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Nuclear DNA characterization of two species of *Vicia*: *Vicia bithynica* L. and *Vicia narbonensis* L.

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Abstract

The species *Vicia bithynica* and *Vicia narbonensis*, from the same subgeneric section of *Vicia faba*, show variations in nuclear DNA content. Nuclear DNAs, extracted from root tips of the two *Vicia species*, were characterized by thermal denaturation, analytical ultracentrifugation and reassociation kinetics. The thermal denaturations of DNA, the number of DNA components reassociating with second order kinetics, the proportion of repeated DNA sequences, the frequency of the repeated DNA classes are reported and compared to previous data on *Vicia faba* DNA. Feulgen absorptions at different thresholds of optical density+ of interphase nuclei in cytological preparations of the root meristems of *V. bithynica* and *V. narbonensis* are determined and compared with *V. faba* analogous determinations. The results, confirming that plant genome is highly flexible, are discussed in relation to other data on the interspecific variations of the nuclear DNA content.

Introduction

Many eukaryotic organisms have more nuclear DNA than is apparently required for genetic and regulatory functions; this excess is supported mostly by repeated sequences and the role of this non coding repetitive DNA is not well understood (Cullis 1983). Moreover data in literature show that nuclear DNA sequence variations occur in the divergence and evolution process of species (Price 1976, 1988, Duhrssen *et al.* 1979, Hutchinson 1980, Narayan 1982, Rao and Rai 1987, Narayan and McIntyre 1989) and such variations may be also the results of developmental and physiological stimuli (Bassi *et al.* 1984, Walbot and Cullis 1985, Cavallini *et al.* 1986, Altamura *et al.* 1987, Cavallini *et al.* 1989, Cionini 1989 and references therein).

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+The term optical density is used according to authors' wish.

The evolutionary importance of these changes, when supported by repeated sequences, was pointed out by several investigations at a biochemical level (Flavell *et al.* 1974, Nagl 1979, Murray *et al.* 1981, Evans *et al.* 1983, Raina and Narayan 1984) and at a cytological level, since it has been demonstrated that the heterochromatic fraction of the genome contains repetitive and satellite DNA (Deumling and Greilhuber 1982, Arnold and Shaw 1985, Bassi 1990 and references therein).

The genus *Vicia* comprises about 120 species and most of them are diploid with the basic chromosome number $2n$=10, 12 or 14. Speciation and evolution of this genus was accompained by variations in chromosome size and number, nuclear DNA content, frequency of repetitive and non repetitive DNA sequences (Chooi 1971a, 1971b, Raina and Rees 1983, Raina and Narayan 1984, Narayan *et al.* 1985, Raina and Bisht 1988). Inside the genus *Vicia*, the section Faba, considered the most advanced, shows DNA values starting from 9.15 pg (2C value) of *V. bithynica*, which is significantly higher than in the other sections, to the highest DNA content present in the genus: 26.65 pg (2C value) of *V. faba*.

Because of this connection it is of interest to compare the results of cytological studies on the genome organization and the amount of repeated sequences in the species of the section Faba. Therefore we have studied two species of this section, *Vicia narbonensis* and *Vicia bithynica* and this paper shows evidences indicating that variations in genome organization are related with highly repeated DNA sequences and heterochromatin component. These results are compared with previous and new data concerning *Vicia faba* genome and phylogenetic relationships between the three species are discussed.

Material and methods

Eu/heterochromatin determination: Root tips obtained by germinating seeds of *Vicia narbonensis, Vicia bithynica* and *Vicia faba* in damp vermiculite, were fixed in ethanol-acetic acid (3:1 v/v). Squashes were made under a cover slip in a drop of 45 % acetic acid after treatment with a 5 % aqueous solution of pectinase (*Sigma*) for 1 h at 37 °C. After remotion of cover-slips by the dry-ice method, the squashes were hydrolysed in 1N HCl at 60 °C for 8 min and Feulgen stained. After staining, the slides were subjected to three 10 min washes in SO_2 water prior to dehydration and mounting in *DPX* (*Fluka*).

Feulgen DNA absorptions in individual cell nuclei, in postsynthetic condition (G_2), was measured at the wavelength of 550 nm using a *Leitz MPV3* integrating microdensitometer. With the same instrument and at the same wavelength, the Feulgen DNA absorption of chromatin fractions with different condensation level was determined by measurements of one and the same nucleus, after selecting different absorption thresholds of optical density in the instrument. The instrument reads all parts of the nucleus where absorption of optical density is greater than the preselected limit, regarding those below this limit as a clear field.

DNA extraction: DNAs were extracted from lyophilized root tissues of *V. bithynica* and *V. narbonensis* according to the method developed by Bendich *et al.* (1983) modified by Durante *et al.* (1985). For each material, roots were ground in a mortar and lysed with a solution containing 0.05 M Tris-HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl and 1 % sodium dodecylsarcosinate. The lysate was heated at 60 °C for 10 min, and preincubated pronase (*Sigma*) was added to a final concentration of 250 μg ml^{-1} After incubation at 37 °C for 3 h, the mixture was centrifuged at 20 000 *g* for 15 min. Solid CsCl and ethidium bromide from a stock solution (10 mg ml^{-1}) were added to the supernatant up to a final concentration of 0.8 g ml^{-1} and 300 μg ml^{-1}, respectively. The solution was centrifuged at 44 000 rpm in a *Beckman L5-65 B* ultracentrifuge for 60 h using the *50 Ti-rotor* and the DNA band, visualized under long-wave UV illuminator, was collected and recentrifuged. Ethidium bromide was then removed by gentle inversion of the solution with *n*-butanol.

Thermal denaturation: Thermal denaturation of DNA samples solubilized in 0.12 M phosphate buffer was carried out in a *Unicam SP 800* spectrophotometer equipped with a *SP 876* temperature programme controller. The increase of hyperchromicity at 260 nm was continuously followed by a *Philips PM 8120* x-y recorder. The GC percentages from Tm values were calculated according to Schildkraut and Lifson (1965).

Analytical ultracentrifugation: Analytical determinations in CsCl density gradient with a refractive index of 1.400 were performed in a *Beckman model E* ultracentrifuge at 44 000 rpm at 20 °C for 20 h. DNA from *Micrococcus lysodeikticus* (density 1.731 g ml^{-1}) was used as a marker.

Reassociation kinetics: DNAs were sheared by sonication in a MSE sonicator at medium output energy for 5 × 5 s with 10 s intervals at 4 °C. Sedimentation in neutral sucrose according to Clevell and Helinski (1969) revealed that the fragment of each sheared DNA had a relatively homogenous length of about 400 bp. Sheared DNAs dissolved in 0.12 M phosphate buffer at a concentration of 50 μg ml^{-1}, were denatured for 10 min at 103 °C and allowed to renature at nearly 25 °C below melting temperatures according to Britten *et al.* (1974). The reassociation processes were monitored in a closed thermostatically-controlled cuvette using the same equipment employed for the analysis of thermal denaturation kinetics. *E. coli* DNA (*Sigma*) was used as a standard after shearing at the same conditions as above. Reassociation experiments were reproduced twice using DNAs deriving from two different extractions. Repetitive DNA frequencies were calculated by the Scatchard-type analysis (Marsh and McCarthy 1974).

Results

Eu/heterochromatin determination: The results of measurements taken on 4C interphase nuclei at different thresholds of absorption optical densiti are reported in

Fig. 1. From these data significative variations in the chromatin structure of *Vicia* species clearly result: while the Feulgen absorption of optical density of *V. bithynica* is reduced to nothing at a threshold absorption of 21 (arbitrary units), those of *V. narbonensis* and *V. faba* are reduced to nothing at the thresholds of absorbance 33 and 36 (a.u.) respectively. These differences are mainly due to the more spiralized DNA fraction.

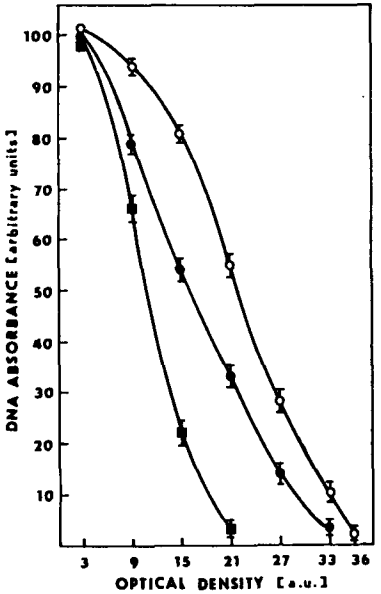

Fig. 1. Absorbance at 550 nm of Feulgen stained DNA at different absorption thresholds of the microdensitometer. *Vicia bithynica* (*squares*), *Vicia narbonensis* (*closed circles*) and *Vicia faba* (*open circles*).

Thermal denaturation of DNA: The values of thermal denaturation and the average GC content of the main peaks and buoyant density from the derivative melting profiles of *V. bithynica* and *V. narbonensis* DNAs are summarized in Table 1.

Table 1. Tm value and average GC content of main peak and buoyant density from the derivative melting profiles of *Vicia narbonensis, V. bithynica* and *V. faba* DNAs.

Species	Tm [°C]	GC [%]	Buoyant density [g cm^{-3}]
Vicia narbonensis	68.0	36.23	1.693
Vicia bithynica	67.5	35.01	1.691
*Vicia faba**	69.4	37.00	1.694

*from Bassi *et al.* 1984.

The Tm values are 67.5 and 68.0 for *V. bithynica* and *V. narbonensis* corresponding to a GC content of 35.01 and 36.23 respectively. For *V. faba* are reported values from Bassi *et al.* (1984).

Analytical ultracentrifugation: Fig. 2 shows the results of the analytical CsCl ultracentrifugation of *V. bithynica* and *V. narbonensis* DNAs. The nuclear DNAs form a symmetrical band with a buoyant density of 1.695 and 1.696 g ml^{-1} respectively. Buoyant density of *V. faba* DNA is 1.693 g cm^{-3} (Bassi *et al.* 1984). These analyses do not reveal DNA satellites.

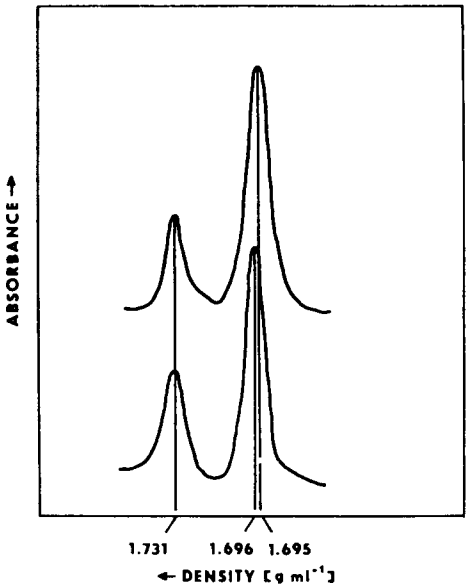

Fig. 2. Analytical CsCl density gradient ultracentrifugation of *Vicia narbonensis* (*above*) and *Vicia bithynica* (*below*) DNAs. *Micrococcus lysodeikticus* DNA (buoyant density 1.731 g ml^{-1}) was used as marker.

Table 2. Analysis of the reassociation kinetics of the DNA samples.

DNA sequence	Species	Frequency	C_0t 1/2 observed	C_0t 1/2 pure	Kinetic complexity	Redundancy
Highly repeated	*V. bithynica*	0.05	6.5 ± 10^{-3}	3.64 ± 10^{-2}	4.14 ± 10^{4}	5.09 ± 10^{3}
	V. narbonensis	0.12	1.8 ± 10^{-2}	2.28 ± 10^{-3}	2.59 ± 10^{3}	3.27 ± 10^{5}
	*V. faba**	0.11	1.7 ± 10^{-2}	1.87 ± 10^{-3}	2.13 ± 10^{3}	6.32 ± 10^{5}
Medium repeated	*V. bithynica*	0.10	2.8 ± 10^{-2}	2.80 ± 10^{-1}	3.08 ± 10^{5}	1.30 ± 10^{3}
	V. narbonensis	0.14	2.0 ± 10^{-1}	2.84 ± 10^{-2}	3.23 ± 10^{4}	2.93 ± 10^{4}
	*V. faba**	0.29	1.4 ± 10^{0}	3.64 ± 10^{-1}	4.14 ± 10^{5}	7.69 ± 10^{3}

*from *Bassi et al.* 1984.

DNA reassociation: The two DNAs and particularly their repetitive fractions, which are most likely involved in the variations in the genome size, were also studied by means of reassociation kinetics. In our system we refer to those reassociating at Cot value < 10^0 as repetitive sequences. Inverted repeats (palindromic sequences) that reassociate very rapidly, were not removed from total DNA. The experimental points for total DNA reassociation curves are derived from three different reassociation kinetics carried out. The reassociation kinetics of DNAs are shown in Fig. 3. The analysis of two components of the reassociation curves, which we consider to represent highly repeated and medium repeated DNA sequences, is detailed in Table 2 and there are also reported the data of *Vicia faba* for comparison (Bassi *et al.* 1984). The redundancy was calculated on the basis of DNA contents in presynthetic nuclei as estimated by our cytophotometric determinations.

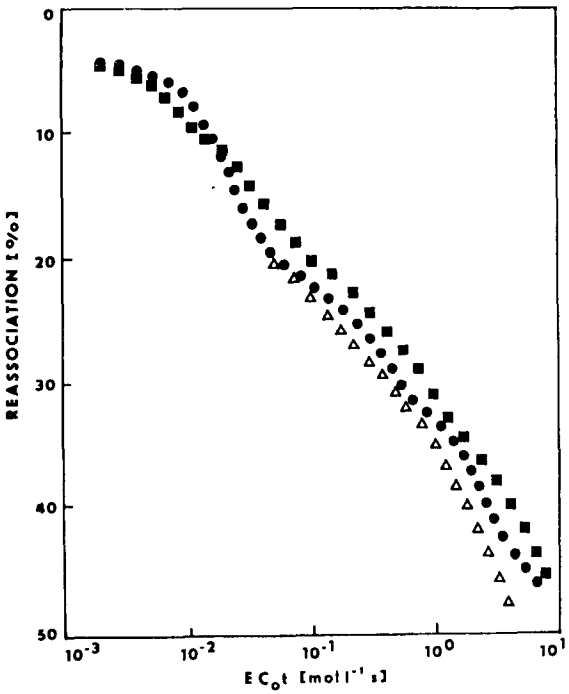

Fig. 3. Reassociation kinetics of *Vicia narbonensis* (*closed circles*) and *Vicia bithynica* (*closed squares*) DNAs. *E. coli* DNA (*open tringles*) was used as marker.

It must be stressed that there is a difference in the frequency of the repeated DNA sequences that is particularly evident in the case of the highly repeated sequences which are progressively less present in the DNA of the two species (12 % and 5 % corresponding to 1.89 and 0.5 pg of DNA per haploid nucleus in *V. narbonensis* and *V. bithynica* respectively: Table 3). The differences in the frequency of repeated

DNA sequences, confirming different organization and size of the two genomes, are also evident by kinetic complexity and redundancy values (Table 2).

Table 3. Nuclear DNA composition in *Vicia* species from C_0t analysis. (HR - highly repeated DNA sequences; MR - medium repeated DNA sequences; NR - non repeated DNA sequences).

Species	HR [%]	[pg]	MR [%]	[pg]	NR [%]	[pg]	Total 2C DNA [pg]
V. bithynica	5	0.45	10	0.91	85	7.77	9.15
V. narbonensis	12	1.74	14	2.03	73	10.62	14.55
*V. faba**	11	2.93	29	7.72	60	15.99	26.65

*from Bassi *et al.* 1984.

Discussion

The variations in DNA content are mainly associated with changes in the amount of repetitive DNA sequences that do not have coding functions. Repeated DNA sequences have been used as a mean to establish relationships between species of different taxa and inside the same taxa, since more closely related species have more families of repeated sequences in common (Rees and Jones 1972, Hirengardner 1976, Flavell *et al.* 1979, Rao and Rai 1987, Vershinin *et al.* 1989).

In this context an approach to elucidate species evolution would be to compare the presence and the organization of a satellite DNA, as in *Vicia faba* (Bassi *et al.* 1984) and in *Vicia melanops* (Narayan *et al.* 1985), but our ultracentrifugation of *V. bithynica* and *V. narbonensis* nuclear DNAs have not revealed DNA satellites (Fig. 2). In the three analysed species of section Faba of *Vicia*, the great differences in DNA content are mainly due to repetitive sequences which represent 15 %, 26 % and 40 % of *V. bithynica*, *V. narbonensis* and *V. faba* respectively. The observed variations in redundancy and kinetic complexity of fast and medium reassociating sequences (Table 2) demonstrate changes in DNA organization of analyzed species proving a greater similarity between *V. faba* and *V. narbonensis* than between *V. faba* and *V. bithynica*. Since heterochromatin has been demonstrated to contain a composite of families of repeated or satellite DNA in higher plants as in animals, the distribution and the role of heterochromatin and repetitive DNA has been studied for taxonomical and phylogenetic correlation (Deumling *et al.* 1976, Schweizer and Ehrendorfer 1976, Greilhuber 1979, Flavell 1986). In this context we have analyzed the more spiralized fraction with high absorption of the genome of the three species. The C-banding method has been commonly used for demonstrating heterochromatin at cytological level (Vosa and Marchi 1972, Comings 1976): as an alternative approach to examine phylogenetical relationships in our report, the determination of the nuclear fraction with different condensation levels by cytophotometric analyses at different thresholds of absorbance after Feulgen staining is used for the first time.

Even if the determination of DNA content by the cytophotometry of Feulgen stained nuclei may be subject to certain reservations (Greilhuber 1986), in regard to our analyses, we can say that the obtained differences in the Feulgen absorption values reflect real differences in the chromatin structure of the three species since we have analyzed nuclei in the same postsynthetic conditions from tissues in the same developmental stage and all squashes were concurrently stained.

From these data, the *V. faba* genome clearly results to be enriched in the heterochromatic fraction in respect to the two others and considering the chromatin structure, it is more similar to *V. narbonensis* than to *V. bithynica*, confirming the biochemical results. Clear differences between the different genomes already appear at low thresholds of optical density absorption: in fact when the threshold of is 9 (a.u.), the residual Feulgen absorptions is reduced to 65 % of the initial value for *V. bithynica* and to 80 % and 95 % for *V. narbonensis* and *V. faba* respectively. At highest thresholds of optical density absorption, the Feulgen absorption of meristematic nuclei from *V. faba* and *V. narbonensis* is still detectable when the Feulgen absorptions of *V. bithynica* is reduced to nothing, thus proving a greater proportion of optically dense chromatin in the first species. Therefore, when the nuclear DNA content increases, both euchromatin and heterochromatin are involved, but the rate of increase is greater for the heterochromatin (Raina and Bisht 1988).

Our biochemical and cytological analyses suggest that *V. narbonensis* and *V. faba* are more genetically related than *V. bithynica* and *V. faba*, confirming also the results obtained by other authors with different methods, including karyotaxonomical as Giemsa C-banding heterochromatin determination (Singh and Lelley 1982) and isozyme pattern analyses (Yamamoto *et al.* 1982). The present study may be a further step on the analyses of the relationships between species inside the genus *Vicia*. Work is in progress in order to reach a better understanding of phylogenesis and evolution in *Vicia*.

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