Cytology of *Vicia* Species. 5. Nuclear Chromatin Structure, Karyomorphological Analysis and DNA Content in Newly Discovered Relatives of *Vicia faba* L.: *Vicia kalakhensis* Khattab, Maxted et Bisby and *Vicia eristalioides* Maxted

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Summary Three species of the *Vicia narbonensis* complex, namely *V. narbonensis* L., *V. kalakhensis* Khattab, Maxted et Bisby and *V. eristalioides* Maxted were analysed for different karyobiological features; the nuclear DNA content and DNA absorption curves were determined, karyomorphometric data were generated using an automated image analysis system and the distribution and staining properties of heterochromatin in metaphase chromosomes, were assessed.

Feulgen absorptions at different thresholds of optical density provided evidence of organisation of the chromatin interphase nuclei and allowed an evaluation of the heterochromatin amount as determined with a cytophotometrical approach. The precise chromosomal measurements were utilised for computing karyomorphological indices.

Key words Chromosome banding, Fluorochromes, Heterochromatin, Interphase nucleus structure, *Vicia* species, Image analysis.

The genus *Vicia* comprises approximately 160 species (Alkin *et al.* 1986) widely distributed throughout the temperate zone of both hemispheres and many of them, mainly of Eurasian origin, are cultivated for food and fodder. The majority shows a basic number of $x=5$, 6 or 7 and only eight species are polyploids ($2n=4x=24$ or 28, Raina and Ogihara 1985).

The speciation in this genus is accompanied by both chromosome size variation and large scale variation in nuclear DNA content, as well as variation in the frequency of repetitive and non repetitive DNA sequences (Cremonini 1990 and references therein). Lately Maxted (1991a) revised the classifications of Chooi (1971), Kupicha (1976) and Hanelt and Mettin (1989). In this classification the genus *Vicia* is subdivided in two sub-genera, namely *Vicia* and *Vicilla*, with 17 and 9 sections respectively, ten rare taxa from both subgenera and new species (Maxted 1988, Khattab *et al.* 1988) were included. In the classification of Maxted *et al.* (1991a) the section *Faba* is composed of three distinct units: two are monospecific: *V. faba* in the former and *V. bithynica* in the latter, while the third is larger and contains the seven species referred to as the *Narbonensis* complex.

In previous papers biochemical and cytophotometric data on *Vicia* species belonging to the *Faba* section have been reported (Cremonini *et al.* 1992, 1993, Frediani *et al.* 1992, Maggini *et al.* 1995). The karyological relationships among the species of this section had been analysed at both

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cytological and banding levels (Schafer 1973, Perrino and Pignone 1981). The conclusion of the reported studies was that for karyobiological parameters the species of the section *Faba* do not form an homogeneous aggregate: some species show a great resemblance to *V. narbonensis*, and therefore they were generally treated together as the *V. narbonensis* group.

Recently two new species (*Vicia kalakhensis* Khattab, Maxted et Bisby and *Vicia eristalioides* Maxted) were discovered, identified and included for their morphological characters in the *V. narbonensis* group by Maxted *et al.* (1991a). Therefore the nuclear DNA content, the chromatin organisation, the chromosomal indices and the distribution and staining properties of chromosomal heterochromatin were analysed.

Materials and methods

Seeds of *Vicia khalakhensis* and *Vicia eristalioides*, kindly provided by Dr. N. Maxted and *V. narbonensis* belonging to the collection held at the Istituto del Germoplasma, Bari, were used.

For cytophotometric analysis seeds of the three species were soaked in running tap water overnight and germinated in Petri dishes at 22°C in darkness for two days and transferred in sterilised water vessels until secondary roots were about 1 cm long. Root tips were fixed in ethanol-acetic 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 hr at 37°C with the addition of 0.001 M EDTA in order to annul the activity of DNase (Berlyn *et al.* 1979). The coverslips were removed by the dry-ice method, and the squashes were hydrolysed in 5 N HCl at room temperature for 30 min and stained by Feulgen reagent. After staining, the slides were subjected to three washes, each of 10 min intervals, in SO₂ water prior to dehydration and mounting in DPX (Fluka). Squashes of the root tips of *Vicia narbonensis* were concurrently stained for each group of slides and used as an internal standard.

Absorptions measured in *V. narbonensis* preparations were also used to convert relative Feulgen arbitrary units into picograms of DNA. Feulgen DNA absorptions in individual cell nuclei, in the postsynthetic condition (G₂), were measured at the wavelength of 550 nm, using a Leitz MPV3 integrating microdensitometer equipped with an HP computer. With the same instrument and at the same wavelength, the Feulgen DNA absorptions of chromatin fractions with different condensation levels were determined by measurements of one and the same nucleus, after selecting different thresholds of optical density according to the method of Di Terlizzi *et al.* (1993). The instrument reads all parts of the nucleus where the optical density is greater than the preselected limit, regarding those below this limit as a clear field. The optical density threshold values were mathematically elaborated in order to obtain the exact position of the inflexion point in the curves and it was possible to discriminate between two areas of integral calculation using Simpson's rule. The residual Feulgen absorption at the inflexion point represents the cytophotometrically determined heterochromatin (Cremonini *et al.* 1993).

The integral calculation was carried out on the best-fit curves obtained from three curves of optical density for each sample. In order to assess whether some chromatin fractions contributed to cytophotometric differences among the species, measurements were taken on interphase nuclei at different thresholds of optical density. Interphase nuclei, with Feulgen absorption values of 4C content and the same surface, were chosen for each species.

Karyotype morphometry was made by an automated image analysis system IBAS 2000 by Kontron-Zeiss. Slides were prepared and analysed according to Venora *et al.* (1991). The classification of Stebbins (1971), the TF% index (Huziwaru 1962) and the Rec and SYi indices (Greilhuber 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

Table 1. Chromosome number, Feulgen absorption (a.u., mean $\pm$ S.E.), surface of interphase nuclei (μm^2 , mean $\pm$ S.E.), nuclear DNA content (pg, mean $\pm$ S.E.) in *Vicia* samples. Nuclear DNA content of *V. narbonensis* is used as internal standard. Each Feulgen absorption value is the mean of 100 determinations carried out in root meristems: twenty prophase for each of five seedlings

Species	Chromosome number	Feulgen absorption	Interphase nuclei surface	DNA amount
<i>V. kalakhensis</i>	14	2580.9 $\pm$ 25.9	2387.5 $\pm$ 98.3	42.22 $\pm$ 0.4
<i>V. eristalioides</i>	14	2351.8 $\pm$ 32.3	2949.8 $\pm$ 51.6	38.58 $\pm$ 0.5
<i>V. narbonensis</i>	14	1770.8 $\pm$ 21.1	2399.3 $\pm$ 53.8	29.10*

*Frediani *et al.* 1992. Biol. Plant **34**: 335–344.

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 index expresses the average of the ratios between the length of each chromosome and that of the longest one. The SYi value indicates 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 computing the arm ratio.

For chromosome banding, actively growing roots of the three species were excised and treated with ice-cold distilled water for 18–24 hr in order to accumulate metaphases. The roots were then fixed for 24 hr in ethanol–acetic acid (3 : 1, v/v). Root tip meristems were squashed under coverslips in a drop of 45% acetic acid, coverslips were removed by the dry-ice method and slides were dried overnight. Staining with bis-benzimide derivative Hoechst H33258 followed Perrino and Pignone (1981), C-banding (BSG) was performed according to Giraldez *et al.* (1979). Banded karyotypes were constructed taking into account chromosomal indices and band position. Karyotypes of the different species were assembled in order to maximize the similarities among the species.

Results

Cytophotometry

The mean Feulgen absorption and the mean surface of early prophase, the chromosome number and the nuclear DNA content in each of the analysed species are listed in Table 1. From the analysis of hydrolysis curve, the optimal time for Feulgen reaction, was 30 min with 5 N HCl at room temperature. The DNA content differs from one species to another as evidenced by statistical analysis: from 42.22 in *V. kalakhensis* to 29.10 in *V. narbonensis* (pg/4C nucleus). A linear relationship between total length of haploid complement and nuclear DNA content was found ($r=0.94$) and the results of the analysis at different thresholds of optical density are summarized in Fig. 1 and in Table 2. At optical density thresholds 21, the Feulgen absorption of *V. eristalioides* and *V. narbonen-*

Fig. 1. Percentage of Feulgen absorption at different thresholds of optical density of 4C, interphase nuclei in *Vicia narbonensis* (○), *Vicia kalakhensis* (●) and *Vicia eristalioides* (□), the arrows indicate the position of inflexion points.

Table 2. Percentages of Feulgen absorption (mean $\pm$ S.E.) at different thresholds of optical density of 4C interphase nuclei in the root meristems of five seedlings for each sample (20 nuclei for each seedling were measured)

Thresholds of optical density	% Feulgen absorption ($\pm$ S.E.)		
	<i>V. kalakhensis</i>	<i>V. eristalioides</i>	<i>V. narbonensis</i>
3	100	100	100
6	78.55 $\pm$ 0.73	73.97 $\pm$ 0.88	72.01 $\pm$ 1.11
9	58.79 $\pm$ 0.93	46.27 $\pm$ 1.29*	43.70 $\pm$ 1.40*
12	37.97 $\pm$ 1.02*	22.18 $\pm$ 1.26	21.80 $\pm$ 1.16
15	23.55 $\pm$ 1.09	7.41 $\pm$ 0.83	8.81 $\pm$ 0.86
18	14.06 $\pm$ 1.53	2.96 $\pm$ 0.55	3.88 $\pm$ 0.56
21	5.80 $\pm$ 0.58	—	—
24	3.03 $\pm$ 0.28	—	—
27	—	—	—

* Indicates the inflexion point position.

Table 3. Prophase nuclei surface (μm^2 , mean $\pm$ S.E.), estimation threshold values, areas (S_1 and S_2 , arbitrary units) from integral calculation by Simpson's rule and ratio S_2/S_1 . The integral calculation was carried out on the best fit curve obtained from three curves of optical density for each for each species

Species	Mean surfaces of prophase nuclei	Threshold values=0	S_1	S_2	S_2/S_1
<i>V. kalakhensis</i>	2508.3 $\pm$ 57.4	27	645.23	94.8	0.144
<i>V. eristalioides</i>	3082.3 $\pm$ 43.5	21	544.93	63.1	0.115
<i>V. narbonensis</i>	2561.8 $\pm$ 46.7	21	545.42	66.1	0.121

sis is reduced to zero, the Feulgen absorption of *V. kalakhensis* is 5.8% and is reduced to zero at the higher threshold of optical density, i.e. 27.

By mathematical elaboration based on Simpson's rule it is possible the determination of the inflexion point of the three curves optimised from the experimental data. This point enables the identification of two areas in each curve (S_1 and S_2) and the value of the second area is found to be similar when comparing the two species *V. eristalioides* and *V. narbonensis* and higher in *V. kalakhensis* (Table 3). A similar pattern is evidenced by the ratio (S_2/S_1) between the areas. Moreover, when the ratio of S_1 over ($S_1 + S_2$) is considered, a similar observation is done. The ratio is in fact the entity of the denser chromatin in relation to the total chromatin as read by the instrument and therefore it describes the cytophotometrically determined heterochromatin in interphase nuclei.

Karyotypes

All karyological data for the three species are reported in Table 4 and karyograms and chromosome banding patterns are reported in Fig. 2 as well as the chromosomal distribution of heterochromatin is reported.

Vicia eristalioides: the individual chromosome lengths ranged from 3.98 to 5.59 μm , with a total length of the haploid complement equal to 33.38 μm . One satellite was located on the short arm of the second chromosome. Based on arm-ratios, the seven chromosome pairs could be divided into three groups (same letter according to Cluster analysis, Table 4). Three chromosome pairs possessed metacentric centromeres and four had submetacentric centromeres.

Vicia kalakhensis: chromosome length varied from 5.0 to 6.93 μm ; the total chromosome length of the haploid set was 42.28 μm . The second pair of chromosomes carried a 2.34 μm long satellite on the short arm. Three chromosomes had the centromere located in metacentric position, three in

Table 4. Chromosome morphometric values (mean $\pm$ S.E.) of *Vicia* samples

Chromosome No.	Relative length (%)	Length (μ m)	Long arm (μ m)	Short arm (μ m)	Satellite (μ m)	Arm ratio long/short	Chromosome type
<i>V. eristalioides</i>							
1	16.72	5.58 aA	3.27 $\pm$ 0.03	2.31 $\pm$ 0.13		1.41 dC	metacentric
2	15.99	5.34 bB	2.30 $\pm$ 0.06	1.06 $\pm$ 0.07	1.98 $\pm$ 0.12	2.16 aA	submetacentric
3	15.59	5.20 cC	3.16 $\pm$ 0.11	2.04 $\pm$ 0.03		1.55 cC	metacentric
4	14.38	4.80 dD	2.91 $\pm$ 0.22	1.89 $\pm$ 0.19		1.54 cC	metacentric
5	13.04	4.35 eE	2.98 $\pm$ 0.16	1.37 $\pm$ 0.18		2.18 aA	submetacentric
6	12.36	4.13 fF	2.71 $\pm$ 0.07	1.42 $\pm$ 0.05		1.92 bB	submetacentric
7	11.92	3.98 gG	2.70 $\pm$ 0.01	1.28 $\pm$ 0.11		2.12 aA	submetacentric
<i>V. kalakhensis</i>							
1	16.39	6.93 aA	4.07 $\pm$ 0.04	2.86 $\pm$ 0.10		1.43 eE	metacentric
2	15.52	6.56 bB	3.22 $\pm$ 0.11	1.00 $\pm$ 0.15	2.34 $\pm$ 0.26	3.21 aA	subtelocentric
3	15.21	6.43 cB	3.85 $\pm$ 0.15	2.58 $\pm$ 0.06		1.49 eE	metacentric
4	14.78	6.25 dC	4.17 $\pm$ 0.13	2.08 $\pm$ 0.09		2.00 bB	submetacentric
5	13.94	5.89 eD	3.79 $\pm$ 0.03	2.10 $\pm$ 0.01		1.80 cC	submetacentric
6	12.35	5.22 fE	3.23 $\pm$ 0.03	1.99 $\pm$ 0.14		1.62 dD	metacentric
7	11.82	5.00 gF	3.36 $\pm$ 0.18	1.64 $\pm$ 0.05		2.05 bB	submetacentric
<i>V. narbonensis</i>							
1	16.46	4.47 aA	2.87 $\pm$ 0.10	1.60 $\pm$ 0.13		1.79 dC	submetacentric
2	15.46	4.20 bB	2.60 $\pm$ 0.17	1.60 $\pm$ 0.05		1.62 eD	metacentric
3	14.81	4.02 cC	2.65 $\pm$ 0.05	1.37 $\pm$ 0.04		1.93 cB	submetacentric
4	14.21	3.85 dD	2.31 $\pm$ 0.20	1.54 $\pm$ 0.09		1.50 fE	metacentric
5	13.85	3.76 eD	2.42 $\pm$ 0.04	1.34 $\pm$ 0.06		1.81 dC	submetacentric
6	13.11	3.56 fE	2.37 $\pm$ 0.30	1.19 $\pm$ 0.03		1.99 bB	submetacentric
7	12.11	3.29 gF	1.53 $\pm$ 0.06	0.74 $\pm$ 0.11	1.02 $\pm$ 0.01	2.07 aA	submetacentric

Values followed by the same letter are not significantly different, according to the Cluster analysis of Scott and Knott (capital letters small letters P=0.05).

Fig. 2. Idiogrammatic representation of the haploid karyotype and bands localization on each species. BSG=C banding.

submetacentric and one in subtelocentric position. According to arm-ratio the seven pairs were grouped in five clusters (Table 4).

Vicia narbonensis: the length of individual chromosomes ranged from 3.29 to 4.47 μ m and the length of the haploid complement was 27.14 μ m. A 1.02 μ m long satellite was located on the short arm of the shortest chromosome pair. Five chromosome pairs possessed submetacentric centromeres and two metacentric centromeres and the seven chromosome pairs were grouped in five clusters according to the arm-ratio (Table 4).

As long as karyomorphological values are concerned, *Vicia kalakhensis* showed the lowest val-

Table 5. Karyotype formulas, set lengths (μm), indices and Stebbins categories in *Vicia* samples

	<i>V. eristalioides</i>	<i>V. kalakhensis</i>	<i>V. narbonensis</i>
Karyotype formula	$\text{sm}^{\text{sc}} + 3\text{m} + 3\text{sm}$	$\text{st}^{\text{sc}} + 3\text{m} + 3\text{sm}$	$\text{sm}^{\text{sc}} + 2\text{m} + 4\text{sm}$
Total length of haploid set	33.38 ± 0.37	42.28 ± 0.10	27.14 ± 2.28
Rec Index	83.02	85.02	84.60
Sy_i Index	56.76	55.52	56.05
TF% Index	34.07	33.73	34.57
Stebbins's categories	All	All	All

Fig. 3. Metaphase chromosomes of *Vicia kalakhensis* (a, d), *Vicia eristalioides* (b, e) and *Vicia narbonensis* (c, f) stained with H33258 (a–c) and C-banding (d–f). $\times 1200$. The numbers on C-banded chromosomes correspond with the numeration of the idiogrammatic representation.

ues of Sy_i (55.52) and TF% (33.73), the longest haploid complement, the longest satellite and the longest pair of chromosome (the first), on the contrary, *Vicia narbonensis* showed the shortest haploid complement. Karyotype formulas, the indices Rec, Sy_i and TF% and the categories according to Stebbins (1971) are summarised in Table 5.

Chromosome banding

In the three species examined the heterochromatin had essentially telomeric distribution (Fig. 3). Large heterochromatic blocks were located mainly at the short arms telomeres, few blocks were seen at the long arms telomeres. Intercalary heterochromatin was not frequent and major intercalary blocks were seen at the satellited chromosomes in all species. Minor intercalary bands were often seen on the long arms, but these bands had generally an occasional nature. The ideograms reporting bands distribution were overlaid on those developed with image analysis, the composed ideograms are given in Fig. 2.

The general distribution of the C-bands was very similar in all species examined and major differences regarded the satellited chromosomes. The chromosomes of *Vicia eristalioides* and those of *Vicia narbonensis* appeared to have a larger proportion of heterochromatin than those of *Vicia*

kalakensis. In fact, if the percentage of chromosomal heterochromatin is calculated according to the method of Venora *et al.* (1995) the three species show 8%, 8% and 6% respectively.

When H33258 staining was applied, the large constant heterochromatin blocks took up a bright fluorescence, the H33258 banding patterns were very constant and species-specific and no intercalary bands were seen with the exception of those associated to the satellited arms. H33258 staining enhanced the similarity among the three karyotypes, indicating that most of the differences in C-banding patterns arose from C-bands which showed indifferent reaction to H33258 staining: in fact, besides the differences in the structure of the satellited chromosomes, the H33258 stained metaphases of the three species were virtually indistinguishable.

Discussion

Although cytophotometric determination of nuclear DNA content using the Feulgen method may be subject to some reservation (Greilhuber 1986, 1988), we have chosen 30 min hydrolysis in 5 N HCl at room temperature after a study of hydrolysis curve. Since nuclei were analysed in the same early prophase conditions from tissues at the same stage of development (we used the meristematic portion from 1 cm long secondary roots), and all squashes were stained together (with *V. narbonensis* meristems used as an internal standard) the observed differences in Feulgen absorption values do reflect real differences in the DNA content.

The result of our cytophotometric analyses indicate values of nuclear DNA content that disagree with the report of Maxted *et al.* (1991b): the divergences are probably due to the different method. When the absorption curves are examined it appears evident that the curve of *V. kalakhensis* differentiates from those of *V. narbonensis* and *V. eristalioides*. On examination of the DNA amount and the nuclear surface (Table 1) it appears evident that this species has more DNA in a smaller nuclear volume, implying a more condensed state of all chromatin and not merely a higher amount of heterochromatin.

As far as karyotype measurements are concerned, the automated image analysis system ensured a high degree of accuracy since the measurements were taken directly in the first image of the chromosomes as projected by the objective. In traditional chromosome measurement methods such as photography, serial treatments are applied to the image, tending to accumulate error in the final determination. The availability of such precise measurement methods allowed the exact determination of karyomorphological indices: the latter are considered to be directly correlated with the evolution of the karyotype as reports on *Cicer* (Ocampo *et al.* 1992) or on *Vigna* (Pignone *et al.* 1995b) can demonstrate.

The three species showed very similar karyotypes. The results obtained on *V. narbonensis* confirm previous reports (Perrino and Pignone 1981, Singh and Lelley 1982) and for *V. kalakhensis* and *V. eristalioides* this is the first report on karyomorphological parameters. Schafer (1973) hypothesised that all karyotypes present in the *V. narbonensis* complex could be derive from an ancestral species by a single translocation. DNA restriction endonucleases fragment analysis was used to obtain information on the genome organization of rDNA in the genus *Vicia* (Raina and Ogihara 1995). The taxa within the *Narbonensis* complex were observed to have fairly similar rDNA monomer size and restriction maps befit a common ancestry.

The cytological data gathered through chromosome banding do not support this hypothesis. At least for *V. kalakhensis* and *V. eristalioides*, a more complicated mechanism needs to be claimed, also to account for the observed variation in nuclear DNA content. The disagreement may probably be due to the fact that the general structure of rDNA regions is fairly well conserved in these species, on the other hand, these regions account for a limited amount of the genome. Conversely the cytophotometric and the chromosome banding methods target a vaster proportion of the genomic DNA.

It is interesting to point out that the two approaches, karyomorphometry and cytophotometry, led to similar conclusions. The results of molecular analysis (Raina and Oghihara 1995) do not support diversity in the *Narbonensis* complex even if the authors have not analysed *V. eristalioides*. On the contrary the results obtained by studying the FokI elements in the genomes of *V. faba* and other *Vicia* species (Maggini *et al.* 1995), using biochemical approaches, support diversity in the *Narbonensis* complex. DNA sequences related to the FokI elements are not detectable even in species (*V. galilea*, *V. hyaenisciamus*, *V. johannis*) that belongs to the *Narbonensis* complex and are represented to a minimum extent in *V. narbonensis*.

It appears evident that in the examined species two classes of heterochromatin are present. The former reacts positively to both C-banding and fluochrome staining and is distributed on the telomeres, the latter is only evidenced by C-banding and is mostly intercalary. It is worth noting that the former class appears less affected by evolutionary changes than the latter. This may be the consequence of a different structural organisation of the two types of heterochromatin and may reflect also differences in the sequences hosted at those sites. A similar observation was made in *Dasypyrum villosum* (Cremonini *et al.* 1994, Pignone *et al.* 1995). The higher evolutionary stability of the heterochromatin class might explain the observation of Perrino and Pignone (1981) who reported limited variation for H33258 staining among the other species of the *V. narbonensis* complex.

One aspect still needs clarification: *V. kalakhensis* shows the highest level of heterochromatin as cytophotometrically determined in interphase nuclei and the lowest heterochromatin content in metaphase chromosomes. It needs to be stressed that the two methods estimate different levels of organization and two different functional stages. Differences in the nuclear heterochromatin might reflect different levels of genomic expression (*V. kalakhensis* has the largest genome) while chromosomal heterochromatin reflects a mode of chromosome packaging. It is possible to hypothesize that *V. kalakhensis* possesses a higher level of interspersed sequences which altogether account for denser nuclei, but features the same level of sequences in heterochromatin localization.

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