

Cytology of *Vicia* species. VI. Nuclear chromatin organization, karyomorphological analysis and DNA amount in *Vicia serratifolia* Jacq.

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SUMMARY - Karyomorphological features of *Vicia serratifolia* Jacq. were studied. the nuclear DNA content was determined in meristematic root cells, karyotype and morphometric data were generated by an automated image analysis system and the distribution and staining properties of heterochromatin were assessed in metaphase chromosomes. Moreover, Feulgen absorption at different thresholds of optical density provided evidence on the organization of the chromatin in interphase nucleus. The collected data were compared with those previously gathered in *V. faba* used as internal standard and *V. narbonensis* considered the reference species of the *Narbonensis* complex. Key words: chromosome banding, heterochromatin, interphase nucleus, *Vicia* species, image analysis, phylogeny.

INTRODUCTION

The genus *Vicia* comprises about 160 species (ALKIN *et al.* 1986) which are widely distributed in the temperate zone of both hemispheres; many of them are cultivated for food and fodder. Evolution and speciation in this genus are accompanied by both variation in chromosome size and number and nuclear DNA content (CHOOI 1971; RAINA and REES 1983; RAINA and NARAYAN 1984).

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In the recent classification of MAXTED *et al.* (1991) the genus *Vicia* is subdivided in two subgenera, namely *Vicia* and *Vicilla* with 17 and 9 sections respectively. The section *Faba* belongs to the sub genus *Vicia* and is composed of three distinct units, two of which are monospecific: *Vicia faba* and *Vicia bithynica*; the third is larger and contains the seven species referred to as the *Narbonensis* complex.

In previous papers biochemical, cytophotometric and karyological data on *Vicia* species have been reported (FREDIANI *et al.* 1987, 1992; DE PACE *et al.* 1991; CREMONINI *et al.* 1993; GALASSO *et al.* 1994; MAGGINI *et al.* 1991, 1995). In order to get a better knowledge of the phylogenetic relationships within the *Narbonensis* complex, a comprehension of the variations at specific level is necessary. Within this framework it is of interest to analyze the nuclear DNA content, the chromatin organization and the karyological parameters and the chromosome banding of *Vicia serratifolia*, a taxon belonging to the *Narbonensis* complex. The data collected are compared with the data from *Vicia faba*, used as internal standard, and of *Vicia narbonensis* that is considered the reference species in the *Narbonensis* complex.

MATERIALS AND METHODS

Cytophotometric analysis. - Seeds of *Vicia serratifolia* Jacq., accession NAR 168, part of the working collections of *Vicia* species held at the Istituto del Germoplasma (Bari, Italy) were used. for cytophotometric analysis 1 cm long roots were fixed in ethanolacetic acid (3:1, v/v), squashes were made under a coverslip in a drop of 45% acetic acid after treatment with a 5% aqueous solution of Pectinase (Sigma) for 1 h at 37°C, with the addition of 0.001 M EDT A in order to inhibit the activity of DNase, if present (BERLYN *et al.* 1979). After coverslips had been removed by the dry-ice method, slides were then simultaneously hydrolyzed in 1N HCl at 60°C for 7 min, Feulgen stained in 0.5% basic fuchsin for 1 h at room temperature, washed twice in SO₂ water for 15 min to bleach out any unbound stain, dehydrated and mounted in DPX balsam (BDH, Chemicals).

The DNA content was estimated by a Leitz MPV3 integrating microdensitometer equipped with a Halogen-Belophot lamp and HP computer at a wavelength of 550nm in individual cell nuclei in post synthetic condition (G₂ phase). Squashes of root tips of *Vicia faba* cv. Aquadulce were concurrently stained for each group of slides and were used as internal standard. Absorption measured in *V. faba* preparations was also used to convert relative Feulgen arbitrary units into picograms of DNA. With the same instrument and at the same wavelength, the Feulgen DNA absorption of chromatin fractions with different condensation levels was measured in the same nucleus after selecting different thresholds of optical density according to the methods of HAVELANGE and JEANNY (1984) and CREMONINI *et al.* (1992). The instrument reads all parts of the nucleus where the optical density is greater than the preselected limit and treats the parts below this limit as a clear field. The results of this analysis were reported as the percentage of Feulgen absorption in comparison with the initial value of 100. The

values of the thresholds of optical density were mathematically elaborated, according to Simpson's rule, in order to obtain the exact position of the inflexion point in the curves. The residual Feulgen absorption at the inflexion point represents the cytophotometrically determined condensed chromatin.

Karyomorphometry. - Roots meristems were pre-treated with a 0.05% colchicine aqueous solution for 4 h at room temperature followed by overnight fixation in 3:1 (v/v) ethanol-acetic acid. Karyotype morphometry was made by an automated image analysis system KSChromo software package by Kontron-Zeiss. Slides were prepared and analyzed according to VENORA *et al.* (1991). The classification of STEBBINS (1971), the TF% value (HUZIWARA 1962) and the Rec and Syi indices (GREILLIUBER and SPETA 1976) were used to perform the analysis. The classification of STEBBINS (1971) is based on the relative frequency of chromosomes with a long arm ratio greater than 2 and on the ratio between the lengths of the longest and the shortest chromosome in the complement. The TF% index is expressed by the ratio between the sum of the lengths of the short arms of individual chromosomes and the total length of the complement. The Rec value expresses the average of the ratios between the length of each chromosome and that of the longest one. The Syi index shows the ratio between the average length of the short arms and the average length of the long arms. Cluster analysis was applied for grouping chromosome pairs (SCOTT and KNOTT 1974), the LEVAN *et al.* (1964) nomenclature was followed excluding the satellite length in the arm ratio computing.

Chromosome banding. - The seeds were surface sterilized with 2% sodium hypochlorite for 5 min, followed by thorough rinsing in running tap water, and allowed to germinate in Petri dishes at 22-25°C. In order to accumulate metaphases, actively growing roots of *V. serratifolia* were excised and treated with a saturated solution of 1,4-dichloro-benzene in distilled water for 2 h at 12°C, or in 0.05% colchicine in distilled water for 4 h; then they were fixed for 24 h in ethanol-acetic acid (3:1, v/v). Root tip meristems were squashed under coverslips in a drop of 45% acetic acid and coverslips were removed by the dry-ice method and slides were dried overnight. C-banding was performed according to GIRALDEZ *et al.* (1979). For fluorochrome banding, the technique of SCHWEIZER (1976) was used: the slides were stained in the dark with Chromomycin A3 ((~MA)) for 50 min; after rinsing and drying the slides were stained with DAPI for 20 min, rinsed and dried. For observation the slides were mounted in pH 7 McIlvaine buffer-glycerol (1:1 v/v).

RESULTS

We have tested two hydrolysis methods (cold hydrolysis: 30 min, 5N HCl at room temperature and hot hydrolysis: 7 min, 1N HCl 60°C) and observed that the different methods gave similar measurements of nuclear DNA content. From the analysis of hot hydrolysis curve the optimal time for Feulgen reaction was 7 min.

Using this hydrolysis procedure, the DNA content estimated for *Vicia serratifolia* is 39.59 pg of DNA in 4C interphase nuclei. The mean Feulgen absorption and the nuclear DNA content are summarized in Table 1 where the values of *Vicia narbonensis* are also reported.

TABLE 1 - Nuclear DNA content of early prophase in the root meristem, surface area of interphase nuclei (4C) and residual Feulgen absorption at the inflexion point from integral calculation by Simpson's rule in *Vicia* samples; each nuclear DNA content and surface area are the mean of fifty determinations carried out in five root meristems.

<i>Vicia</i> species	Surface area (μ^2 $\pm$ S.E.)	DNA content (pg, mean value)	Residual Feulgen absorption (a.u.)
<i>serratifolia</i>	2651 $\pm$ 81	39.59	50.78
<i>faba</i>	3619 $\pm$ 95	53.31*	45.29
<i>narbonensis</i> ^o	2399 $\pm$ 53	29.10	43.70

* BENNETT and SMITH 1976; ^oFREDIANI *et al.* 1992.

The results of the analysis, on 4C interphase nuclei, at different thresholds of optical density are reported in Table 2 and in Figure 1. In order to compare the values of the curves of optical density we have chosen, in each sample, interphase nuclei having Feulgen absorption values corresponding to 4C content and the same surface area. Statistical analysis (t-student, data not reported) does not show significant differences either within the 4C DNA amounts in the interphase nuclei of the same slide or between roots collected from different seeds.

TABLE 2 - Percentage of Feulgen absorption (mean $\pm$ S.E.) at different thresholds of optical density of interphase nuclei in the root meristems of five seedlings for each sample. Twenty nuclei for each sample were measured.

Thresholds of optical density	% Feulgen absorption in interphase nuclei	
	<i>V. serratifolia</i>	<i>V. faba</i>
2	100	100
4	87.38 $\pm$ 0.73	96.87 $\pm$ 1.25
6	70.51 $\pm$ 1.34	89.94 $\pm$ 1.91
8	50.78 $\pm$ 1.43*	81.08 $\pm$ 1.11
10	39.68 $\pm$ 1.63	69.33 $\pm$ 0.82
12	27.77 $\pm$ 1.58	57.89 $\pm$ 1.42
14	15.05 $\pm$ 1.19	45.29 $\pm$ 1.28*
16	9.68 $\pm$ 1.24	30.63 $\pm$ 1.81
18	5.46 $\pm$ 0.98	15.73 $\pm$ 1.47
20	2.24 $\pm$ 0.48	7.18 $\pm$ 0.95
22	—	2.04 $\pm$ 0.20
24	—	0.91 $\pm$ 0.11
26	—	—

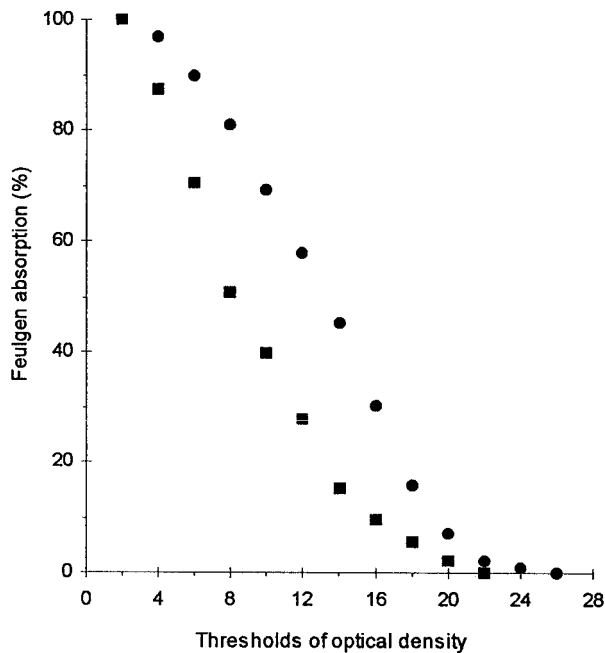


Fig. 1. - Percentage of Feulgen absorption at different thresholds of optical density of 4C interphase nuclei in *Vicia serratifolia* (●) and *Vicia faba* (■).

At optical density threshold 22 the Feulgen absorption of *V. serratifolia* is reduced to zero and the Feulgen absorption of *V. faba* is reduced to zero at threshold of optical density 26. For *V. serratifolia* the inflexion point is at optical density threshold 8 and the residual absorption is 50.68%, for *V. faba* the inflexion point is at optical density threshold 14 and the residual Feulgen absorption is 45.29%.

The C-banded heterochromatin determined by the BSG method has in *V. serratifolia* a mainly terminal position. In fact, all the telomeric regions of the short arms and the telomeric region of the long arm of the satellited chromosome harbour prominent heterochromatic blocks. In four cases the heterochromatic block was actually formed by two adjacent bands, which were more easily resolved in less contracted metaphases (Fig. 2). All prominent telomeric bands showed a bright fluorescence after DAPI staining (Fig. 3a). Few intercalary bands were observed mostly appearing as faintly stained dots. Only one clear band could be located on the long arm of one of the shorter chromosomes. The secondary constriction showed a positive reaction to the C-banding technique and the whole region was darkly stained; in those metaphases where the secondary constriction was more stretched the heterochromatin appeared located at the two sides of the constriction, becoming visible as two separated bands. This region showed also positive reaction to the staining with CMA₃ (Fig. 3b).

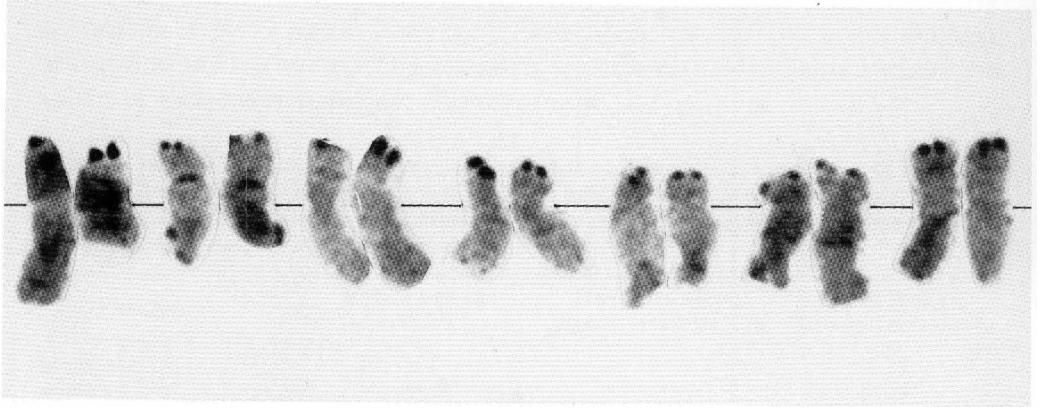


Fig. 2 - C-banded karyotype of metaphase chromosomes of *Vicia serratifolia* Giemsa, 400x. Five metaphases for each of five seedlings were analysed

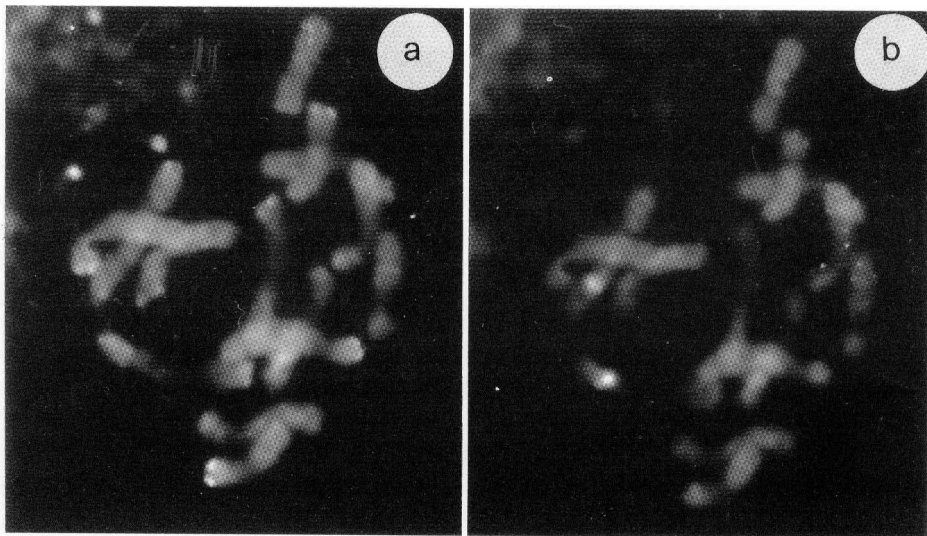


fig 3- Metaphase chromosomes of *Vicia serratifolia*. a) CMA, b) DAPI. 320x

The somatic chromosome number $2n=14$ was observed, the general characteristics of the chromosomes are summarized in Table 3. The individual chromosome length ranged from 4.77 and 5.98 μm . One satellite, 1.91 μm long, was located on the short arm of the second chromosome. Based on length, the seven chromosome pairs could be divided into three groups (same letter according to Cluster analysis), if the arm-ratio was used as the discriminating character, five groups were obtained. Six pairs are submetacentric and one is metacentric, according to the classification of LEVAN *et al.* (1964, Tab. 3 and Fig. 4).

TABLE 3- Chromosomemorphometric data.

Chrom. No.	Relative length %	Chrom. length (μm)	Long arm (μm)	Short arm (μm)	Satellite (μm)	Arm Ratio long/short	Chromosome type
1	15.67	5.98 aA	3.95±0.31	2.03±0.22		1.95 dC	submetacentric
2	15.12	5.77 aA	2.44±0.14	1.42±0.12	1.91±0.15	1.72 eD	submetacentric
3	14.91	5.69 aA	3.93±0.44	1.76±0.11		2.23 bB	submetacentric
4	14.49	5.53 aA	3.52±0.24	2.01±0.16		1.75 eD	submetacentric
5	13.92	5.31 bB	3.75±0.20	1.56±0.12		2.40 aA	submetacentric
6	13.39	5.11 bB	3.44±0.27	1.67±0.09		2.06 cC	submetacentric
7	12.50	4.77 cC	2.75±0.24	2.02±0.15		1.36 fE	metacentric

Values followed by the same letter are not significantly different, according to the Cluster analysis of SCOTT and KNOTT 1974 (capital letters P=0.01, small letters P=0.05). Thirty metaphase plates in five seedlings were analysed

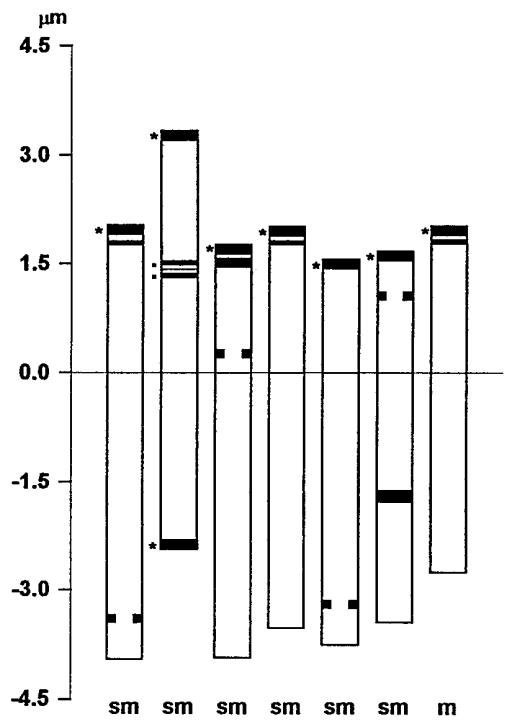


Fig. 4- C-banded idiogram of metaphase chromosomes of *Vicia serratifolia*. ■ major bands, □ inconstant bands, * DAPI bands, • CMA bands.

The total length of the haploid complement is 38.16 m, the indices and the STEBBINS' categories are reported in Tab. 4.

TABLE 4- Set length, indices and Stebbins' categories in *Vicia* samples.

<i>Vicia</i> species	Haploid set length	Rec index	Syi index	TF% index	Stebbins' categories
<i>serratifolia</i>	38.2±2.1	89.7	52.4	32.7	2A
<i>faba</i>	58.1±5.0	44.3	16.4	13.4	3B
<i>narbonensis</i> ^o	27.1±2.3	84.6	56.1	34.6	2A

^o CREMONINI *et al.* 1998.

DISCUSSION

Our determination of nuclear DNA content ($4C=39.59$ pg) differs from the value reported by RAINA and BISHT (1988, $4C=30.06$ pg) but this divergence may be due to the different method they used: i.e. hydrolysis for one hour and *in toto* staining of meristems prior to squashing. In our opinion the procedure used by RAINA and BISHT (1988) may yield less reliable data since effect hydrolysis and staining are not uniform over the meristematic cells of the whole root tip. Additionally the hydrolysis time may be excessive and therefore their results might not reflect the real DNA content but a smaller amount due to a partial removal of DNA. Moreover, we have analyzed nuclei in the same early prophase conditions from tissues at the same stage of development and all squashes were stained together with *Vicia faba* squashes used as an internal standard.

The determination of the nuclear chromatin fractions with different condensation levels by cytophotometric analysis at different thresholds of optical density is used as an alternative method for the determination of the heterochromatin fraction. Only nuclei from meristematic cells were chosen since the percentage of heterochromatin is higher and more constant in meristematic cells than in differentiated cells (BASSI 1990 and references therein). Since the instrument, at lower thresholds of optical density, reads all the chromatin and at higher thresholds only the more condensed chromatin fraction, we have formulated the hypothesis that the residual Feulgen absorption at the inflexion point may represent the cytophotometrically determined heterochromatin component (CREMONINI *et al.* 1993, 1994) and our method may be considered a more objective determination in comparison with HA VELANGE and JEANNY method (1984).

It is interesting to notice that the cytophotometrically determined interphase heterochromatin in *V. serratifolia* represents a larger proportion of the

genome (50.78%) than in *V. faba* and *V. narbonensis* which showed values of 45.29% and 43.70% respectively.

As far as karyotype measurements are concerned, the automated image analysis system allowed a high degree of accuracy since measurements were taken directly in the first image of the chromosomes as projected by the objective onto the CCD focal plane. In traditional chromosome measuring methods such as the photographic one, serial treatments are applied to the image, tending to accumulate error in the final determination. The availability of such precise measures allowed the exact determination of karyomorphological indices that are considered to be directly correlated with the evolution of the karyotype as studies carried out in *Cicer* (OCAMPO *et al.* 1992), in *Vigna* (VENORA and PIGNONE 1995; VENORA and PADULOSI 1997) can demonstrate. The karyotype herein proposed for *V. serratifolia* is quite different from that described by SCHAFER (1973) for the same species, in fact she reported the satellited pair to be the 4th longest pair, while in our karyotype it is the 21st longest pair. As additional points of difference, the total haploid length is longer and the seventh pair is metacentric in our karyotype, while SCHAFER (1973) reported the seventh pair to be submetacentric. These differences might be partially due to the use of different pretreatments which influence chromosome contraction and length, but differences in the karyotype of the samples analyzed might not be excluded. In fact, karyomorphological indices suggest such an evident similarity between the two species (*V. serratifolia* and *V. narbonensis*), since the values of Rec and Syi are quite alike and both species fall in close categories according to STEBBINS (1971). Such a similarity suggests that the two materials could be two different accessions of the same species. The karyotype of *V. serratifolia* is rather similar to that of *V. narbonensis*, although a few clear differences make the two karyotypes easily distinguishable. Both species possess large heterochromatin blocks at the short arm telomeres even if *V. serratifolia* has a lower number of bands than *V. narbonensis*; the similarity with *V. narbonensis* is reinforced by the amount and distribution of DAPI bands. In both species only the prominent telomeric bands show a positive reaction to fluorochrome staining, and the most bands are located on the short arms. A similar observation was made by PERRINO and PIGNONE (1981), using the distribution of Hoechst 33258 bands along the karyotype also brought up the marked resemblance of the karyotypes of these two species. The amount of C-banded heterochromatin in *V. serratifolia* appears to be slightly lower than in *V. narbonensis*; this observation might appear in contrast with the difference denoted by cytophotometric determination (50.78% and 43.70% respectively). Nevertheless, it is necessary to consider that the binding of Giemsa stain to chromatin is not stoichiometric. Moreover, C-banding and cytophotometry analyse cells at different functional stages: C-banded heterochromatin represents a level of packaging DNA into chromosomes, while cytophotometric heterochromatin delineates condensed DNA in interphase.

nuclei..YAMAMOTO (1986) by means of hybridization experiments and isozyme analysis, concluded that these two species are similar. Our analysis, integrating observations made at different levels of the genome organization, reinforce this perception.

In comparison to the reference species *V. narbonensis* the C-bands appear to be similar in number, size and distribution. Occasionally intercalary or centromeric bands could be observed in some metaphases, but due to their erratic nature they were not considered in the present analysis

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