An increment in the Duranta repens L. (Verbenaceae) knowledge: DNA content, karyology, meiosis and palynology

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Abstract — Meiotic behavior, pollen morphology, interphasic nucleus pattern and karyotype description for *Duranta repens* (Verbenaceae) are presented. The species had semi-reticulate interphasic nucleus and in mitotic metaphase, chromosome number was $2n = 34$ (32 m + 2 sm), with secondary constriction in one chromosome pair. Additionally to the A complement, we observed B chromosomes, varying to 0-3 in some cells of several individuals. Meiotic behavior was regular and pollen grains were isopolar and tricolporate, being classified as prolate-spheroidal with psilate-perfurate exine ornamentation.

Key words: cytogenetics, DNA content, *Duranta repens*, palynology, Verbenaceae.

INTRODUCTION

The genus *Duranta* is a distinctive genus in the Verbenaceae with around 17 species distributed primarily in seasonal forests and ecotones of tropical Americas, and naturalized in similar areas of the Paleotropics, with the center of diversity in the middle and lower elevations of the northern Andes of South America (Munir 1995; Sanders 2001).

Among the species, *Duranta repens* L. is the most widespread, apparently occurring naturally from tropical Florida in the United States, around the Caribbean basin and at places along the American Pacific Coast and in east-central South America, where is widely cultivated (Sanders 2001). This species is distinguished from other similar species by the more numerous axillary racemose inflorescence, by the membranaceous and sparsely puberulent leaves, moderately long calyx teeth apicules and by the relatively short corolla tube with 7-9 mm. Nevertheless, the wide variation in habit, thorns and the shape and margins of the leaves in this species has led to the publication of about 20 intra and intergeneric synonyms as: *Duranta erecta* L., *Duranta plumieri* Jacq. and *Duranta xalapensis* Kunth., for example (Martinez and Múlgura de Romero 1997; Sanders 2001).

In accordance to Sanders (1984; 2001) unresolved taxonomic complexities in *Duranta* involve species limits and the continuity of variation among populations. Species limits need more detailed studies and the same author indicated that in areas of sympatry, some herbarium specimens are morphologically intermediate, being possible the hybrid formation among different species.

The determination of chromosome number in plants is a critical step to detect processes that make feasible abrupt speciation such as polyploidy, aneuploidy and dysploidy (Briggs and Walters 1997; Guerra 2008). More yet, the knowledge of chromosome numbers and morphology plus the meiotic behavior may help to differentiate between allopolyploidy or autopolyploidy considering the differences or similarities between the involved genomes (Stebbins 1971; Guerra 2008).

Some other chromosome markers are too very important in the plant species studies, the

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C-banding, Ag-NOR and fluorescent staining methods performed with counter-staining reagents added to Fluorochrome *in situ* Hybridization (FISH) and Genome *in situ* Hybridization (GISH) are useful to mark species-distinctive regions of chromosomes. They can also be applied to cytotaxonomy and chromosome evolution, including the comparison among individuals of the same species distributed into and among different populations for example (KOKUBUGATA and KATSUHIKO 1996; SOUSA *et al.* 2009; SOUSA *et al*. 2010).

Additionaly to cytogenetics, estimation of nuclear DNA content by flow cytometry and pollen characters may provide useful information by taxonomists in the delimitation of species (LOUREIRO *et al.* 2007; MACLUF *et al.* 2010; MARQUEZ *et al.* 2010, SOUSA *et al*. 2010). Significant developments in systematics atributable to flow cytometry and palynology include: improved detection and delineation of species; improved data related to the contribution of chromosomal speciation to species diversity, and increased availability of characters for the inference of phylogenetic relationships and polarity of character evolution (KRON *et al.* 2007). For the genus *Duranta* there isn't information about DNA content and palynological studies are scarce, being these two tools important approaches for characterization of the species, principally for those with taxonomical problems.

In this work we described for the first time the chromosome morphology, and DNA content of *Duranta repens*. Adittionally we also reviewed some palynological characters and compared the same with previous descriptions.

MATERIALS AND METHODS

Plant materials - Flowers buds, leaves and seeds of *D. repens* were collected of around 20 individuals in a great population from Juiz de Fora, Minas Gerais (MG) state, Brazil. The numbers of identification are deposited at the Herbarium CESJ of Universidade Federal de Juiz de Fora (UFJF).

Mitotic chromosome preparation - Meristematic cells from root tips of seeds were pre-treated with 8-hydroxyquinoline (2mM) during 6-8h at 4°C and fixed in 3:1 methanol: acetic acid solution at least 24h before the slide preparation. Pre-treated root tips were macerated in an enzymatic solution [4% cellulase (Sigma) plus 40% pectinase (Sigma) diluted in 0.001M citric acid-sodium citrate pH 4,8 buffer] at 37°C (4h). The slides were prepared according to CARVALHO and SARAIVA (1993, 1997). A total of 50 individuals were used for analysis.

Karyotype analysis - At least 10 metaphases were used to determine the length of the short (*s*) and long arm (*l*) of each chromosome. In the following, the chromosome length ($t = l + s$) and arm ratio ($r = l / s$) were calculated. The chromosomes were classified on the basis of arm ratio using the standard nomenclature (LEVAN *et al.* 1964): m, median (r = 1,01-1,69); sm, submedian (r = 1,70-3,00); st, subterminal (r = 3,01-7,00); and t, terminal (r = 7,01-8,00). Chromosomes were measured with the Image ProPlus (Media CyberneticsTM) software and the ideograms arranged in order of decreasing short arm length.

Meiosis, cell suspensions and slides preparation - In order to obtain suspensions with meiotic stage cells appropriate to chromosome counts and analyses of meiotic behavior, the immature inflorescence were divided into arbitrary sizes and the cells of each one were observed. Cell suspensions were made based on the previously defined size that showed meiotic cells in different stages of development. The cell suspensions used were prepared according to VICCINI *et al.* (2005) with some modifications. Approximately 40 anthers were excised from 12 flower buds and placed in a special microtube (0.5 ml) with a nylon screen attached (60µm). The material was washed in distilled water to remove fixative. The tube containing the anthers was immersed in enzymatic solution (Pectinex Novozymes, Bagsvaerd, Denmark) and incubated at 34°C for 20 min. After enzymatic maceration, the anthers were washed in distilled water and mechanically fragmented to remove the pollen mother cells (PMCs). The cellular suspension obtained was centrifuged at 2000rpm for 12 min. For slides preparation, drops of the suspension were added to clean slides. Following the slides were air-dried and stained with Giemsa solution for three min. Cell images were analyzed using Image Pro Plus software (Media Cyberneticsä, Silver Spring. MD, USA). Around 10 suspensions were obtained with a mixture of buds of all individuals of the population. Around 12 slides were prepared by suspension.

Flow cytometry analysis - For DNA content determination, nearly 20-30 mg of *D. repens* fresh leaf and the same weight of fresh leaf of internal reference standard (*Pisum sativum*) were chopped with a razor blade in the presence of 1 mL LB01 ice buffer for nuclei releasing

(DOLEZEL *et al.* 1989). The chopped tissue was aspirated through two layers of cheesecloth with a plastic pipette and filtered through a 50 µm nylon filter and collected in a polystyrene tube. suspension was stained with 25 µL of a solution of propidium iodide 1% (w/v) and 5 µL of RNase (20mg.L-1) was added to each sample. The samples were stored in a dark refrigerator and analyzed after 1 to 2h. For each sample, at least 10,000 nuclei were analyzed using a logarithmic scale display. The analysis was performed with a FacsCalibur cytometer (Becton Dickinson). Each flow cytometric histogram was saved using Cell Quest software and analyzed with WinMDI 2.8 software. The 2C DNA content of the sample was calculated as the sample peak mean, divided by the Pisum sativum peak mean, and multiplied with the amount of Pisum sativum DNA (9.09 pg).

Palynology - For palynology, mature anthers were placed in glacial acetic acid for at least 24h and then acetolysed according to ERDTMAN (1960). The slides were mounted in glycerin jelly and examined using a BX 51 Olympus microscopy and the images analyzed using Image Pro Plus software (Media Cyberneticsä, Silver Spring. MD, USA). At least 20 slides were prepared and measures of pollen diameters were obtained from at least 20 grains by slides while for other characters about 10 grains were used by slide. The pollen classifications were made according to PUNT *et al.* (2007).

RESULTS AND DISCUSSION

Apparently, for *Duranta* genus, only *D. erecta* and *D. repens* are known cytogenetically, nevertheless, only the chromosome number is described in the literature, and the two taxa are considered the same species for some authors. The counts 2n = 16, 24 and 32 are the numbers registered for *D. erecta* (MUNIR 1995) while 2n = 34 is the number counted for *D. repens* (COLEMAN 1982). Our chromosome counting in agreement with the number observed by COLEMAN (1982) are indicators that these two taxa shows genetical differences and that hypothesis of synonymy among them must be reviewed.

The karyotype of *D. repens* is symmetrical, consisting of 34 median to submedian chromosomes, with arm ratios varying from 1.03 to 1.85 µm (Table 1). A chromosome pair bearing a subterminal secondary constriction was often ob-

TABLE 1 — Chromosomal morphology of *Duranta repens* obtained by median of 10 mitotic metaphases.

Chromosome pair	s	l	t = l+s	r = l/s	classification
1**	2.56	2.65	5.21	1.03	m
2	1.95	2.10	4.05	1.07	m
3	1.78	2.80	4.58	1.57	m
4	1.70	1.85	3.55	1.09	m
5	1.48	1.91	3.39	1.29	m
6	0.91	1.09	2.00	1.20	m
7	0.86	1.10	1.96	1.28	m
8	0.85	1.28	2.13	1.50	m
9	0.84	0.99	1.83	1.18	m
10	0.83	1.54	2.37	1.85	sm
11	0.83	0.93	1.76	1.12	m
12	0.79	1.08	1.87	1.38	m
13	0.77	1.05	1.82	1.36	m
14	0.72	0.94	1.66	1.30	m
15	0.70	1.08	1.78	1.54	m
16	0.62	1.14	1.76	1.84	sm
17	0.59	0.94	1.53	1.59	m

** Chromosome pair with secondary constriction; s = length of the short arm; l = length of the large arm; t = chromosome length, r = arm ratio; m = median; sm = submedian. All measures are computed in µm.

served in high numbers of metaphases analyzed (Figure 1A-D). In all metaphases where the secondary constrictions were visible these structures were observed in the short arm of the bigger chromosome (Table 1, Figure 1F). Respect to chromosome size, we observed that chromosomes varied from 1.53 to 5.21 µm (Table 1, Figure 1F), and the nucleus showed a semi-reticulate classification (Figure 1E). Additionally to the normal chromosome complement (A chromosomes) we also observed the presence of B chromosomes in metaphases of some individuals varying to 0-3 B chromosomes (Figure 1 A-D, Figure 2A). These chromosomes, also referred to as supernumerary or accessory chromosomes are "additional dispensable chromosomes" present in some individuals of some species, which probably arisen from the A chromosomes but

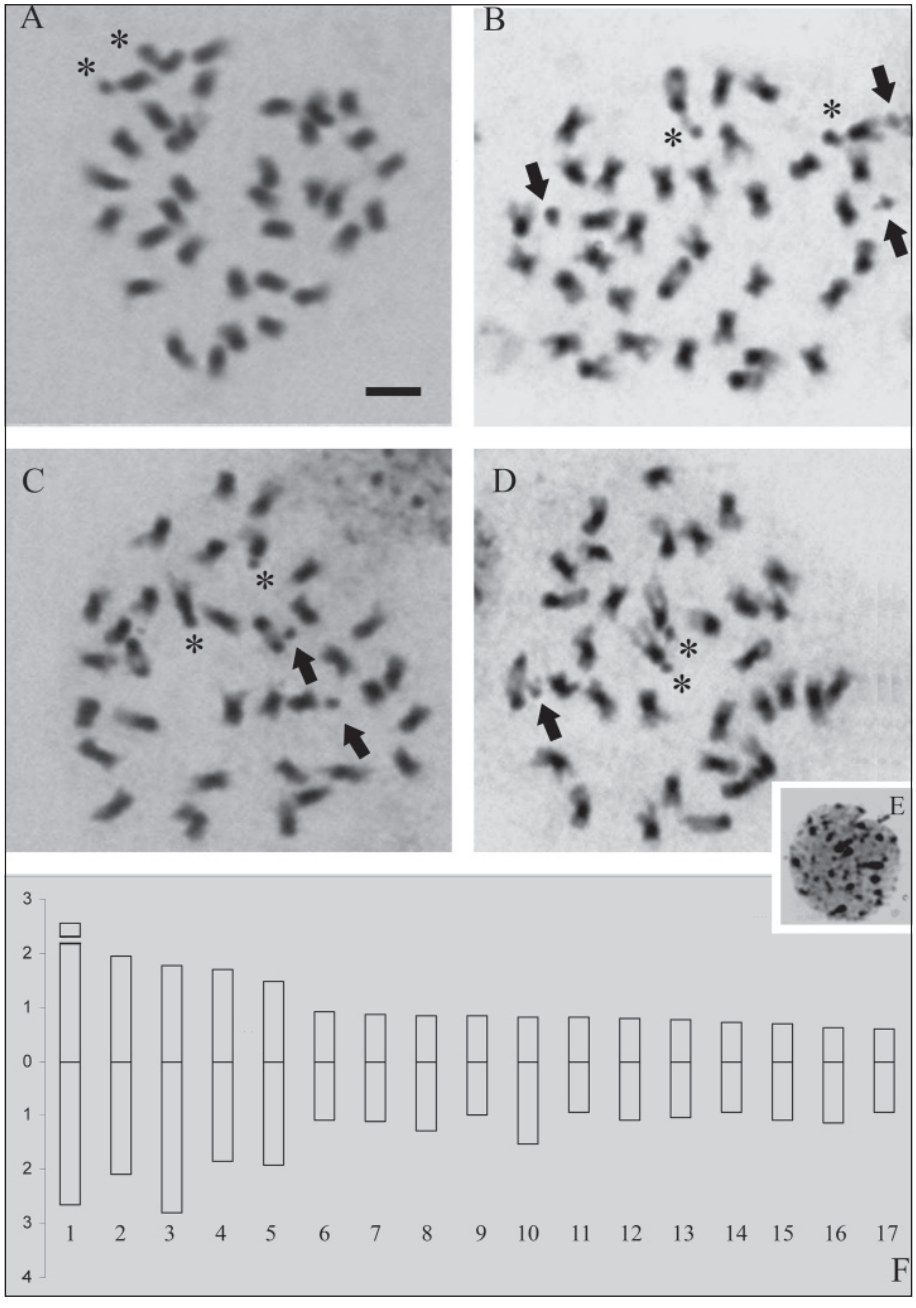

Fig. 1 — Karyological observations in *Duranta repens*: (A-D) Mitotic metaphases with 2n = 34 (arrows shows the B chromosomes; * shows the chromosomes with secondary constrictions), (E) semi-reticulate interphasic nucleus, (F) *Duranta repens* idiogram. Bar = 5 µm.

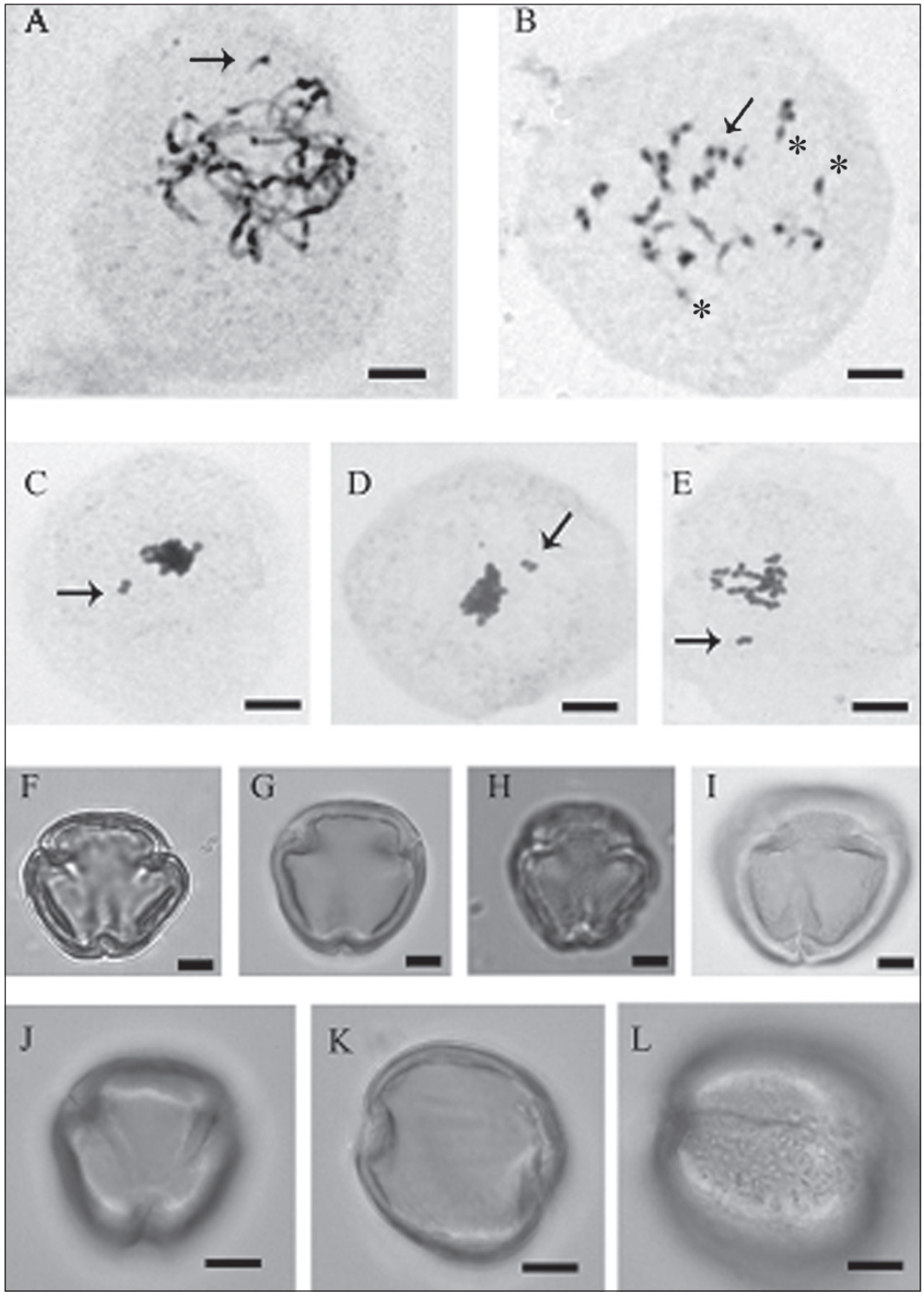

Fig. 2 — Meiotic configurations of *Duranta repens*: (A) pachytene (arrows shows a possible B chromosome), (B) diakinesis (arrows shows trivalents and * shows univalents), (C-E) metaphase I (arrows show lost chromosomes); Pollen morphology of *D. repens*: (F-J) polar view, (K-L) equatorial view. Bar = 5 µm.

follow their own evolutionary pathway. Their irregular mitotic and meiotic behavior allows them to accumulate selfishly in the germline, enabling non-Mendelian inheritance with transmission rates exceeding those of normal chromosomes (JONES and HOUBEN 2003). They have

been found in all major groups of animal and plants, and for *Duranta* this is the first relate.

In the literature, little are the works related to karyomorphology of Verbenaceae species. Maybe these failures can be due the difficulty in the obtainment of meristematic root cells as observed by VICCINI *et al.* (2005) in the genus *Lippia*. Nevertheless, some chromosomal morphological works can be observed in *Lippia alba* where root are easy to obtain (BRANDÃO *et al.* 2007; SOUSA *et al.* 2009). In *Duranta repens* these problems no exist once seeds have a great percentage of germination (98%).

In general, meiotic behavior of *D. repens* was normal, showing 17 bivalents in diakinesis, with some little cells showing meiotic alterations. The principal alterations observed were metaphase I with lost chromosomes (22%), trivalent (7.3%) and univalent (13%) formations observed in diakinesis (Figure 2A-E), every all phases showed normal development. Around 32,765 cells were analyzed.

Respect to DNA content, there are just few previous report of nuclear 2C DNA content in Verbenaceae, such as *Lantana camara* (5.50pg), *Tectona grandis* (0.96pg), *Verbena rigida* (2.37pg) and *Aloysia triphylla* (1.47pg) (Plant DNA C-values Database, http://data.kew.org/cvalues/homepage.html; HANSON *et al.* 2005; LOUREIRO *et al.* 2007). This is not surprising since it was estimated that only 1% of angiosperm species show a DNA content determined (LOUREIRO *et al.* 2007). For *D. repens* we observed a DNA content of 4.40pg, with this observation, *D. repens* is the second Verbenaceae species with bigger DNA content among the Verbenaceae descibed until the moment. The histogram obtained for DNA content of *D. repens* can be seen in Figure 3.

TABLE 2 — *Duranta repens* pollen grain measure (µm) in equatorial and polar view and index for pollen classification, m = mean size.

Equatorial view	Polar view
equatorial diameter (E)	Equatorial diameter (D)
Range of variation m	**Range of variation m**
16.03-20.23 17.66	16.01-22.52 19.31
Polar axis (P)	**Aperture number**
Range of variation m	3
17.84-20.22 18.80	**Endexine =** 1.67 (± 0.23)
P/E	**Ectexine =** 2.12 (± 0.78)
1.06	**Exine** = 3.84 (**±** 0.42)

The morphological characterization of *D. repens* pollen grain is represented in Table 2. As P/E ratio is within 1.00-1.14µm, the pollen grain can be classified as prolate-spheroidal according to PUNT *et al.* (2007), Figure 2 (F-L). The grains are isopolar and tricolporate and can be considered with a median size, with the equatorial diameter (E) of 17.66 µm and polar axis (P) of 18.80 µm. The exine showed around 3.48 µm with the ectexine (2.12 µm) highest in size when compared with the endexine (1.67 µm) (Table 2). The grains were tricolporate, with a triangular amb and intraspecific variation were not observed. In the literature RAJ (1983) observed different values for the pollen diameter of *D. repens* whose variation was 24-27 µm for the polar axis and 29-31 µm for the equatorial diameter. These difference can be occurred by the treatment and fixation process utilized for that author. We observed a psilate-perfurate exine ornamentation being similar to RAJ (1983) observations.

In view of the presented features, the chromosome number, nuclear structure, DNA content and chromosome and pollen morphology can be useful in taxonomic and evolutionary studies in the genus *Duranta*.

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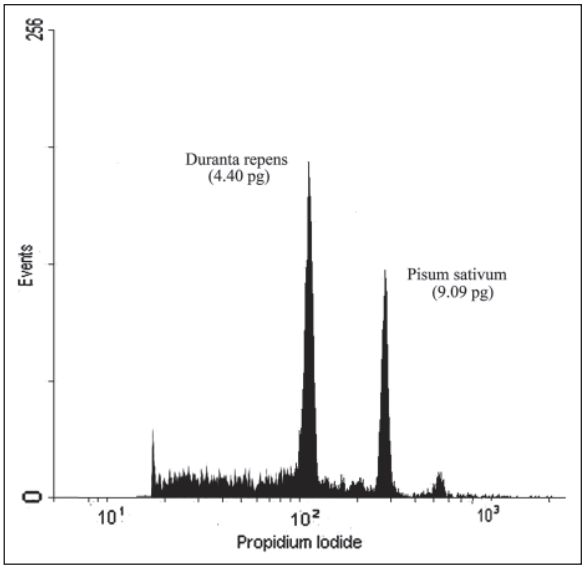

Fig. 3 — Histogram representing the DNA content of *Duranta repens* (4.40 pg). The species *Pisum sativum* (9.09 pg) was used as intern pattern.

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