specialist on the *Herreriaceae* family, Prof. Rosana Conrado Lopes (Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro, RJ).

Karyotype analysis

Cuttings were obtained from plants grown under shading, rooted in washed sand, and used for root tip collection. Root tips (average 1 cm length) were washed for 20 min and placed for 2 h in distilled water. Root tips were transferred to test tubes containing 3 μ M amiprophos-methyl (APM) solution for 4 h at 34°C, then washed with distilled water for 10 min, and fixed in fresh methanol : acetic acid (3 : 1) solution. The fixative was changed three times and stored at -20°C. For enzymatic maceration, root tips were washed with distilled water and transferred to Eppendorf

tubes containing 1 : 12 Flaxzyme (Novo FermentTM): distilled water, for 90 min at 34°C, in water bath. Subsequently, roots were washed for 15 min with distilled water, post-fixed in fresh methanol:acetic acid solution (3 : 1), and stored again at -20°C. Slides were prepared by meristematic cellular dissociation, air-dried, and placed on a hot plate at 37°C for 20 min. The slides were stained with 3% Giemsa solution in phosphate buffer (pH 6.8) for 4 min, and then washed in distilled water and air-dried at room temperature (Carvalho and Saraiva 1993, 1997).

Figures of chromosomes were randomly captured with a Photometrics CCD 12 bits video camera coupled to an OlympusTM BX 60 microscope with a 100× immersion lens. The digitized images were analyzed by Image-Pro Plus SoftwareTM (Version 4.5, MediaCybernetics) and by NIH Image public-domain program (Rasband 1998). The arms of each chromosome were measured in pixel units and then converted to a micrometer scale. Chromosome images processing was carried out on a Power Macintosh G4 computer. The full lengths (μm) of short and long arms of chromosomes were measured and the relative length [(RL (%))], centromeric rate (CR), and arms ratio (AR) were calculated. The centromeric index was determined according to the criteria for morphologic classification of chromosomes as proposed by Guerra (1986).

Flow cytometry

A two-step flow cytometric analysis was carried out to determine DNA nuclear content of *H. salsaparilha* basically as described Doležal and Göhde (1995). Leaf discs (10 mm diameter), removed from fully expanded leaves derived from shading house-grown donor plants, were placed in a Petri dish (60 mm diameter), together with a similar amount of leaf tissue from a known internal DNA (9.09 pg) standard, *Pisum sativum* cv. Ctirad. Leaf discs were chopped with a sharp razor blade for 2 min in 0.5 ml of nuclei extraction buffer—NEB (0.1 M citric acid and 0.5% Tween 20), and filtered through a 30 μm pore-size nylon sieve nested into a 3.5 ml tube (Partec, Germany), and centrifuged for 5 min at 250×g. An additional 0.5 ml extraction buffer aliquot was added throughout the filter and the final volume adjusted to 1 ml. The suspension was centrifuged for 5 min at 1200 rpm. Then the supernatant was discarded, and the pellet was briefly vortexed and resuspended in 100 μl NEB, and stored for 10 min at 5°C in the darkness. Samples were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma Chem. Co., USA) or PI (propidium iodide; Sigma Chem. Co., USA). A 1.5 ml aliquot of a staining buffer solution (500 μl of 15 μM DAPI and 0.4 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ or 500 μl of 15 mM PI, 500 μl of 1 mg ml⁻¹ RNase and 0.4 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) was used to stain the samples with DAPI or PI. The samples were incubated at room temperature for 10–15 or 30 min in the dark for staining with DAPI or PI, respectively.

After staining the samples were transferred to reading tubes and run through a Partec PAS II/III Flow Cytometer (PARTEC GmbH, Munster, Germany), equipped with laser sources and a high pressure mercury arc lamp (HBO-100 W) and filters KG-1, BG-38 and UG-1 for excitation; the last one used for 200–700 nm UV excitation. The GO/G1 peaks and coefficients of variation (CV) were calculated using the FlowMax software (Partec). *Pisum sativum* L. (2C=9.09 pg) was used as internal standard (Doležal *et al.* 1992). Nuclear DNA content was estimated according to Doležal (1991). The peak running in channel 100 corresponds to *H. salsaparilha*. The results presented in pg were transformed into base pairs. At least 5,000 nuclei per plant sample were analyzed. Samples with CV above 5% were not used in the analyses.

Results and discussion

Karyotype analysis

The procedures applied for chromosome pre-treatment and preparation resulted in morphologically defined metaphases, which were scattered at the same focal plane of the slide, allowing the assembly of the first karyogram published for the species. *Herreria salsaparilha* has 29 pairs of ho-

Table 1. Morphometric data and classification of the chromosomes of *Herreria salsaparilha* Martius ($2n=58$)

Chromosome	Full length (μm)	Average length		R*	CI [#]	Class	RL (%)**
		Short arm	Long arm				
1	10.05	0.70	9.35	13.36	7.0	A	10.51
2	8.35	0.65	7.70	11.85	7.8	A	8.73
3	7.10	0.65	6.45	9.92	9.2	A	7.42
4	5.15	0.65	4.50	6.92	12.6	A	5.38
5	4.95	0.50	4.45	8.90	10.1	A	5.18
6	4.15	0.75	3.40	4.53	18.1	A	4.34
7	4.05	0.60	3.45	5.75	14.8	A	4.23
8	3.95	0.95	3.00	3.16	24.1	A	4.13
9	3.45	1.05	2.40	2.29	30.4	SM	3.61
10	3.40	0.65	2.75	4.23	19.1	A	3.55
11	3.25	0.55	2.70	4.91	16.9	A	3.40
12	3.05	0.65	2.40	3.69	21.3	A	3.19
13	2.80	1.30	1.50	1.15	46.4	M	2.93
14	2.65	0.80	1.85	2.31	30.2	SM	2.77
15	2.55	0.70	1.85	2.64	27.5	SM	2.67
16	2.50	0.65	1.85	2.85	26.0	SM	2.61
17	2.40	0.70	1.70	2.43	29.2	SM	2.51
18	2.25	0.70	1.55	2.21	31.1	SM	2.35
19	2.10	0.70	1.40	2.00	33.3	SM	2.20
20	2.05	0.70	1.35	1.93	34.1	SM	2.14
21	1.95	0.75	1.20	1.60	38.5	SM	2.04
22	1.85	0.65	1.20	1.85	35.1	SM	1.93
23	1.80	0.55	1.25	2.27	30.6	SM	1.88
24	1.75	0.30	1.45	4.83	17.1	A	1.83
25	1.75	0.65	1.10	1.69	37.1	SM	1.83
26	1.75	0.70	1.05	1.50	40.0	SM	1.83
27	1.65	0.65	1.00	1.54	39.4	SM	1.73
28	1.65	0.65	1.00	1.54	39.4	SM	1.73
29	1.30	0.50	0.80	1.60	38.5	SM	1.36
Total	95.65	20.00	75.65				
Average				3.78	20.9		100.00

*R: ratio between arms, [#]CI: Centromeric rate, **RL: Relative length, A=acrocentric, SM=submetacentric, M=metacentric.

mologous chromosomes ($2n=58$) (Fig. 1) in all analyzed cells: 12 acrocentric pairs (with total arm length varying from 1.75 to 10.51 μm), 16 submetacentric pairs (1.30 to 3.45 μm), and 1 metacentric pair (2.8 μm) (Table 1).

After an extensive search in specialized literature, the somatic chromosome number for species within the genus *Herreria* was reported only by Satô (1942). A somatic chromosome number of 54 was determined for the species examined including the species in Liliaceae. The number of chromosomes found by us in *H. salsaparilha* ($2n=58$) differs from the results of Satô (1942) ($2n=54$). Additionally, the relative high somatic chromosome number found for *Herreria* that ranged from 26 to 32, for instance, *S. nipponica* Miq., *S. arisanensis* Hayata, *S. chapaensis* Gagnep., *S. discotis* Warb, except for *S. china* L. (64, 96) as reviewed by Goldblatt and Johnson (1996, 2000). The determination of the chromosome number is an important insight to strengthen the recently proposition of its inclusion within Herreriaceae (Lopes and Andreato 2003), since the species has been previously included within families Liliaceae, Smilacaceae and Dioscoreaceae (Satô 1942,

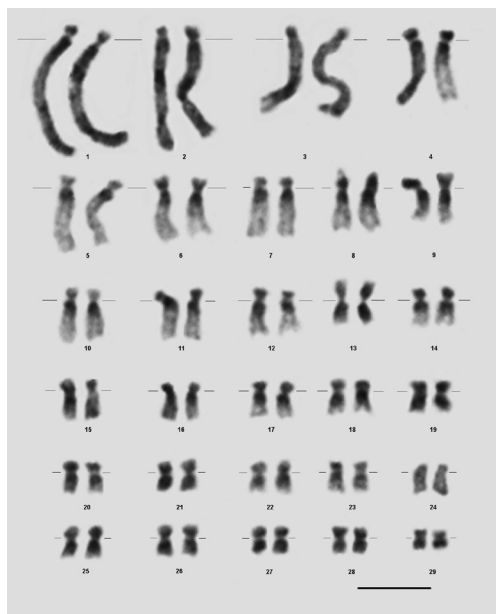


Fig. 1. Karyogram of *Herreria salsaparilha* Martius. Metaphase chromosomes pre-treated with 3 μ M APM, for 4 h, and stained with 3% Giemsa, organized in 29 homologous pairs ($2n=58$). Bar=5 μ m.

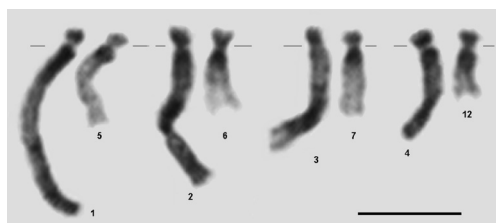


Fig. 2. Some examples of chromosomes (taken from figure 1) that suggest that a fusion process can be involved in the evolution of *Herreria* karyotype. Note the similar morphology of chromosome pairs (1–5, 2–6, 3–7 and 4–12) between the one-half proximal regions (left chromosomes) as compared to the other chromosomes (positioned at right side). Bar=5 μ m.

reason for the divergent results between the studies. Indeed, based on air-dry technique (Carvalho and Saraiva 1993, 1997), the species displayed a relatively high somatic chromosome number ($2n=58$), and a marked variation in chromosome size (Fig. 1), which is not evident in work by Satô.

In the present work, the use of the mitotic blocking APM and fixation in methanol:acetic acid solution provided metaphase chromosomes with high morphological integrity. According to Plan-chais *et al.* (2000), APM causes microtubule depolymerization, hindering cells to enter anaphase, while another advantage of this herbicide is its high efficiency at micromolar concentrations. The

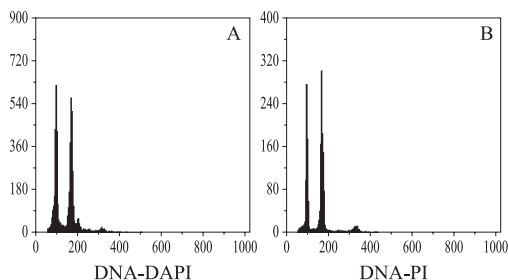


Fig. 3. Histograms of nuclei stained with fluorochromes (A) DAPI (4',6-diamidino-2-phenylindole) and (B) PI (propidium iodide) using *Pisum sativum* L. ($2C=9.09$ pg) as internal standard. Vertical and horizontal axes represent the amount of nuclei and fluorescence intensity, respectively. GO/G1 peak for *Herreria salsaparilha* Martius corresponds to channel 100 in both histograms (DAPI and PI), and for *P. sativum* to channel 166 in histogram A (DAPI, $2C=5.5$ pg) and 166 in histogram B (PI, $2C=5.5$ pg), respectively.

Lopes and Andreata 2003). Based on a thorough analysis of morphological characters, collections and bibliography, it was concluded that *Clara* is an independent genera and establish a new circumscription (Lopes and Andreata 2003).

According to Satô (1942), *H. salsaparilha* has four pairs of long chromosomes with sub-terminal constriction, and 23 pairs of short chromosomes, analogous to the *Yucca-agave* type (5 long and 25 short) (Satô 1935). The genera *Yucca* sp. and *Agave* sp. belong to the family Agavaceae, closely related with the family Herreriaceae (Dahlgren and Clifford 1982). It is important to point out that the methodology adopted by Satô (1942), in which the root tips were fixed, embedded in paraffin, sectioned (10–25- μ m thick sections), and stained with gentian violet, having the results documented by drawings with camera lucida. This methodology, different from the one used in the present work, most probably could be the

Table 2. Nuclear DNA content (picograms) of *Herreria salsaparrilha* Martius using fluorochromes DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide), and *Pisum sativum* L. as internal standard

Fluorochrome	Species	Chanel	DNA (pg)
DAPI	<i>H. salsaparrilha</i>	100	5.51
	<i>P. sativum</i>	165	9.09
PI	<i>H. salsaparrilha</i>	100	5.48
	<i>P. sativum</i>	165	9.09

fixation solution avoids losing nucleic acids and proteins, preserves morphology and causes precipitation of nucleases that can damage chromosomes (Leitch *et al.* 1994). Besides, the cellular dissociation methodology provided slides with metaphases in well-individualized chromosomes. The captured images were used for carrying out detailed analyses of each pair of homologous, and the consequent suitable karyogram assembly. In agreement with other works (Carvalho and Saraiva 1993, 1997, Caixeta and Carvalho 1998, Clarindo and Carvalho 2006, Clarindo *et al.* 2007), the meristem enzymatic maceration and cellular dissociation method provides high-quality chromosome preparations.

Interestingly, the morphology of the chromosomes showed a marked variation in size (Table 1). This karyotype asymmetric type may suggest an evolutionary process with involvement of polyploidization, justified by the relative increased chromosome number ($2n=58$) and fusions (Fig. 2). Allopolyploidization followed by chromosome rearrangements is a common process in plants. The structural rearrangements among chromosomes, after hybridization and allopolyploidization, could possibly have an important contribution for genetic diploidization (Ma and Gustafson 2005), while maintaining genome stability.

Flow cytometry analysis and DNA quantification

The 2C value was determined for *Herreria salsaparrilha* (5.51 pg) using *Pisum sativum* cv. Ctirad as an internal DNA standard (9.09 pg), previously determined by Doležal *et al.* (1992) (Table 2). The established DNA content was calculated to be equivalent to 5.31×10^9 base pairs. The flow cytometry analysis, for both DAPI and PI staining techniques, generated histograms (Fig. 3) with coefficients of variation (CV) bellow 3%. The relatively low CV value indicates a high resolution analysis due to a very homogenous DNA staining and excellent instrumental performance in detecting the fluorescent signal. Samples comprising at least 5,000 nuclei each were analyzed. The applied procedure can be therefore considered well suited for estimating nuclear DNA content in samples of *H. salsaparrilha*.

In our study, fresh plant material was processed without any fixation. In addition the material was stained with DAPI or PI. It has been used extensively to label DNA for excitation with UV, and is often the stain of choice in instruments employing a mercury arc lamp (Ormerod 1999).

In plants, the ploidy level, the number and size of chromosomes and the amount of nuclear DNA are usually different among the taxa. Currently, flow cytometry has been successfully used for plant DNA content determination (Schwenke *et al.* 1998, Bennett *et al.* 2000). The technique is accurate, rapid, and shows little variation among results from experiments repeated in different laboratories (Doležal *et al.* 1998), besides being compatible with other similar methodologies such as photometric cytometry and image cytometry (Vilhar *et al.* 2001). This method is based on the isolation of cells or nuclei in suspension and the use of specific DNA fluorochromes. The fluorescence emission per nucleus is measured with a flow cytometer. The method was originally applied in animal cells and it has been extended to studies with plants (Doležal 1991).

The nuclear DNA content of *H. salsaparilha* ($2C=5.5$ pg) is larger than the content found in most monocotyledonous plants analyzed by Bennett *et al.* (2000). However, there is tremendous variation in the DNA content of plants, particularly in monocotyledons. For example, the amount of DNA found in *Brahea dulcis* (Palmae) was $2C=112.6$ pg, whereas in *Carex nubigera* ssp. *albata* (Cyperaceae) was $2C=0.3$ pg of nuclear DNA (Bennett *et al.* 2000).

In *H. salsaparilha*, the amount of DNA (pg) found with both DAPI and PI was very similar, with all CVs below 3%. According to Doležel (1997), CVs between 1% and 3% indicate a high-resolution estimation of nuclear DNA content in plants. The efficiency of a particular dye and staining protocol is evaluated by the CV of the GO/G1 peak of the histogram generated by the cytometer. The CV is an indicator of the sharpness and resolution of the peak. If the CV becomes higher and its peak becomes wider the difficulty in discriminating two closely placed, becomes more difficult, resulting in lower accuracy of estimating S-phase region of the DNA histogram (Yanpaisan *et al.* 1999).

The same values found with DAPI and PI ($2C=5.5$ pg) are due to non-significant difference in the AT/CG basis ratio in relation to the fluorochrome base specificity. In view of this similarity, both DAPI and PI can be used for flow cytometry analysis of *H. salsaparilha*. Concurrently, Contim *et al.* (2005) reported similar responses for Rosewood (*Aniba rosaeodora*). The $2C$ value of rosewood showed a nuclear DNA content of 2.36 pg using DAPI or 2.32 pg using PI as fluorochromes. However, in accordance to Shapiro (2003), the authors reported that the DNA value based on PI staining should be adopted, since has no bias for AT or CG-rich sequences within genomes.

The fluorochromes used in flow cytometry can be classified in two types: intercalating, such as PI, and those with preferential binding to nitrogen bases such as DAPI (ATTN bases) (Doležel 1991). The same author comparing the response of DAPI, PI and mitramycin (fluorochrome with preferential binding to CG nitrogen bases) in *Raphanus sativus* L., *Lycopersicon esculentum* Mill., *Zea mays* L., *Pisum sativum* L., *Vicia faba* L. and *Allium cepa* L. found that results obtained with PI were the same as those obtained by other authors using other techniques. However, the values obtained with DAPI and mitramycin were significantly different. The author suggests that this is caused by differences in AT/CG ratios, and the differences in species-specific recognition of DNA by these fluorochromes. The use of intercalating fluorochromes, coupled with those with preferential binding to nitrogen bases, can provide additional information on DNA organization.

The data taken together provide new insights on the genus presenting the first detailed karyotype for this medicinal species. This karyotype asymmetric type may suggest an evolutionary process with involvement of polyploidization, as justified by the relative increased chromosome number ($2n=58$) and fusions.

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