Theor Appl Genet (1984) 187–192

Changes in DNA composition in the evolution of *Vicia* species

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Received January 8, 1984; Accepted January 18, 1984
Communicated by D. von Wettstein

Summary. The composition of nuclear DNA in 3 *Vicia* species are compared. The species *V. eriocarpa, V. johannis* and *V. melanops* are from three separate subgeneric sections of *Vicia* and show a fourfold variation in their amounts of nuclear DNA. DNA melting experiments, buoyant density gradient analysis and Cot reassociation experiments show that the quantitiative change in nuclear DNA between the three species is achieved by changes in the amounts of both repetitive and nonrepetitive DNA sequences. It is suggested that while the increase in the repetitive fraction is achieved by the proliferation of repetitive base sequences the increase in the nonrepetitive fraction is due to the steady accretion of highly diverged base sequences resulting from mutations, deletions, insertions and base sequence rearrangements among families of repetitive sequences.

Key words: *Vicia* – nuclear DNA – Evolution – Base sequence

Introduction

The genus *Vicia* (family Leguminosae) has about 120 species distributed in the temperate zones of both hemispheres. Ninetyfive percent of species within this genus are diploids with basic chromosome numbers 2 n = 10, 12 or 14. Speciation and evolution within this genus has involved large changes in chromosome size and in nuclear DNA amounts (Martin and Shanks 1966; Raina and Rees 1983). The DNA variation is five fold among diploid species which suggests extensive amplification or deletion of base sequences during evolution. Despite this large scale variation in the amount of nuclear DNA the distribution of DNA within the chromosome complements of several diploid species have remained remarkably similar (Raina and Rees 1983). This would suggest constraints upon changes in DNA amounts. Similar constraints upon DNA distribution in chromosome complements during evolution are also reported in *Lathyrus,* (Narayan 1982; Narayan and Durrant 1983) *Lolium* and *Festuca* (Seal and Rees 1982). In the following investigation the molecular composition of the nuclear DNA of 3 *Vicia* species are compared. The results tell us about the changes in the composition of nuclear DNA during the evolution of *Vicia* species.

Material and methods

The three species *V. eriocarpa* (2 n = 14), *V. johannis* (2 n = 14) and *V. melanops* (2 n = 10) used in this investigation come from three separate subgeneric sections, *Cracca, Faba,* and *Vicia* respectively. The difference in the amounts of nuclear DNA between *V. eriocarpa* (4.5 pg) and *V. melanops* (20.04 pg) is fourfold. *V. johannis* (14.14 pg) has an intermediate DNA value.

DNA extraction and purification

Surface sterilised seeds of *Vicia* species were grown in the dark under axenic conditions for three to four weeks. Young shoots from seedlings were collected and ground to a fine powder under liquid nitrogen using mortar and pestle. The powder was suspended in an extraction buffer which contained 0.1 M NaCl, 10 mM EDTA, 50 mM tris pH 8.5, 1 M Na ClO_4, 0.5% diethyl pyrocarbonate and 2% Sarkosyl. After lysis the solution was shaken with redistilled phenol and then with chloroform – octonol (24 : 1). The aqueous extract which contained DNA was separated each time by centrifugation and this step was continued until no protein precipitate was visible in the interphase. The DNA extract was layered under two volumes of 98% ethyl alcohol. The DNA precipitate was spooled out, washed in 70% ethanol, air dried and then dissolved in 50 mM tris, 1 mM EDTA buffer (pH 8.5).

The DNA was further purified by digesting with RNAse (75 µg per ml at 37 °C for 1 h) and with pronase (100 µg/ml at 37 °C for 3 h). To remove contaminating polysaccharides the

DNA was coupled with ethidium bromide (600 μg/ml, from a stock solution of 10 mg/ml of ethidium bromide). The solution was made up to a final density of 1.515 g/ml by adding solid CsCl and centrifuged to equilibrium (40,000 rpm for 48 h) in a preparative ultra centrifuge. The DNA band was separated under UV and recentrifuged to equilibrium in CsCl solution as before. Ethidium bromide was removed from the purified DNA by n-butanol extraction. DNA was dialised into 1 mM tris, 0.25 M EDTA buffer (pH 8.5) and stored at 0 °C.

DNA melting experiments

DNA melting was done in a fully automated SP 1800 spectrophotometer with electronically heated cell blocks. The absorbance measurements made at present time intervals were automatically recorded in a digital print out. Purified DNA sample were dialised into 0.1 × SSC (SSC = 0.15 NaCl, 0.015 M trisodium citrate, pH 7) in a final concentration of 20 μg/ml. At least two DNA samples were melted simultaneously for each species and the results were compared. The increase in the optical density of DNA (hyperchromicity) was monitored and recorded for each 0.25 °C rise in temperature. The absorbance measurements were corrected for solvent expansion using the table of Mandel and Marmur (1968). The corrected absorbance values were plotted as a ratio A_t / A_{25} (absorbance at temperature 't' divided by the initial absorbance at 25 °C) against temperature. From the slope of the melting profile, T_m (temperature corresponding to half the final increase in relative absorbance) was calculated. The average G+C content and the average base compositional heterogeneity for the DNA samples were estimated using the equations of Mandel and Marmur (1968).

Analytical density gradient analysis

DNA samples (1.5 μg) of *Vicia* species were centrifuged to equilibrium in neutral CsCl solution (density 1.71 g/ml) in a centriscan – 75 analytical ultracentrifuge. 0.25 μg of *Micrococcus lysodeicticus* DNA of known buoyant density was added to each sample as an internal standard. At equilibrium the density gradients were optically scanned at 254 mμ and plotted. The mean buoyant density of *Vicia* DNA was calculated with reference to the buoyant density of *M. lysodeikticus* DNA. From the buoyant density the G+C contents of *Vicia* DNA was calculated using the modified equations of Schilkraut et al. (1962) as given by Kemp and Sutton (1976).

Identification and isolation of cryptic satellite DNA sequences using actinomycin-D

0.5 μg of actinomycin-D was added to each microgram of nuclear DNA in solution and the final density was made up to 1.65 g/ml using 60% neutral CsCl solution. The final concentration of DNA was 50 μg/ml. DNA-CsCl solution was centrifuged to equilibrium (40,000 rpm at 24 °C for 24 h) in 20 ml tubes in a preparative ultra centrifuge. The DNA solution was fractionated into 1 ml samples. The optical density of each fraction measured at 260 mμ was plotted. The satellite DNA peak was separated and dialysed into 20 × SSC for 24 h (Brown et al. 1971) and then into 0.1 × SSC for 6 h.

The DNA solution was made up to 1 M using Na Acetate crystals and then layered with three volumes of 70% ethanol. The solution was cooled to 0 °C for 3 h and the DNA precipitate was collected by centrifugation. The DNA was dissolved in 10 mM tris 1 mM EDTA buffer (pH 7.4) and stored at 0 °C.

Cot reassociation of nuclear DNA

The percentage reassociation of dissociated DNA sequences was monitored optically at regular Cot intervals. The details of this experiment are already described by Narayan and Rees (1976). The DNA samples were sheared to an average fragment size of 450 base pairs using a virtis-60 homogeniser. The fragment size was confirmed using agarose gel electrophoresis. Sheared DNA samples were dialised into 0.12 M phosphate buffer (equimolar mixture of NaH_2PO_4 and Na_2HPO_4, pH 6.8). The sheared DNA was dissociated by heating to 100 °C for 7 min in a water bath and its reassociation started by bringing quickly to 62 °C (Tm-20). The reassociation of DNA between Cot 0.001 and Cot 0.1 was done in 0.04 M phosphate buffer and the reassociation between Cot 1,000 and 20,000 in 0.4 M phosphate buffer. The Cot values were standardised using the tables given by Britten et al. 1974.

When thermally dissociated DNA is cooled to the reassociation temperature there is a rapid loss in absorbance due to the increase in the secondary structure of DNA. This is often referred to as collapse hypochromicity which is distinctly different from the regular loss in absorbance due to reassociation. The mean collapse hypochromicity was estimated by dissociating and cooling DNA samples to the reassociation temperature in the spectrophotometer. The mean collapse hypochromicity obtained in our experiments is 10.4% of the total. This value is closely similar to a theoretical estimate (9.7%) obtained using the equations of Bendich and Anderson (1977).

Results and discussion

1 Changes in the base composition of nuclear DNA

The DNA melting profile for the three species was similar and the melting profile for the DNA of *V. melanops*, which is typical for the three species, appear in Fig. 1. The Tm, average G+C content and the base compositional heterogeneity derived from the melting profiles are in Table 1. The Tm for the three species are similar and the average G+C content varies from 34.89% in *V. eriocarpa* to 38.06% in *V. melanops*. *V. johannis* has an intermediate value of 37.80%. The base compositional heterogeneity, which is a measure of the base pair distri-

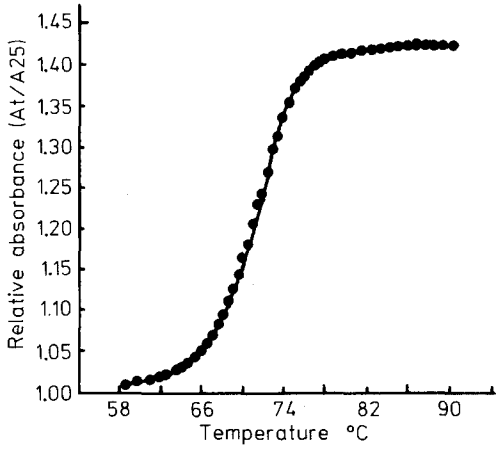

Fig. 1. DNA melting profile for *V. melanops*

Table 1. DNA melting experiments in *Vicia* species

	Tm (°C)	% G+C Content	Base compositional heterogeneity
V. eriocarpa	68.2	34.89	19.52
V. johannis	69.0	37.80	19.52
V. melanops	69.5	38.06	17.08

Table 2. Buoyant density analysis in *Vicia* species

	Density of the main band (g/ml)	% G+C content	Estimated 5 methyl cytidine content (%)
V. eriocarpa	1.689	36.22	3.48
V. johannis	1.688	35.20	8.09
V. melanops	1.688	35.20	9.95

bution in nuclear DNA, is the same for *V. eriocarpa* and *V. johannis*. *V. melanops* has a slightly lower value. The results would suggest that the base composition of the extra DNA responsible for the fourfold variation in nuclear DNA among the three species is not significantly different from that of the total DNA. Moreover, the large variation in nuclear DNA between the three species did not result in significant changes in their base pair distribution. More detailed information regarding the distribution of base sequences in the three species is obtained from the buoyant density gradient analysis in CsCl.

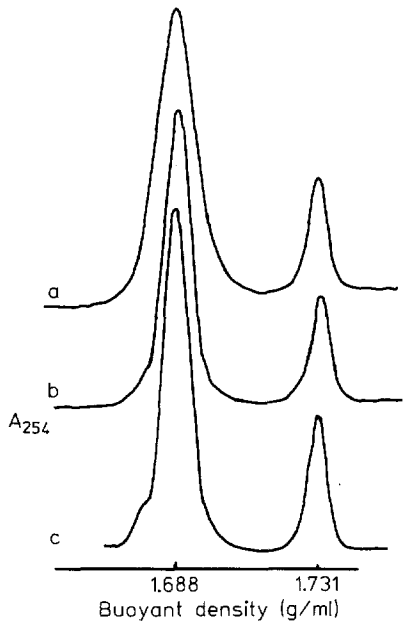

Fig. 2a – c. Buoyant density gradient profile for *Vicia* DNA in neutral CsCl: **a)** *V. eriocarpa*, **b)** *V. johannis*, **c)** *V. melanops*

The buoyant density gradient profile for the three *Vicia* species appear in Fig. 2. The average buoyant densities and the G+C contents estimated from them are in Table 2. While *V. eriocarpa* and *V. johannis show* narrow and symmetrical DNA density gradient profiles, *V. melanops* shows the presence of a satellite fraction with an average buoyant density of 1.680 g/ml. This light satellite fraction makes up approximately 5.1% of the total DNA.

A comparison of Tables 1 and 2 would show that the average G+C contents, estimated independently from T_m and buoyant density, differ on average by 2.26%. This difference could result from the presence of unusual base pairs or due to the methylation of the deoxycytidine residues in the DNA. In DNA from higher plants, 5-methyl deoxycytidine constitutes up to 7.1%, which is substantially higher than the 0.7 to 2.8 mol%, in animal DNA (Ehrlich and Wang 1981). While T_m of DNA remains unaffected by the 5-methyl deoxycytidine bases, the buoyant density is lowered by 0.004 g/cm^{-3} for every 6.3 mol% of 5-methyl deoxycytidine (Kemp and Sutton 1976). Using their equation, the mole fraction of 5-methyl deoxycytidine was calculated, which also is in Table 2. The amount of 5-methyl deoxycytidine estimated by this method varies from 3.48% in *V. eriocarpa* to 9.95% in *V. melanops* (mean value 7.17%). In proportional terms as well as in absolute amounts the 5-methyl deoxycytidine content appears to increase with increasing DNA amounts in the three *Vicia* species.

The density gradient profile for the DNA of *V. johannis* coupled with actinomycin-D showed the presence of a distinct satellite fraction. This fraction was isolated and purified by repeated centrifugations in neutral CsCl. The details regarding the molecular composition of satellite DNA sequences and their distribution in the chromosome complements of *Vicia* species will be reported at a later date.

2 Quantitative changes in the DNA components

The Cot reassociation curves for the *Vicia* species and of *E. coli* for comparison appear in Fig. 3. Based on their reassociation kinetics the total nuclear DNA of each species was divided into 3 components, a) a fast fraction made up of highly repetitive sequences reassociating prior to Cot 10^{-2}, b) an intermediate fraction (middle repetitive fraction) which reassociated between Cot 10^{-2} and Cot 4×10^{2} and c) the single copy sequences which reassociated between Cot 4×10^{2} and 10^{4}. The maximum reassociation obtained in this experiment was 92%.

Quantitative estimates for the three components are in Table 3. This table shows that in absolute amounts the three DNA components increase with increase in total nuclear DNA amounts. In proportional terms, however, the three DNA components differ in the three species and show no correlation with their total DNA amounts.

In Fig. 4 the amounts of highly repetitive DNA, middle repetitive DNA and the non repetitive DNA for

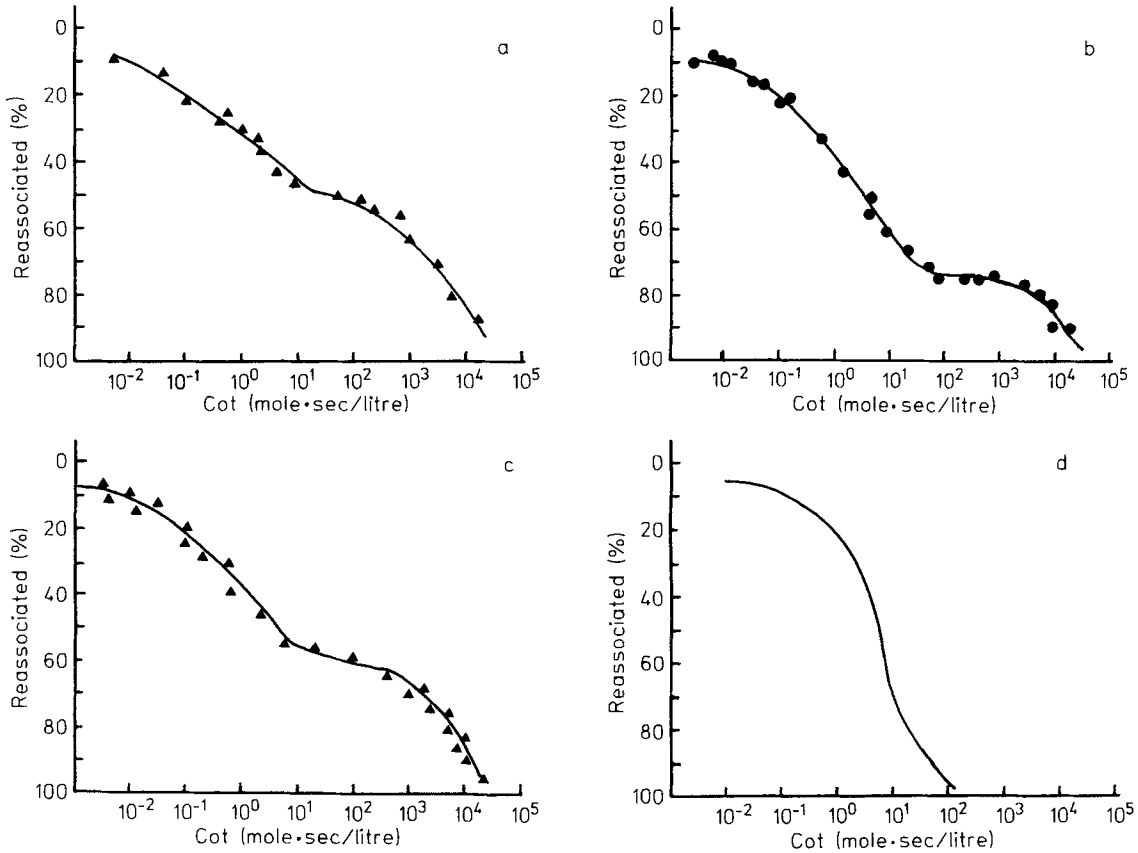

Fig. 3a – d. Cot reassociation curves for *Vicia* species and *E. coli* for comparison: **a)** *V. eriocarpa*, **b)** *V. johannis*, **c)** *V. melanops* and **d)** *E. coli*

Table 3. Nuclear DNA composition in *Vicia* species

	Highly rept DNA		Mid rept DNA		Non rept DNA		Total DNA (*pg*)
	%	Amount (*pg*)	%	Amount (*pg*)	%	Amount (*pg*)	
V. eriocarpa	5	0.23	48	2.16	47	2.11	4.50
V. johannis	3	0.43	69	9.93	28	3.96	14.14
V. melanops	8	1.60	54	10.81	38	7.60	20.02

the three species are plotted against their total DNA. While *V. eriocarpa* and *V. johannis* have lower amounts of highly repetitive DNA (0.225 pg and 0.43 pg, respectively), *V. melanops* has a significantly large amount (1.60 pg). It is important that in neutral CsCl density gradient analysis *V. melanops* alone gave a satellite DNA fraction which makes up 5.1% of the genome. In a separate Cot reassociation experiment (unpublished results) this fraction reassociated between Cot 10^{-5} and Cot 10^{-2} with a Cot ½ value of 4×10^{-3}. This satellite DNA fraction would make up 62% of the highly repetitive sequences in *V. melanops*. It is clear from Fig. 4 that while both middle repetitive and non repetitive DNA increase with increasing DNA amounts, the rate of increase is greater for the middle repetitive fraction. In the closely related genus *Lathyrus* nuclear DNA variation among diploid species is fourfold (Narayan 1982). The fraction of DNA responsible for the DNA variation within this genus is made up of both middle repetitive DNA and non repetitive DNA in a consistent ratio of 4.0 to 1.0. (Narayan and Rees 1976; Rees and Narayan 1977). As in *Lathyrus* many other plant and animal genera show consistent ratios of repetitive and non repetitive DNA sequences in the extra DNA contributing to the DNA variation among species (Hutchinson et al. 1980).

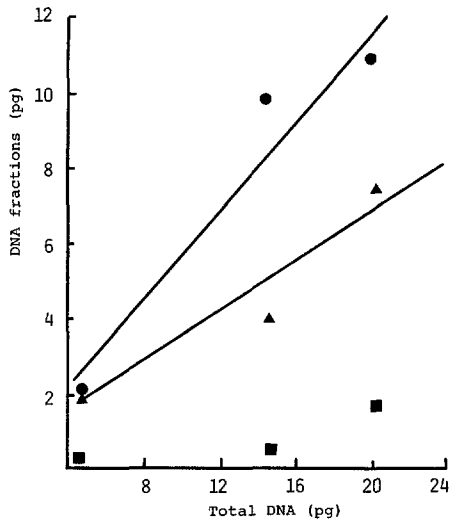

Fig. 4. The amounts of highly repetitive DNA (■) middle repetitive DNA (▲) and non repetitive DNA (●) are plotted against the total DNA of the three species

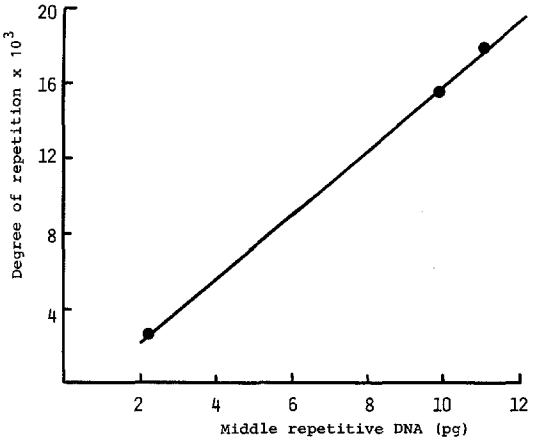

Fig. 5. The degree of repetition in the middle repetitive DNA of each species is plotted against their absolute amounts (*pg*)

An increase in the repetitive DNA is readily explained as due to the saltatory replication of repetitive base sequences (Britten and Khone 1968). The increase in the single copy DNA cannot occur by this process because extensive replication of non repetitive DNA would make them repetitive. It is logical therefore to assume that the increase in the nuclear DNA among species was initially restricted to the repetitive fraction. Extensive divergence in the repetitive DNA due to mutations, insertions, deletions or base sequence rearrangements would have resulted in the generation of additional non repetitive DNA sequences. Experimental evidence which confirms the above assumption comes from the results of Thompson and Murray (1980). They have shown that most single copy DNA sequences in Mungbean and Pea genomes are in fact short interpersed elements from ancient repetitive sequences.

3 Changes in the base sequence complexity of repetitive and non repetitive DNA

The Cot reassociation data for the middle repetitive and non repetitive DNA sequences were normalised to 100% and the reassociation rate constants (Cot ½) were estimated for each fraction. The *E. coli* DNA (genome size 4.6×10^6 base pairs) gave under similar experimental conditions a Cot ½ value of 6. Using this value as a standard, the average kinetic comlexity as number of base pairs was estimated for each fraction. The average kinetic complexity is a theoretical estimate of base sequence divergence which limits the in vitro reassociation of dissociated DNA. The results are in Table 4. When converted into picograms the estimated kinetic complexity for the non repetitive DNA is similar to the analytical estimates (Table 4) obtained by subtracting the amount of repetitive DNA from the total. The ratio between the analytical estimate and the kinetic estimate for the non repetitive DNA is approximately equal to 1.0 (mean value 1.09) which confirms that the amounts of non repetitive DNA estimated by this method are made up predominantly of singly copy DNA or sequences of very low repeats.

The middle repetitive fraction of the three species have closely similar Cot ½ values with a mean of

Table 4. Base sequence complexity for repetitive and non repetitive DNA fractions. The figures in parenthesis are the amount of non repetitive DNA (in *pg*) estimated from the kinetic complexity. 0.917 *pg* of DNA has 10^9 base pairs

	Non repetitive DNA		Mid repetitive DNA	
	Cot ½	Average kenetic complexity (base pairs)	Cot ½	Average kinetic complexity (base pairs)
V. eriocarpa	5×10^3	3.51×10^9 (3.16 *pg*)	1.0×10^0	0.76×10^6
V. johannis	6×10^3	4.22×10^9 (3.79 *pg*)	0.8×10^0	0.61×10^6
V. melanops	1×10^4	7.02×10^9 (6.32 *pg*)	0.8×10^0	0.60×10^6

0.8×10^0. This would convert to approximately 0.6×10^6 base pairs. Knowing the amount of middle repetitive DNA in each species and their average kinetic complexity in base pairs it was possible to estimate the average degree of repetition in the middle repetitive DNA of the three species. In Fig. 5 the degree of repetition in the middle repetitive DNA of the three species are plotted against their absolute amounts in picograms. The regression is linear and significant ($P<0.001$) which suggests that the increase in the middle repetitive DNA was achieved by extensive replication in the repetitive DNA sequences. However, this fraction reassociates over a broad range of Cot values (from Cot 10^{-2} till Cot 10^2) which indicates extensive base sequence divergence among families of repetitive sequences, during evolution.

Conclusions

1. The quantitative changes in nuclear DNA in the evolution of diploid *Vicia* species stems from changes in the amounts of both repetitive and non repetitive sequences.

2. The increase in the repetitive fraction is readily explained as being due to the saltatory replication of DNA base sequences. The increase in the non repetitive fraction, on the other hand, would have resulted from the steady accretion of highly diverged repetitive sequences.

3. Some of the highly repetitive sequences resolved as satellite DNA using analytical ultra centrifugation have very low base sequence complexity. This would suggest that these sequences would have proliferated relatively recently during the evolution of the genus.

4. The average mole % of 5-methyl deoxycytidine bases for the three species is closely similar to the average value (7.1%) reported for higher plants. However, there appears to be a slight increase in the amount of methylated deoxycytidine bases with increasing DNA amounts.

Acknowledgements. We are grateful to Professor H. Rees for his encouragement. S. N. Raina received a Commonwealth University Fellowship during the investigation. Present address: Dr. S. N. Raina, Department of Botany, The University of Jodhpur, India.

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