The distribution of satellite DNA in the chromosome complements of *Vicia* species (Leguminosae)

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Abstract

When centrifuged to equilibrium in neutral CsCl approximately 5% of the total nuclear DNA of *V. melanops* separated into a light satellite fraction. Buoyant density gradient analysis, thermal denaturation analysis and Cot reassociation experiments were used to find out the base sequence organisation of the satellite DNA. Using the method of *in situ* hybridisation its distribution in the chromosome complements of *V. melanops* and three other *Vicia* species were compared.

Introduction

Among the 120 species in the genus *Vicia* (family Leguminosae) 95% are diploids with 2n = 10, 12 or 14. Speciation and evolution within this genus have involved a fivefold increase in the amounts of nuclear DNA (Raina & Rees, 1983). This variation in DNA amounts stems from quantitative changes in both repetitive and non repetitive DNA sequences (Raina & Narayan, 1984). Buoyant density gradient analysis of the nuclear DNA using analytical ultracentrifugation has shown the presence of a distinct satellite DNA fraction in the genome of *Vicia melanops*. This paper describes the molecular composition of this satellite DNA and its location in the chromosome complements of *V. melanops* and other related species.

Material and methods

V. melanops Sibth & Sm. (2n = 10) belongs to the subgenus *Vicia* and has 20.04 pg of DNA in its 2C nuclei. Other species investigated are *V. villosa* Roth. subsp. *eriocarpa* (Hansskn.) Hal. (2n = 12) *V. johannis* Tamamchian (2n = 14) and *V. hybrida* L. (2n = 10). *V. eriocarpa* and *V. johannis* are from separate subgenera, *Cracca* and *Faba* respectively. *V. hybrida* is taxonomically more closely related to *V. melanops* than to *V. eriocarpa* and *V. johannis* and is included in the same subgenus *Vicia*. The 2C nuclear DNA amounts are 4.5 pg, 14.14 pg, and 16.46 pg for *V. eriocarpa, V. johannis* and *V. hybrida* respectively (Raina & Rees, 1983). Seeds of *V. eriocarpa* and *V. johannis* were originally from Turkey. The seeds of *V. melanops* are from France. They were supplied by Dr. R. Allkin, Southampton University, England.

DNA extraction and purification

Young shoots from seedlings grown in the dark under axenic conditions were ground to a fine powder under liquid nitrogen. The powder was suspended and lysed in a buffer which contained 0.1 M NaCl, 10 mM EDTA, 50 mM Tris (pH 8.5), 1 M $NaClO_4$, 0.5% diethyl pyrocarbonate and 2% sarkosyl. After lysis the solution was shaken with redistilled phenol and chloroform-octanol (24:1). The aqueous extract was removed each time by centrifugation and this step was repeated until no protein precipitate was visible in the interphase. The crude DNA was precipitated using ethanol. The DNA

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precipitate was dissolved in 50 mM Tris, 1 mM EDTA buffer (pH 8.5).

RNA and protein impurities were removed by digesting with RNAse (75 μg/ml, 37 °C for 1 h) and pronase (100 μg/ml, 37 °C for 3 h). To remove the polysaccharides the DNA was coupled with ethidium bromide (600 μg of ethidium bromide per 1 ml of DNA). Adding CsCl crystals the final density of the solution was made up to 1.515 g/ml. It was centrifuged to equilibrium in a preparative ultra centrifuge (40,000 rpm at 24 °C for 48 h). The DNA band was separated under UV and recentrifuged in neutral CsCl as before. Ethidium bromide was removed using n-butanol extraction. Purified DNA was dialysed into 10 mM tris (pH 8.5), 0.25 mM EDTA buffer (pH 8.5) at 4 °C.

Analytical density gradient analysis

1.5 μg of DNA was centrifuged to equilibrium in neutral CsCl (1.71 g/ml) in a centriscan-75 ultracentrifuge. 0.25 μg of *Micrococcus lysodeikticus* DNA of known buoyant density was added as marker. At equilibrium the density gradients were scanned at 254 nM. The mean buoyant density of the satellite component was estimated with reference to the marker DNA.

To separate the satellite DNA from the main band 1 mg of purified DNA from *V. melanops* was centrifuged to equilibrium in 20 ml neutral CsCl solution in a preparative ultracentrifuge (at 40,000 rpm at 24 °C for 48 h). The optical densities of fractions collected from the density gradient were measured in a spectrophotometer. The fractions which contained the satellite DNA were pooled and purified by repeating the centrifugation as before. The purified satellite DNA was dialysed against 10 mM tris, (pH 8.5) and stored at 0 °C.

Thermal denaturation analysis for DNA

Thermal denaturation was done in an SP 1800 spectrophotometer with electronically heated cell blocks. The increase in absorbance was monitored at 260 nm and recorded automatically in a digital print-out for each 0.25 °C rise in temperature. The data were corrected for expansion of the solvent using the table of Mandel and Marmur (1968). The melting profile for the DNA was plotted as a ratio A_t/A_{25} (absorbance at temperature 't' divided by initial absorbance at 25 °C). DNA melting was done in 0.1 × SSC (SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

Cot reassociation of satellite DNA

The satellite DNA was dialysed against 0.04 M phosphate buffer (equimolar mixture of Na_2HPO_4 and NaH_2PO_4, pH 6.8) and sheared to an average fragment size of 450 base pairs using a Virtis-60 homogeniser. The reassociation of heat-denatured DNA sequences was monitored optically in the SP 1800 spectrophotometer. The Cot values were standardised for 0.12 M phosphate buffer using the table given by Britten *et al.* (1974) *E. coli* DNA (genome size, 4.6×10^6 bp) and T_4 DNA (genome size 17.5×10^4 bp) were also reassociated simultaneously for comparison.

Preparation of H^3 labelled complementary RNA (c-RNA) and in situ *hybridisation to chromosomal DNA*

H^3 labelled c-RNA was transcribed from satellite DNA using *E. coli* RNA polymerase (from Sigma Ltd.). In each reaction 3 μg of satellite DNA was used as template. H^3 labelled nucleotides (UTP, 46 Ci/mMol, and ATP 32 Ci/mMol) were purchased from Amersham Radiochemicals. The labelled c--RNA had a specific activity of 6×10^7 cpm/μg. It was hybridised *in situ* to the chromosomal DNA using the method given by Dennis *et al.* (1980). Eight μl of labelled c-RNA (100,000 cpm) in 3 × SSC and 50% formamide was placed on each slide. It was covered with a coverslip and sealed with rubber solution. The chromosomal DNA was denatured by dipping the slides for 5 min in a water bath maintained at 75 °C. The slides were then incubated for 24 h at 37 °C. After removing the coverslips the excess c-RNA was removed by extensive washing in several changes of SSC. The nonspecifically bound c-RNA was removed by treating with RNAse. The slides were then dehydrated in two changes each of 75% and 98% ethanol. After air drying the slides were dipped in Ilford photographic emulsion (K_2 emulsion diluted 1:1 with distilled water) and exposed for 14 to 21 days. The slides were developed in Kodak D-19 developer and stained with 3% Giemsa solution.

Giemsa C-banding of metaphase chromosomes

Metaphase chromosomes prepared from root meristems were C-banded using the method suggested by Vosa (1974).

Results and discussion

The composition of satellite DNA

The buoyant density gradient profile for the nuclear DNA of *V. melanops* is in Figure 1. The average buoyant density is 1.688 g/ml which translates to an average G + C content of 35.20% (Kemp & Sutton, 1976). The density gradient profile is narrow but shows the presence of a distinct satellite DNA fraction. This light satellite DNA fraction makes up approximately 5.1% of the total genome. The buoyant density gradient profile for the purified satellite DNA is also in Figure 1. The satellite DNA has a buoyant density of 1.680 g/ml which would correspond to 27.4% G + C content.

The melting profile for the satellite DNA is in Figure 2. As would be expected from the results of the density gradient analysis the slope for the melting profile is steep, which suggests homogeneity in base pair distribution. T_m (the temperature corresponding to half the final increase in relative absorbance) was 66 °C. An independent estimate of G + C content for the satellite DNA was obtained from T_m using the equations of Mandel and Marmur (1968). The G + C content estimated by this method

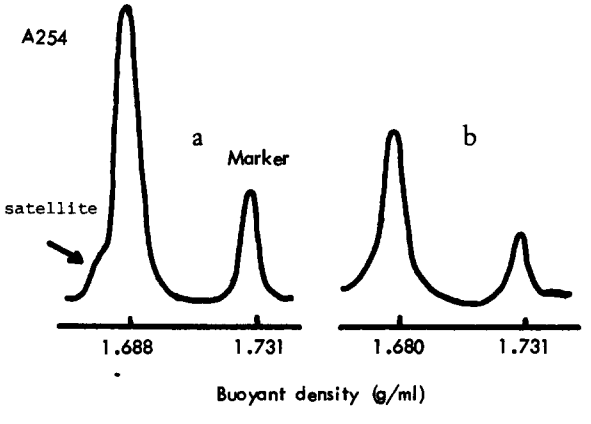

Fig. 1. Buoyant density gradient profile: (a) for the total nuclear DNA; (b) for the satellite DNA.

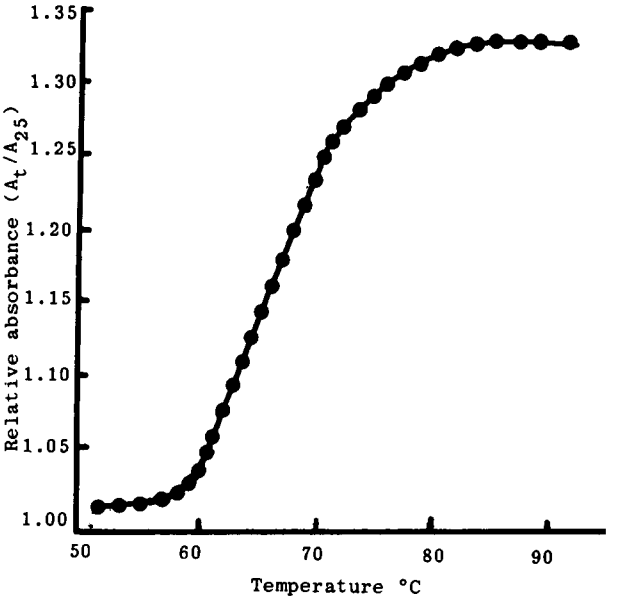

Fig. 2. Melting profile for purified satellite DNA.

was 29%, which is closely similar to the value obtained from the buoyant density analysis.

The Cot reassociation curve for the satellite DNA and of T_4 phage DNA for comparison appear in Figure 3. The satellite DNA reassociated between Cot 10^{-5} and 10^{-2}. The reassociation constant 'Cot ½' (Cot value at which 50% of the dissociated DNA duplexes have reassociated) for the satellite DNA was 4×10^{-3}. Under identical experimental conditions *E. coli* DNA (genome size, 4.6×10^6 bp) gave a Cot value of 6.0. T_4 DNA (17.5×10^4 bp) gave a 'Cot ½' value of 0.4. We may conclude therefore that the satellite DNA has an average kinetic complexity 1500 times smaller than that of *E. coli* DNA or about 100 times smaller than that of T_4 phage DNA. The low base-sequence complexity of the satellite DNA would imply that these sequences have proliferated relatively recently during the evolution of the genus.

Eight percent of the total DNA in *V. melanops* reassociates prior to Cot 10^{-2} and are classified as highly repetitive sequences (Raina & Narayan, 1984). The light satellite DNA described here makes up approximately 62% of the highly repetitive fraction.

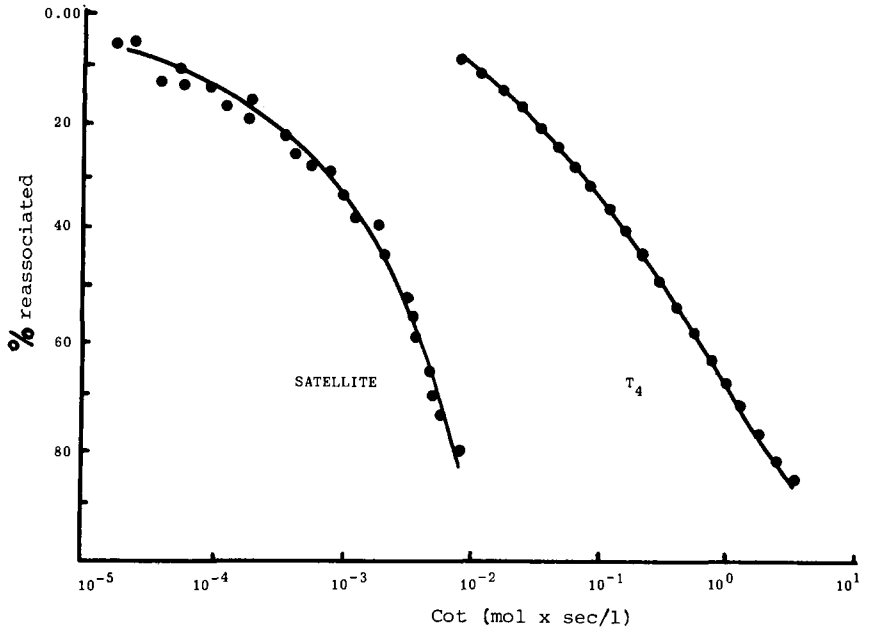

Fig. 3. Cot reassociation of the satellite DNA. The reassociation curve was normalised to 100%. The cot reassociation curve for T_4 phage DNA is given for comparison.

Distribution of constitutive heterochromatin in the chromosome complements of V. melanops

The haploid chromosome complement of *V. melanops* is in Figure 4. The chromosomes are arranged in order of size. All chromosomes are submedian in shape with the largest chromosome having the nucleolar-organiser region in its short arm. The C-banded chromosomes and karyotype appear in Figure 4. All chromosomes have distinct C bands at or near the telomeres of their short arms. Analysis of less condensed premetaphase chromosomes has shown faint intercalary bands in the long arm of chromosomes 1, 2 and 3. These intercalary bands are not normally visible in fully condensed metaphase chromosomes. Heterochromatic C-bands are also found in the terminal region of the long arm of chromosome 5 and near the centromere of chromosome 1.

The distribution of satellite DNA sequences in the chromosome complements of Vicia *species*

H^3 labelled c-RNA was hybridised to the chromosomal DNA of *V. melanops, V. eriocarpa, V. johannis* and *V. hybrida.* At least three complete metaphase plates were analysed for each species to confirm the distribution of silver grains in each chromosome.

The distribution of silver grains in the chromosome complement of *V. melanops* is in Figure 5. Even though the satellite DNA represents only 5% of the total DNA it is fairly evenly distributed on all chromosomes within the complement. The figure shows that the satellite DNA sequences are located at specific sites in each chromosome. While it is located at the centromeres of all chromosomes, greater aggregation of silver grains at the centromeres is found in chromosomes 1, 3 and 5. The labelled sites are also found in the intercalary regions of all chromosomes. In chromosomes 1, 2 and 5 these sites would correspond to the faint intercalary C-bands visible in the premetaphase mitotic chromosomes. In several plant and animal species satellite DNA sequences are located in their C-band heterochromatin (John & Miklos, 1979). In the closely related genus *Lathyrus* a satellite-DNA isolated from *L. tingitanus* was located in the centromeric heterochromatin of all chromosomes (Narayan & Durrant, 1983). However, in several

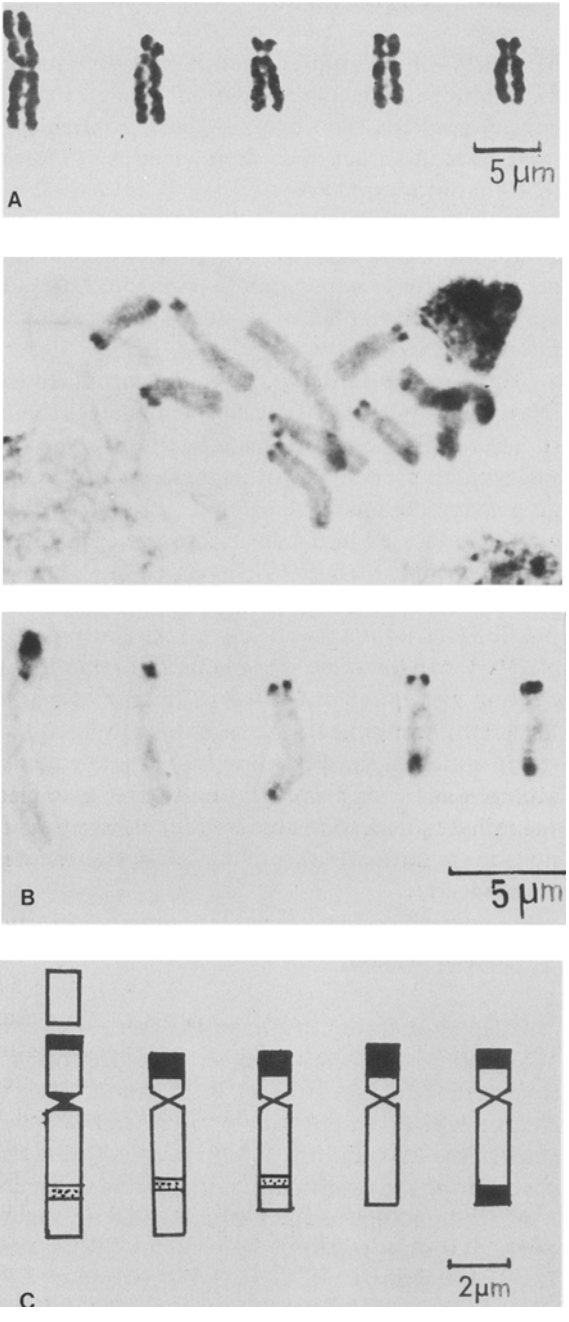

Fig. 4. Vicia melanops: (a) Haploid chromosome complement; – (b) C-banded chromosomes; – (c) karyotype showing the relative positions of the C-bands.

diploid plant species the distribution of satellite DNA sites does not correspond well with their C-banding pattern (Bedbrook *et al.*, 1980; Teoh *et al.*, 1983) All chromosomes of *V. melanops* have well

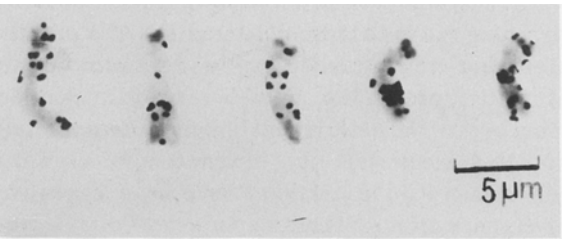

Fig. 5. In situ hybridisation of satellite DNA to *V. melanops* chromosomes.

defined C-bands at their terminal regions. But very few labelled sites are found in these regions. It is fairly well established that the Giemsa-positive C-band regions contain several different highly repetitive sequences with substantial variation in copy numbers. The light satellite DNA sequences in *V. melanops* appear to be present in very few copies in their terminal C-bands. Timmis *et al.* (1975) separated an Ag^+/Cs_2SO_4 satellite DNA fraction from *V. faba*. They reported a generalised distribution of satellite DNA sites in the chromosome complements of *V. faba*. They have also observed a relatively low number of labelled sites in the terminal regions of chromosomes.

The satellite DNA did not hybridise *in situ* with the chromosomal DNA of *V. eriocarpa* and *V. johannis*. The molecular processes involved in the origin of satellite DNA sequences and the mechanisms by which they are distributed in the chromosome complements of closely related species during evolution are not clearly understood. Molecular hybridisation experiments have made possible direct comparison of nucleotide sequences in related species. It is confirmed from these experiments that related species have the same complements of highly repeated DNA sequences. However the amount of any one particular sequence differed greatly even among closely related species (Peacock *et al.*, 1981). Singh *et al.* (1976) have shown that when a repeated DNA sequence is present over an extended range of taxa (e.g. the repeated DNA sequences in the X chromosomes of many animals) its repeat number varies from species to species. Gosden *et al.* (1977) and Fry and Salser (1977) have shown that even when a major satellite DNA is present only in very low repetitive frequency, in a related species the nucleotide sequence of the basic repeat units are maintained.

The method of *in situ* hybridisation is not sufficiently sensitive to identify similar DNA sequences present in very low frequency in the chromosomes of related species. It is possible that the nucleotide sequences of the basic repeat unit of *melanops* satellite DNA are present only in very few copies in the genomes of *V. johannis* and *V. eriocarpa*. Extensive divergence among repetitive sequences due to mutations, deletions, base substitutions or due to large scale interspersion within the genome could reduce the hybridisation of *melanops* satellite DNA to the chromosomal DNA of *V. johannis* and *V. eriocarpa*. The three species are from three separate subgenera of *Vicia* and therefore taxonomically more diverged than species included in the same section. It was significant therefore to find out whether species included in the same subgenus (*Vicia*) have repetitive base sequences with greater homology to the *melanops* satellite DNA. The H^3 labelled c-RNA was therefore hybridised to the chromosomal DNA of *V. hybrida* (Fig. 6). The figure shows that the satellite DNA is located in all chromosomes of *V. hybrida*. The silver grains are located predominantly at the telomeric and centromeric regions of chromosomes. All hybridisation experiments were done simultaneously under indentical experimental conditions. The average number of silver grains estimated for the chromosome complement of *V. hybrida* was approximately 16% of the average value estimated for *V. melanops*. This variation could result from the differences in the degree of amplification of the basic repeat unit of the satellite DNA in the two species or from the divergence in DNA base sequences during the evolution of the genus.

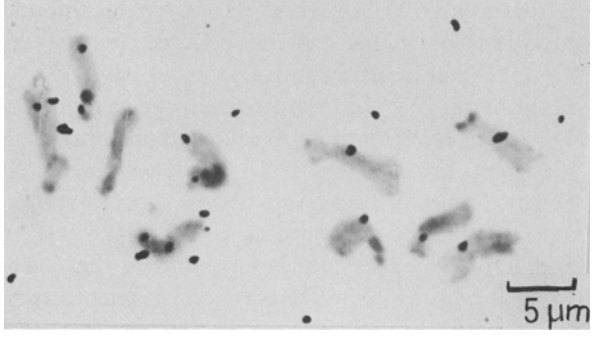

Fig. 6. In situ hybridisation of satellite DNA to the chromosomes of *V. hybrida*.

Summary and conclusions

(1) 5.1% of the total DNA in *V. melanops* makes up a light satellite DNA fraction in neutral CsCl density gradients. The average G + C content estimated for this fraction is 28% which is 7% lower than that of total DNA.

(2) The satellite DNA is homogeneous in base composition and showed low base sequence complexity in Cot reassociation experiments. It made up approximately 62% of the highly repetitive DNA in *V. melanops*.

(3) When hybridised *in situ* to the chromosomal DNA the satellite DNA was located in all chromosomes within the complement of *V. melanops*. It was located predominantly at the centromeric and intercalary regions. The satellite DNA sequences were also located in the chromosome complement of *V. hybrida* which is closely related to *V. melanops* and belongs to the same subgenus *Vicia*. The satellite DNA did not hybridise to the chromosomal DNA of two other *Vicia* species; *V. johannis* (section *Faba*) and *V. eriocarpa* (section *Cracca*). Differential amplification of satellite DNA sequences in different species as well as base sequence divergence during the evolution of the genus are suggested to be responsible for the observed variations in the amounts of satellite DNA sequences in *Vicia* species.

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