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DNA Sequence organization In finger millet *(Eleusine coracana)**

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Abstract. Approximately 39 to 49% of the genome of finger millet consists of repetitive DNA sequences which intersperse with 18% of single copy DNA sequences of 1900 nucleotide pairs. Agarose gel filtration and electrophoresis experiments have yielded the sizes of interspersed repeated sequences as 4000-4200 nucleotide pairs and 150-200 nucleotide pairs. Approximately 20% of the repeated DNA sequences (4000-4200 nucleotide pairs) are involved in long range interspersion pattern, while 60% of the repeated DNA sequences (150-200 nucleotide pairs) are involved in short period interspersion pattern.

Based on the data available in literature and the results described here on DNA sequence organization in plants, it is proposed that plants with haploid DNA content of more than 2.5 pg exhibit mostly the short period interspersion pattern, while those with haploid DNA content of less than 2.5 pg show diverse patterns of genome organization.

Keywords. DNA sequence organization; repetitive DNA; millet species.

Introduction

DNA sequence organization refers to the arrangement of repeated and non-repeated DNA sequences in a genome. Such information is expected to throw light on several important questions such as gene regulation. To date, DNA sequence organization has been studied in a large number of animal and plant species (Nagl, 1978). Based on these data, it is now known that there is an interspersion of repeated and single copy DNA sequences and that there are two main patterns of DNA sequence interspersion viz., short period and long period. Most of the eukaryotic species studied so far have revealed the presence of short period interspersion pattern, whereas a few insects such as *Drosophila* exhibit the long range interspersion pattern (Manning *et al*., 1975; Crain *et al*., 1976). Plant genomes differ from those of animals in two important respects, firstly, they have a relatively greater proportion of repetitive DNA sequences; secondly, the lengths of the interspersed repeated sequences are highly variable in the different species.

We have recently described the genome characterization of three Gramineae species, namely, finger millet, pearl millet and rice, with rather low DNA content

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Abbreviations used: UV, ultra violet.

(Deshpande and Ranjekar, 1980). We have also carried out the·DNA sequence organization studies in these species to determine whether they differ from the other well characterized Gramineae species, namely, rye (Smith and Flavell, 1977) and wheat (Flavell and Smith, 1976) in the mode of arrangement of repeated and single copy DNA sequences. In this paper, we report the data on DNA sequence organization in finger millet (*Eleusine coracana* Gaertn).

Materials and methods

All the chemicals used throughout the work were of Analytical Reagent (AR) grade or Guaranteed Reagent (GR) grade obtained from BDH, Sarabhai Chemicals and E. Merck. Certain chemicals such as RNAse, PIPES, Tris were obtained from Sigma Chemical Corporation, USA. λDNA, S_1 nuclease, EcoRI and DNA molecular weight markers were secured from Boehringer Mannehiem, West Germany.

Isolation ofDNA

DNA of finger millet was isolated from shoots of eight day old plants as described earlier (Deshpande and Ranjekar, 1980).

DNA shearing and sizing

DNA fragments of an average length of 550 nucleotide pairs were obtained by sonication for three min using Biosonic III (Bronwill model, 250 W with 20 KHz) fitted with a half inch probe. Sonication with a ¼ inch probe for one min at 20 setting yielded DNA fragments of 1500 nucleotide pairs. Larger DNA fragments (4900 nucleotide pairs) were obtained by homogenizing the DNA solution in a Sorvall Omnimixer (Model No. 17106) for 6 min at 25,000 rpm. DNAs of fragment size in the range of 1000-3000 nucleotide pairs were obtained using a Virtis 60 homogenizer by varying the conditions of speed and time in the range of 29,500 to 58,000 rpm and 4 to 45 min respectively. The average size of the DNA fragments after shearing was determined by sedimentation through neutral sucrose gradients (5-25% in 0.15 M NaCl+0.015 M Trisodium citrate, pH 7.0). in a Beckman preparatory ultracentrifuge (McConkey, 1967; McEwen, 1967; Van der Schans, *et al*., 1969).

Kinetics of DNA reassociation

The reassociation kinetics of finger millet DNA of all sizes were studied essentially by the method of Britten, *et al*. (1974). The separation and estimation of denatured and reassociated DNA fractions were carried out as described earlier (Ranjekar and Murthy, 1973; Ranjekar *et al*., 1974).

Thermal denaturation

Thermal denaturation studies of native finger millet DNAs of all sizes and of the repetitive fractions were carried out in a Gilford Spectrophotometer 250 equipped with a thermoprogrammer (Model No. 2527), an analog multiplexer (Model No. 6046) and a reference compensator (Ranjekar *et al*., 1976). In these studies, the data were used without any correction.

Single strand collapse was determined by melting unreassociated unique finger millet DNA in 0.12 M sodium phosphate buffer, pH 6.8. This value was observed to be 1-1.5%.

Digestion with S_1 nuclease

Native DNA of fragment size 5700 nucleotide pairs was used for S_1 nuclease digestion according to the procedure detailed earlier (Seshadri and Ranjekar, 1980).

Size fractionation of S_1 nuclease resistant reassociated duplexes

The size distribution of S_1 resistant duplexes was determined by gel filtration on an Agarose A_{50} (100-200 mesh size, Biorad Laboratories) column (92×1.5 cm), previously calibrated with calf thymus DNA of known duplex lengths. Approximately 70-100 μg of S_1 nuclease-resistant DNA duplexes, separated by hydroxyapatite chromatography, were loaded on the column and the elution was carried out with 0.12 M sodium phosphate buffer, pH 6.8 (Sachs and Painter, 1972; Wimpee and Rawson, 1979).

The fractions from this column corresponding to the peaks on the ultraviolet (UV) monitor were then analyzed by Agarose gel electrophoresis for the determination of size. Electrophoresis was carried out as described by Thomas and Davis (1975) with some modifications. λDNA, digested with EcoRI was used as a molecular weight marker in these experiments.

Results

Reassociation kinetics of 550 nucleotide pair long finger millet DNA

Figure 1 represents the reassociation kinetics of 550 nucleotide pair long DNA from finger millet. The solid line is the experimental curve, while the dashed line through the data points reveals the computer fit for second order kinetics using a nonlinear least square analysis method. A standard optimization subroutine STEPIT (written by J. P. Chandler and distributed by Quantum Chemistry Programme Exchange, Indiana Universitity, Bloomington, Indiana, USA) was used for this analysis keeping all the parameters to free float. It clearly indicates the presence of two kinetic components with a root mean square value of 0.0261. The fast reassociating component represents 49% of the genome and is repetitive. The latter 42% of the genome reassociates slowly with a rate of 0.00194 mol^{-1} s^{-1}. A part of the genome 8.5% fails to reassociate at Cot 10,000 mol×s/1. The lower dashed curves in figure 1 represent the predicted reassociation kinetics of the individual components if they existed alone.

Reassociation kinetics of DNAs of different fragment lengths

The reassociation kinetics of DNAs from finger millet, of four different fragment lengths in the range of 550-5700 nucleotide pairs were studied (figure 2). As no labelled DNAs were used throughout the work, the reassociation experiments were carried out in the Cot range of 10^{-2} to2.5×10 mol.s.1^{-1}. By Cot 2.5×10 mol. s.1^{-1}, almost all repetitive DNA sequences and very few single copy sequences are assumed to reassociate (Deshpande and Ranjekar, 1980). The extent of hydroxy-

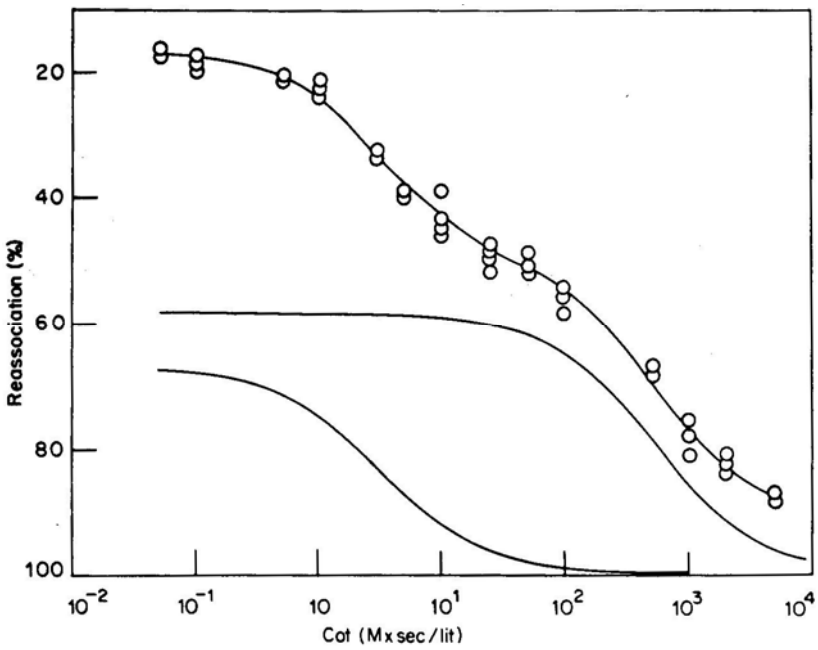


Figure 1. Reassociation kinetics of 550 nucleotide pair long finger millet DNA by hydroxylapatite fractionation.

The line through the data points represents the least square fit for two component system allowing all the parameters to free float (root-mean-square, 0.026). The lower dashed curves represent the reassociation kinetics of pure components. The DNA solutions (25-200 μg/ml) in 0.12 M sodium phosphate buffer (pH 6.8) were first denatured at 100°C and incubated at 62°C to obtain a desired Cot value. The unreassociated and reassociated DNA fractions were isolated at 0.12 M and 0.4 M sodium phosphate buffer (pH 6.8) respectively.

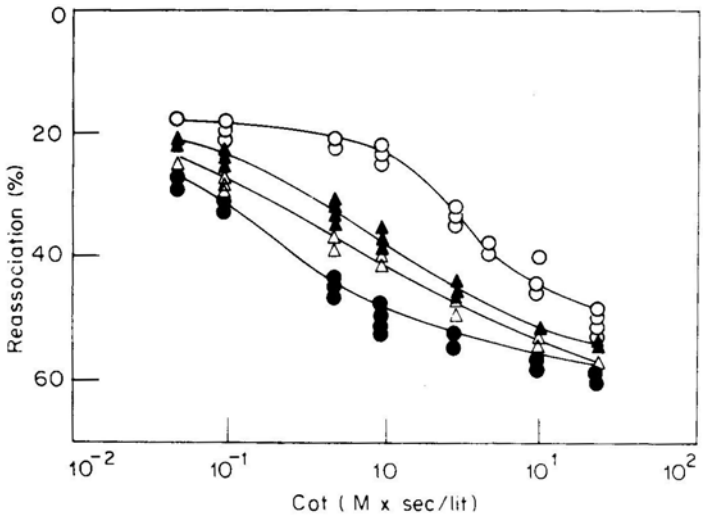


Figure 2. Hydroxyapatite reassociation kinetics of finger millet DNA at fragment lengths 550 nucleotide pairs (O), 1500 nucleotide pairs (—▲—), 4900 nucleotide pairs (Δ), and 5700 nucleotide pairs (●).

apatite binding increases from 18% to 28% and from 49% to 58% at Cot 0.5 and 25 mol. s.1^{-1}, respectively as the fragment length increases from 550 nucleotide pairs to 5700 nucleotide pairs. The increase in the binding of reassociated duplexes to hydroxyapatite can be either due to interspersion of repeated and single copy sequences or due to the effect of DNA fragment length alone. If there is no interspersion of different kinetic components, then the reassociation rate will solely be affected by the fragment length and the observed rate constants of repetitive DNA fractions will only vary as a function of the square root of the ratio of the fragment lengths of the two fractions being compared as represented by the following formula (Wetmur and Davidson, 1968):

$$K_1 / K_2 = (L_1/L_2)^{0.5}$$

where L_1 = shorter fragment size as number of nucleotide pairs

L_2 = longer fragment size as number of nucleotide pairs

K_1 = rate constant for reassociation of shorter (L_1) fragments

K_2 = rate constant for reassociation of longer (L_2) fragments

However, DNA fragments containing both single copy and repetitive sequences will have rate constants greater than those predicted as above. Table 1 gives the observed and the predicted rate constants for fragments of different lengths. The ratios, of observed rates to predicted rates vary in the range of 3.0 to 6.0 suggesting the presence of single copy DNA sequences in an unreassociated form contiguous to repetitive duplexes. Thus, it appears that about 58% of the total genome of finger millet (5700 nucleotide pairs) consists of interspersed sequences.

Table 1. Comparison of the experimental and the predicted rate constants (K) of the repeated DNA fractions of different fragment lengths.

Fragment length nucleotide pairs	Cot 1/2 observed[a] mol. S. l^{-1}	Observed K[b] l. $mol^{-1} S^{-1}$	Predicted[c] l. $mol^{-1} S^{-1}$	K observed / K. predicted
550	1.4×10^{0}	0.714	—	—
1500	2.0×10^{-1}	5.000	1.179	4.440
4900	1.25×10^{-1}	8.000	2.1319	3.7525
5700	7.5×10^{-2}	13.00	2.2994	5.784

[a] Calculated from the reassociation curves upto Cot 25 in figure 2.

[b] $$K = \frac{1}{\text{Cot 1/2 observed}}$$

[c] Calculated using $$\frac{K1}{K2} = \frac{L1^{0.5}}{L2}$$

where K1 and K2 are the rate constant for the reassociation of short (L l) and long (L2) fragments, respectively.

Hyperchromicity studies of reassociated repetitive DNA fragments

As observed in figure 3, the melting profiles of Cot 25 fractions of different fragment lengths of finger millet DNA are smooth. There is an increase in the

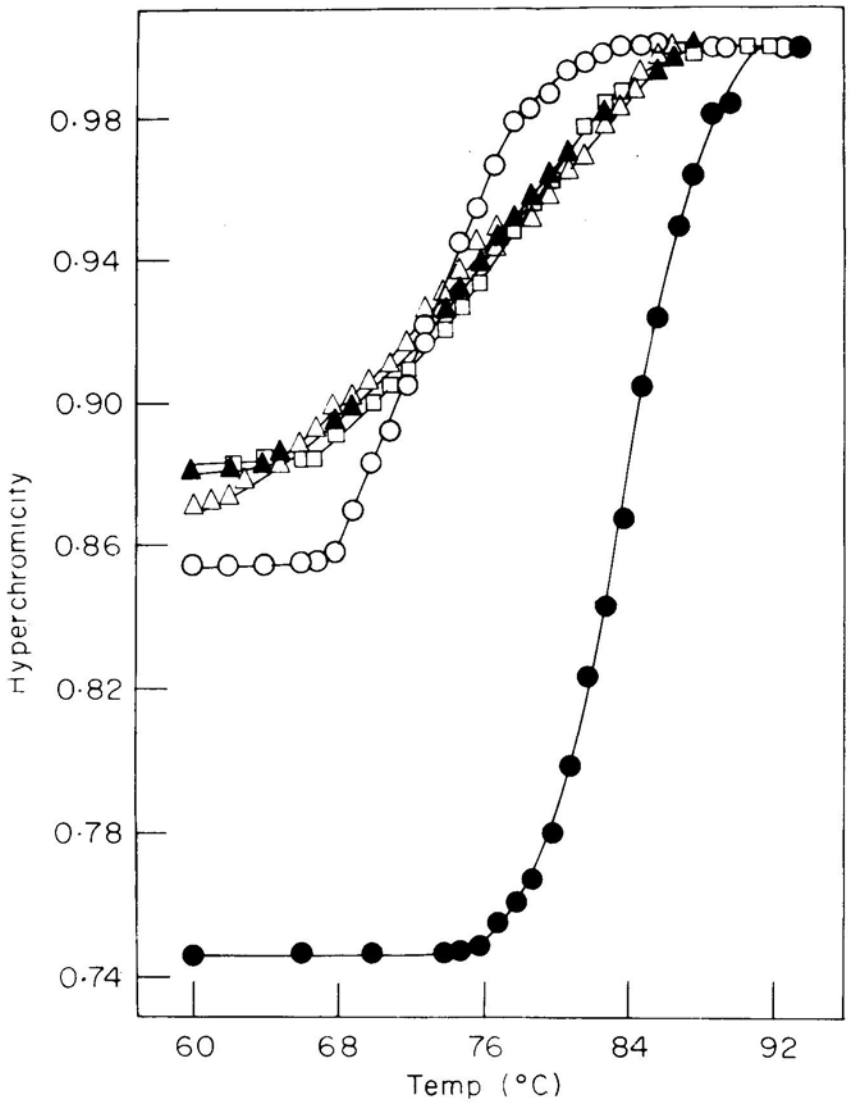


Figure 3. Thermal denaturation profiles of finger millet Cot 25 DNA of various fragment lengths, compared to those of native, 550 nucleotide pair long DNA.

The reassociated duplexes at Cot 25 were separated on hydroxyapatite. These fractions were dialyzed against 0.12 sodium phosphate buffer (pH 6.8) and were melted in a Gilford 250 spectrophotometer by increasing the temperature at a rate of 1°C/min. the graphs of relative absorbance (A260(Tl)/A260(98°C) *vs* temperature were plotted where A260 (Tl) is the absorbance at the corresponding temperature. (O) 550 nucleotide pairs; (Δ), 1500 nucleotide pairs; (▲), 4900 nucleotide pairs (□), 5700 nucleotide pairs; (●), native, 550 nucleotide pair long DNA. Each point in these graphs represents an average of atleast five experiments.

melting temperature of the reassociated duplexes (1.5°-4.0°C) while the hyperchromicity decreases with increasing fragment length (14% to 11.6%). Such decrease in the hyperchromicity of a repetitive DNA fraction with increase in the size of the DNA fragment is attributed to the increasing presence of single strand regions in the reassociated fragments. These single stranded regions are, most likely, unique sequences which remain unreassociated at Cot 25 and contribute very little (about 1-1.5%) to DNA hyperchromicity. These data, thus provide an additional evidence for the existence of repetitive and nonrepetitive DNA sequences on the same strand in finger millet genome.

Using hyperchromicity values, we calculate that about 50% of the 550 nucleotide pair long DNA is base-paired while only 39% of the 5700 nucleotide long fragments is base-paired. The later value is slightly lower as compared to the repetitive DNA content revealed from the reassociation curve of the 550 nucleotide pair long DNA (figure 1). The average size of the interspersed repetitive DNA sequences which reassociate within the 5700 nucleotide pair fragment is computed to be 2223 nucleotide pairs (table 2).

Table 2. Melting analysis of Cot 25 DNA isolated from DNAs of different fragment lengths.

DNA fragment size (nucleotide pairs)	550	1500	4900	5700	Native sonicated DNA (550 nucleotide pairs)
Fraction bound to HA at Cot 25 [a]	0.49	0.535	0.575	0.58	—
Hyperchromicity [b]	0.142	0.13	0.118	0.114	0.27
Tm °C [c]	73.0	75.0	76.5	77.0	—
Duplex content (D) [d]	0.498	0.459	0.403	0.390	—
Average duplex length [e]	273	676	1979	2223	—
Duplex content from S_1 nuclease [f]	—	—	—	0.41	—

[a] Obtained from figure 2.

[b] Obtained from figure 3. Hyperchromicity (H) was calculated using the formula

$$H = \frac{A260\ (98°C) - A260\ (60°C)}{A260\ (98°C)}$$

(Zimmerman and Goldberg, 1977; Wimpee and Rawson 1977)

[c] Obtained from figure 3.

[d] The average duplex content (D) of bound fragment was estimated using the formula:

$$D = \frac{H - \text{single strand collapse}}{H\ (\text{native sonicated DNA}) - \text{single strand collapse}}$$

The hyperchromicity of the denatured single copy DNA was determined in order to obtain a value for single strand collapse and was of the order of 1. 5% (Graham *et al*., 1974)

[e] The average length of duplex region is the product of the duplex content (D) and the fragment length of DNA.

[f] Duplex content of S_1 nuclease treated repetitive DNA fragment was obtained as:

$$\frac{\mu g \text{ of S1 nuclease resistant repetitive duplexes bound to hydroxyapatite column}}{\mu g \text{ of S1 nuclease treated total DNA loaded on hydroxyapatite column}}$$

S_1 nuclease studies of reassociated repetitive DNA fragments

An independent approach to determine the presence of single strand regions in the reassociated fractions is to-carry out S_1 nuclease experiments. The hyperchromicity of Cot 25 fraction isolated from 5700 nucleotide pair DNA is 11.6% while that of the S_1 nuclease resistant Cot 25 DNA is 21.5% (figure 4). This further indicates that the single strand regions in the DNA fraction are mostly digested by S_1 nuclease.

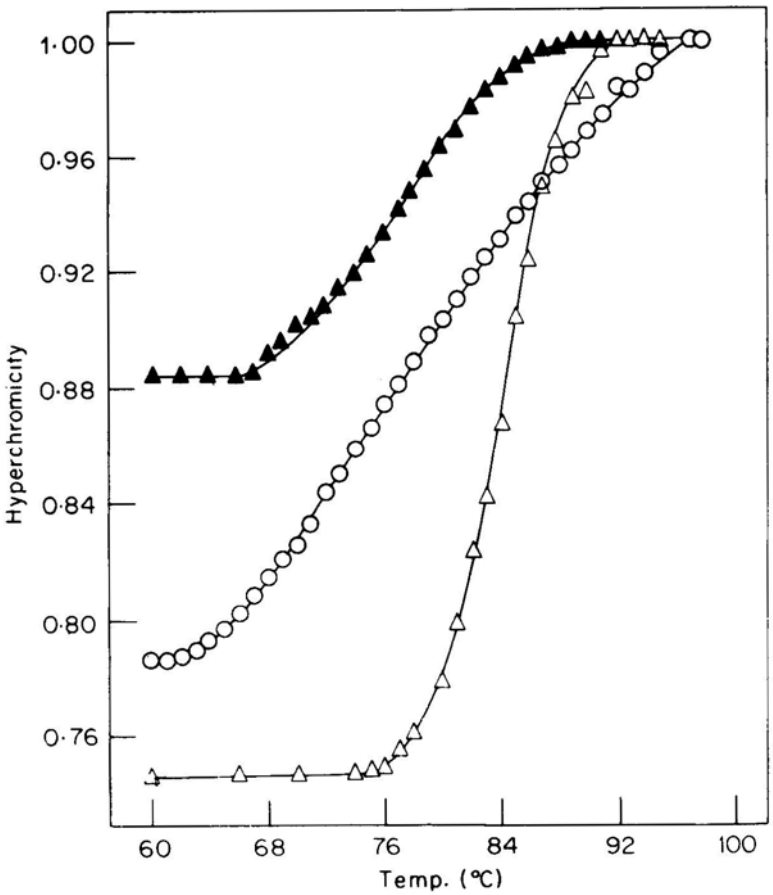


Figure 4. Thermal denaturation profiles of Cot 25 finger millet DNA before and after S_1 nuclease digestion along with native, 550 nucleotide pair long DNA.

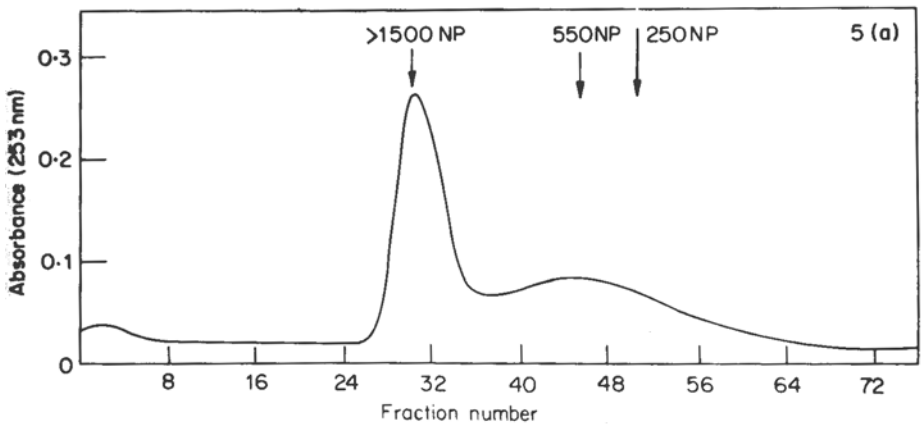
Native DNA of 5700 nucleotide pairs was dialysed against 0.18 M NaCl/0.006M PIPES buffer, pH 6.8. The DNA samples were denatured at 100°C for 10 min and then reassociated to specific Cot values. "After the required incubation, the DNA solution containing mixture of reassociated and unreassociated nucleotide fragments was incubated with S_1 nuclease for 1 h at 37°C to digest the single stranded fragments. The S_1 nuclease resistant duplexes were isolated by hydroxyapatite column chromatography and were dialyzed against 0.12 M sodium phosphate buffer (pH 6.8). The hyperchromicity curves were plotted as described in figure 3. Before S_1 nuclease treatment, (▲); after S_1 nuclease treatment, (O) and native 550 nucleotide pairs long DNA (Δ). In these experiments, each point represents an average of three different experiments using three different DNA preparations.

S_1 nuclease experiments can also be used to provide an independent estimate of the fraction of nucleotides which are in duplex form in the hydroxyapatite binding DNA fraction. Approximately 41% of 5700 nucleotide pair long DNA fraction, which is capable of binding to hydroxyapatite at Cot 25 is S_1 nuclease resistant and hence is in duplex form. This estimate compares well with the estimated value of 39% obtained from hydroxyapatite measurements. Since we assume that all the repetitive DNA sequences reassociate by Cot 25, the fraction of fragments which could bind to hydroxyapatite (5700 nucleotide pairs) and which are in duplex form, most probably represent the per cent of repetitive sequences. The proportion of single copy DNA will, therefore, be 59-61%. This value is in good agreement with that obtained in the experiment on reassociation kinetics of 550 nucleotide pair long DNA (figure 1).

Agarose gel column chromatography and Agarose gel electrophoresis of S_1 nuclease-resistant repetitive DNA

A direct estimate of the size is distribution of repetitive DNA sequence was obtained by analyzing S_1 nuclease resistant repetitive duplexes using Agarose column chromatography.

Since the exclusion limit of the Agarose A_{50} column is 1500 nucleotide pairs, all the DNA sequences having a length greater than 1500 nucleotide pairs are excluded from the column. The first peak in figure 5b indicates these sequences. By comparison with the column chromatographic profile of calf thymus DNA of known duplex lengths, the second fraction in figure 5b appears to consist of DNA sequences of length 150-200 nucleotide pairs. The proportion of the excluded DNA fraction (greater than 1500 nucleotide pairs) ranges from 18% to 23%; while that of the second fraction varies from 57% to 64%. The remaining 18% to 20% of the repetitive duplexes have a broad size distribution in the range of 500 to 1000 nucleotide pairs. The precise lengths of these fractions were then determined by Agarose gel electrophoresis (figure 6). From the mobility of the excluded fraction in gel electrophoresis, the molecular weight was estimated to be 4000-4200 nucleotide pairs. The low molecular weight traction of repetitive DNA shows a very high mobility corresponding to a length of less than 500 nucleotide pairs. Considering Agarose column chromatography and electrophoresis results together, the length of the low molecular weight DNA fraction of finger millet is determined to be 150-200 nucleotide pairs.



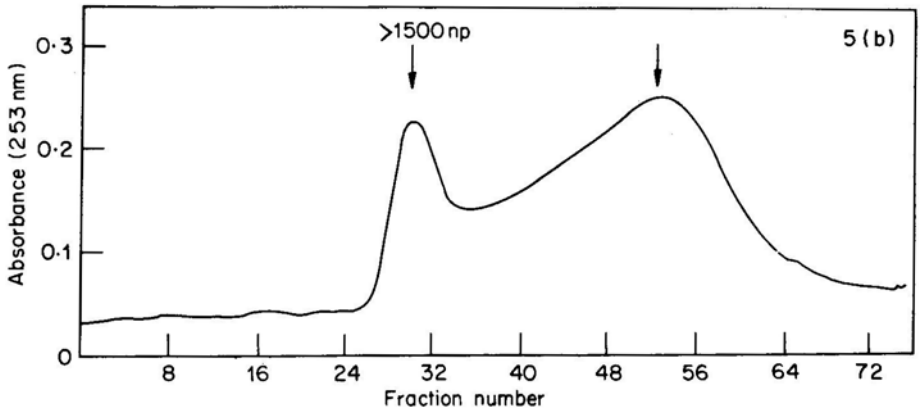
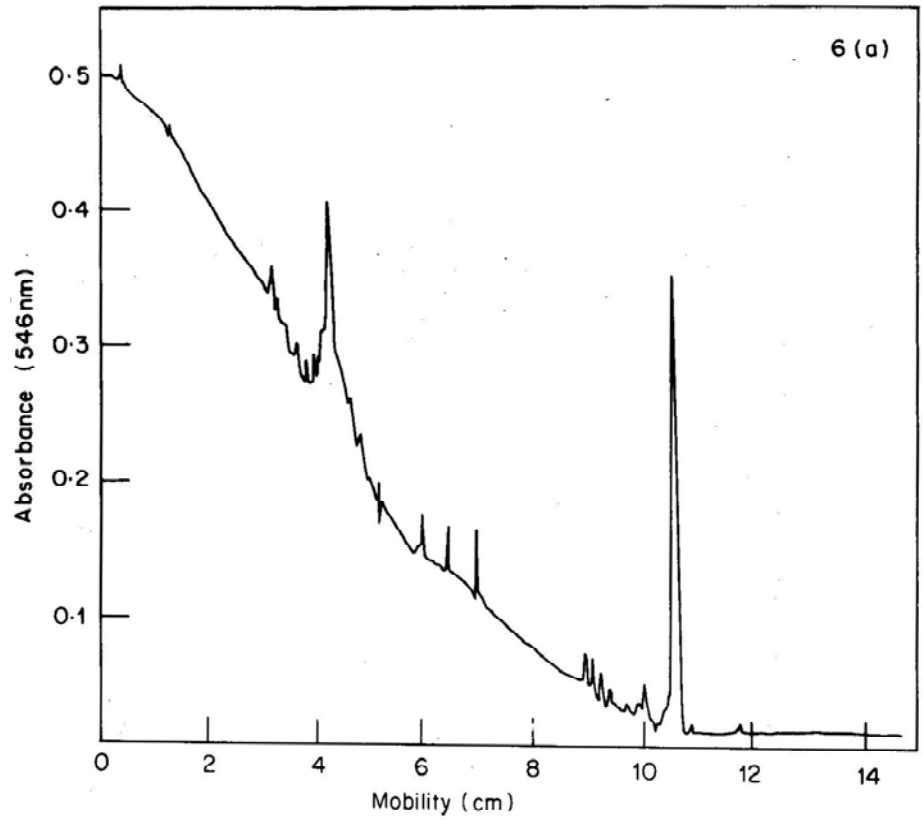


Figure 5. Agarose A50 column chromatographic profile of (a) calf thymus DNA of known duplex lengths (b) S1 nuclease resistant Cot 25 finger millet DNA.

The size distribution of S_1 resistant duplexes was determined by gel filtration on an Agarose A50 (Bio-Rad) column using 0.12 sodium phosphate buffer (pH 6.8) and the scans were obtained by monitoring the effluent with the help of an LKB Univord at 253 nm.



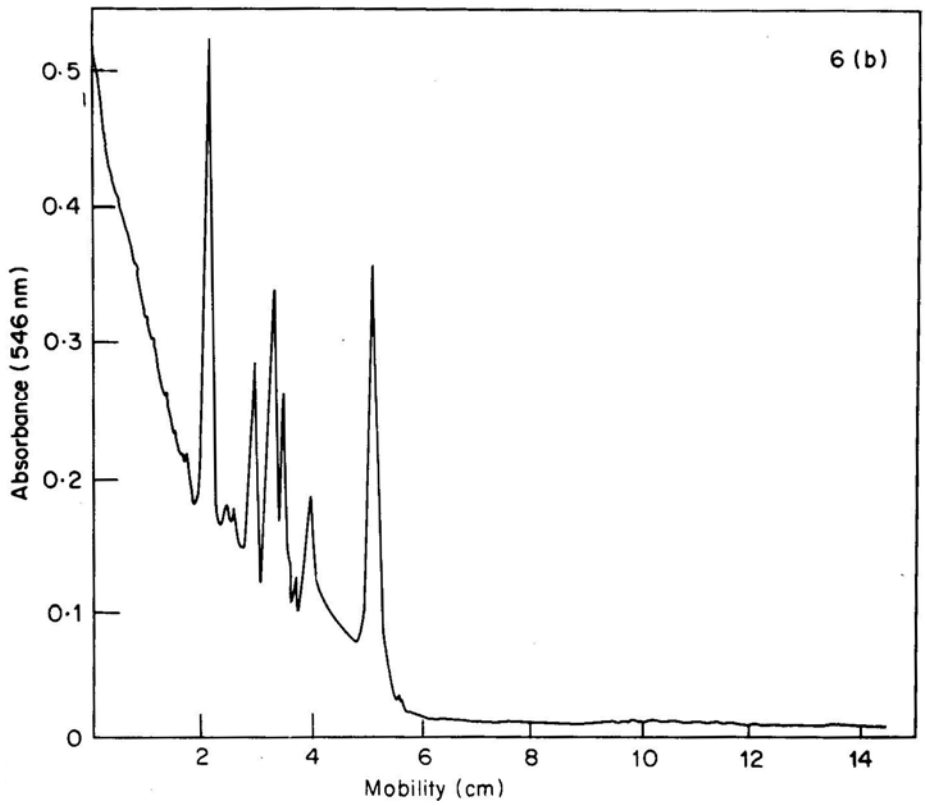


Figure 6. Agarose gel electrophoresis of S_1 resistant repetitive duplexes.

The fractions eluted from Agarose column were loaded on Agarose gels (0.6 cm×15 cm) and electrophoresis was carried out at 50 V for 5 h at room temperature. The profiles were obtained by scanning the gels at 546 nm in a Gilford 250 spectrophotometer equipped with a gel scanner, (a) Finger millet repetitive duplexes, (b) λ DNA digest with EcoRI as a DNA molecular weight marker. This electrophoretic scan compares very well with that of Thomas and Davis (1975) who have used ethidium bromide to stain the DNA bands and have taken the photographs in UV fluorescence.

Spacing of repetitive sequence elements

The length of the interspersed single copy sequences can be estimated from a curve relating the fraction of DNA fragments binding to hydroxyapatite and the fragment length . From figure 7, it is observed that two slopes are present and the change in the slope occurs at a fragment length of approximately 1800-2000 nucleotide pairs. Upto 1900 nucleotide pairs, there is a linear relationship between fragment length and per cent binding to hydroxyapatite and thereafter the increase in the binding is more gradual. Thus the length of the single copy DNA of finger millet DNA is in the range of 1800-2000 nucleotide pairs.

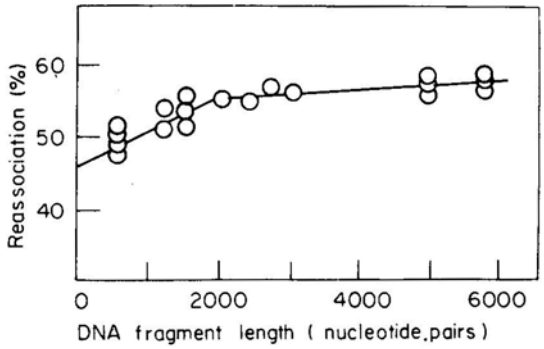


Figure 7. The percentage binding of finger millet DNA to hydroxyapatite at Cot 25 as a function of DNA fragment length. DNAs were sheared to various fragment lengths and adjusted to 0.12 M sodium phosphate buffer (p H 6.8). The percentage binding was calculated as described in figure 1.

An estimate of the percentage of repetitive DNA content can be obtained by extrapolating the curve of greater slope to a fragment length of zero. The intercept reveals the percentage of repetitive DNA as 46% which is in close agreement with the values obtained from reassociation kinetics, hyperchromicity and S_1 nuclease measurements (table 3).

Table 3. Duplex content (D) of repetitive DNA fractions of finger millet from various independent methods

Method	Duplex content
Hyperchromicity measurements (table 2)	0.39
S_1 nuclease resistance measurements (table 2)	0.41
Reassociation (%) vs fragment length (figure 7)	0.46
Reassociation curve of 550 nucleotide pairs (figure 1)	0.49

Discussion

We have employed the most commonly used approach for studying the DNA sequence organization for the analysis of the genome of finger millet. We have not been able to obtain labelled DNA of sufficiently high specific activity and hence have not used labelled tracer DNA. Vorobev and Kosjuk (1974) and Baldari and Amaldi (1976) have clearly shown that their results obtained from sea urchin and *Xenopus*, respectively, using unlabelled DNAs are in good agreement with the results in sea urchin (Graham *et al*., 1974) and *Xenopus* (Davidson *et al*., 1973) where labelled DNA was used. In plants, Wimpee and Rawson (1979) have used unlabelled DNA for studying DNA reassociation kinetics in pearl millet. Since only unlabelled DNA was used in our experiments, we have been unable to estimate the zero time binding fraction and to apply the corresponding correction to the reassociation data.

From our data, it is clear that 58% of the total genome of finger millet consists of interspersed repetitive and nonrepetitive DNA sequences. About 18% of the single copy DNA sequences (1900 nucleotide pairs) are interspersed with 20% of 4000-4200 nucleotide pairs, 60% of 150-200 nucleotide pairs and 20% of intermediate size repetitive DNA sequences.

The information of DNA sequence organization in finger millet provides an additional strong evidence that plant genomes exhibit a great diversity in the length of the interspersed repeated DNA sequences. In fact, finger millet is the first plant genome where the lengths of the interspersed repeated DNA sequences are as large as that seen in pearl millet (Wimpee and Rawson, 1979) and smaller than those exhibiting typical short period interspersion pattern as in the case of wheat (Flavell and Smith, 1976), rye (Smith and Flavell, 1977), Soybean (Goldberg, 1978 and Gurley *et al*., 1979), tobacco (Zimmerman and Goldberg 1977) and pea (Murray *et al*., 1978). Thus the DNA sequence interspersion pattern in finger millet is different from that in other plants studied so far.

From the available data on plant species, a relationship can be postulated between the DNA content and the DNA sequence organization pattern. Plant species with a haploid genome size of more than 2.5 pg exhibit mostly the short period interspersion pattern which is typical of animal genomes; examples are wheat (17.3 pg), rye (7.9 pg), tobacco (3.9 pg), soybean (6.5 pg per cell) and pea (4.9-5.2 pg). On the other hand, plant species with a haploid DNA content of less than 2.5 pg show a diverse nature of genome organization. This is evident in cotton (0.795 pg per cell, Walbot and Dure, 1976), pearl millet (2.5 pg, Wimpee and Rawson, 1979), mung bean (0.5 pg, Murray *et al*., 1979) and french bean (1.8 pg, Seshadri and Ranjekar, 1980). In these species, the length of the interspersed repeated sequences varies in the range of 1200-5000 nucleotide pairs. Finger millet falls under this category. In view of its wide occurrence, short period interspersion patten in eukaryotic genomes in assumed to be preferred to long period interspersion pattern during the course of evolution. Further, Britten and Davidson, (1969), and Davidson and Britten (1979) have proposed a model which is based on the significance of short period interspersion pattern in the coordinate regulation of transcription. However, considering the recent data on plant genomes, where interspersion patterns are not typically short period, it appears that the above model may have to be either altered or modified.

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