CHAPTER 3

GENOME TURNOVER IN GREAT MILLET AND RELATED MILLETS
SUMMARY

Based on optical reassociation studies of total nuclear DNAs at 55°C, 62°C, 69°C and 75°C, it is concluded that repeat families in great millet, little millet, barn yard millet and finger millet are heterogeneous while those of fox tail millet are homogeneous. In great millet, almost one third of the sequences that behave as single copy at standard conditions are actually fossil repeats. Such "fossil" repeats are not a prominent feature of the genomes of the other four millets. The ratios of sequence complexities of repeats isolated at 75°C to those isolated at 55°C are 2.2, 3.5, 81 and 0.3 in case of little millet, finger millet, fox tail millet and great millet respectively.

On the basis of the above three observations, it is suggested that among these millets, the rate of turnover of the genome of fox tail millet is the slowest while that of great millet is the fastest.
A substantial proportion of the nuclear genomes of eukaryotes is comprised of repeated DNA sequences. Since this feature of the genome has not been observed in prokaryotes, these repeated DNA sequences would appear important to the eukaryotic way of life. Dramatic differences in genome composition and organisation that have been observed in organisms of apparently similar biological and developmental complexity have been attributed to DNA sequences which serve no strongly sequence dependent functions [9]. These differences have been discussed by Thompson and Murray in an evolutionary context [22]. Figure 3.1 presents a model for genome evolution based on suggestions first made by Britten and Kohne [4] and later elaborated by Flavell et al. [7] and Thompson and Murray [22]. An important element of this model is the concept that single copy sequences are the end products of divergence through base substitutions of repeated sequences.

DNA reassociation kinetic experiments have provided valuable information about the structure and sequence organisation of eukaryotic genomes. Most of the reassociation experiments are performed at a temperature 25°C below the melting temperature of the native DNA, a criterion at which optimal reassociation rates are generally achieved [3,13,17,25]. Experiments conducted on a few plant DNAs have shown that the fraction of the genome consisting of repetitive elements is a function of the reassociation temperature [1,2,14]. When the reassociation temperature is reduced below that of the standard criterion to a more permissive criterion such as Tm - 35°C, more mismatched duplexes are allowed to
Fig. 3.1. MODE OF GENOME EVOLUTION

This figure presents a model for genome evolution based on suggestions first made by Britten and Kohne [4] and recently elaborated by Flavell et al. [7] and Thompson and Murray [22].
FIG 3.1

AMPLIFICATION EVENTS

OLD REPEATS
Imprecise copies
Short, interspersed

NEW REPEATS
Precise copies
Large clusters

MUTATION
TRANSLOCATION

SINGLE COPY

DELETION EVENTS
form and the fraction scored as repetitive increases. On the other hand, when the reassociation temperature is increased to about Tm - 5°C or Tm - 10°C, only well matched duplexes form and the fraction of the genome scored as repetitive decreases. It was thus pointed out by Kohne [10] that the terms repetitive and single copy are largely operational definitions.

Repetitive sequences are defined as being present, in more than a few copies, per haploid genome at the standard criterion (Tm - 25°C) while single copy sequences refer to those sequences which exhibit kinetics as if they were present in one copy per haploid genome at the standard criterion. Fossil repeats are sequences which exhibit single copy kinetics at standard criterion but show repetitive kinetics at permissive temperatures such as Tm - 35°C. True single copy sequences are defined as those sequences which exhibit single copy kinetics at standard as well as permissive criterion [16].

In another independent approach, Bendich and Anderson [2] have classified repeat families in a given genome into homogeneous or heterogeneous. A family is defined as a group of sequences similar enough to form stable duplexes under specified conditions of measurement. A homogeneous family contains member sequences of the same similarity. For example in one homogeneous family, the members are all related by 80% sequence homology, in a second by 70% homology and so on. A heterogeneous family contains member sequences of varying similarity ranging from nearly perfect replicas to sequences which are highly diverged. In homogeneous families, entire families are removed from the reassociation pool when the temperature of reassociation is raised. However, the copy number
of the remaining families remains unaltered. Since the rate of reassociation depends on concentration of interacting sequences, no change is observed in the rate of reassociation, with increase in temperature. In case of heterogeneous families, the highly diverged members of each family will no longer be able to form duplexes when the reassociation temperature is raised, thereby resulting in a decrease in the size of the family. This results in a reduction in the rate of reassociation at elevated temperatures.

Both the above approaches namely determination of percentage of true single copy as well as nature of repeat families in a genome are useful in identifying the relative rates of genome turnover. A comparative estimate of differences in the turnover rates of genomes of related species/genera generates useful data about evolution of genomes. We have used both the above strategies in the present work on five millets namely fox tail millet, little millet, barn yard millet, finger millet and great millet. The effect of temperatures, much above (75°C, stringent) and below (55°C, permissive) that of the standard temperature of 62°C, on the total nuclear DNA reassociation was determined. Such studies are useful in determining the nature of the repeat families in the genome. In addition, the kinetic behaviour of total repeats (i.e. repeats isolated at limit Cot values) isolated at more stringent criterion (75°C) was contrasted with repeats isolated at permissive criterion (55°C) by self reassociation of these repeats at standard criterion (62°C).
3.2. MATERIALS AND METHODS

3.2.1. Chemicals and seed material

Pipes buffer [Piperazine - N - N'-bis (2-ethane sulfonic acid), calf thymus DNA and purines and pyrimidines like adenine, guanine, cytosine, thymine and 5-methyl cytosine were obtained from Sigma Chemical Co., USA. S1 nuclease was procured from Bethesda Research Laboratories (BRL), USA, while methanol for HPLC was from Loba Chemie Industrial Co., Bombay. All other chemicals as well as the seed material were procured as described in Chapter 2.

3.2.2. Isolation of DNA and its shearing and sizing

DNAs were isolated from the shoots of great millet, little millet, barn yard millet, fox tail millet and finger millet by the procedure outlined in Chapter 2. Native high molecular weight DNAs dialyzed against 0.12 M sodium phosphate buffer (pH 6.8) were fragmented to a modal length of 550 bp using Sonic Oscillator (Bronwell Model, Biosonik III, 250 W, 20 KHz) fitted with a half inch probe (Green). Prior to sonication, nitrogen gas was bubbled through the DNA solution for 5-10 mins [24]. Approximately 30 ml of DNA solution was sonicated at a maximum intensity for 3 minutes, with 10 minutes interval between each successive one minute pulse of sonication. During sonication, the temperature of the solution was not allowed to rise above 4°C.

DNA fragments of about 5 kbp were obtained using a Vir Tis 60 K homogenizer at a speed of 45,000 rpm for 20 minutes at 4°C. The DNA
concentrations during sonication were kept constant at 400 µg/ml. The average fragment length of the DNA fragments was determined by agarose gel electrophoresis [12].

3.2.3. DNA reassociation Kinetics

Separation of two DNA strands by heat denaturation and reassociation of complementary strands at an appropriate temperature are two important steps in a typical reassociation experiment of DNA. A proper choice of incubation temperature (62°C, Tm - 25°C) and salt concentration (0.12M sodium phosphate buffer, pH 6.8; Na+ 0.18M) provides optimum conditions for DNA reassociation. The following techniques were used to measure DNA reassociation kinetics.

Optical reassociation

Since DNA strand separation produces a hyperchromic shift in absorbance which is reversed during reassociation, the latter may be followed by monitoring a decrease in absorbance (also known as hypochromic shift) as a function of time.

Gilford 250 spectrophotometer equipped with a thermoprogrammer (Model No.2527), analogue multiplexer (Model No.6046) and automatic reference compensator was used to monitor optical reassociation of millet DNAs. Thermal cuvettes were filled with approximately 0.3 ml of sonicated DNA solution (25-50 µg/ml) in 0.12M sodium phosphate buffer (pH 6.8) and temperature of the solution was raised to 100°C. The absorbance change occurring during the heating of DNA samples from 62° to 100°C was monitored at 260 nm and their hyperchromicity was calculated as described in Chapter 2.
The denatured DNA samples were maintained at 100°C for 4-5 min to ensure complete denaturation and then were quickly cooled to 62°C by circulation of cold water. The drop in temperature to 62°C was achieved in 60-90 seconds and the change in DNA absorbance was continuously recorded with time. For determination of zero time or start of reassociation, the mid point (80°C) between 100°C and 62°C was considered [6]. A Cot value at different time intervals is defined as half the product of absorbance of dissociated DNA x hours of incubation i.e. \( \text{Cot} = \frac{A \times t}{2} \) [4, 11]. Thus a Cot value of one is equivalent to incubation of DNA solution of concentration 83 µg/ml (approx. 2 O.D.) for one hour [19]. The percentage reassociation of DNA at different Cot values was calculated using the formula:

\[
\% R = \frac{A_{260} \text{ of denatured DNA} - A_{260} \text{ at a given time}}{A_{260} \text{ of denatured DNA} - A_{260} \text{ of native DNA at 62°C}} \times 100
\]

The optical reassociation of DNA was monitored in the Cot range of 1 x 10^{-3} to 1 x 10^{1} M.S. at four different incubation temperatures namely 55°C, 62°C, 69°C and 75°C. The zero time varied according to the incubation temperature and was the mid-point between 100°C and the incubation temperature.

Hydroxyapatite column chromatography

This is a convenient procedure to determine DNA reassociation kinetics in the Cot range of 10^{-2} to 10^{4}.

Hydroxyapatite was prepared essentially according to the procedure of Tiselius et al. [23] and was stored at 4°C with a few drops of chloroform. The hydroxyapatite preparation usually formed columns with good
flow characteristics. The extent of DNA recovery from each batch of hydroxyapatite was checked and was always more than 95%.

The hydroxyapatite slurry was poured into a double jacketed glass column (15 x 2.5 cms) to a bed volume of approximately 15 ml. The column was equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) and maintained at 62°C with a thermostatic circulating water bath.

The hydroxyapatite chromatography was done essentially according to Kohne and Britten [11] and Britten et al. [5]. Sonicated DNA samples ranging in concentration from 30 μg/ml for low Cot values to 300 μg/ml for higher Cot values in 0.12 M sodium phosphate buffer (pH 6.8) were sealed in boiling tubes and denatured by boiling for 10 minutes at 100°C. These were then incubated to specific Cot values at 62°C and then were loaded on hydroxyapatite columns pre-equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) at 62°C. Single stranded DNA was eluted with 0.12M sodium phosphate buffer (pH 6.8) and double stranded DNA was isolated with 0.4M sodium phosphate buffer (pH 6.8). The relative amounts of single and double stranded DNA were determined by measuring absorbance of DNA fractions at 260 nm on a Shimadzu double beam spectrophotometer (Model US -210 A) assuming that one absorbance unit (A_{260}) is equal to 50 μg of DNA/ml. The extent of DNA reassociation at a specific Cot value was calculated using the formula:

\[
% R = \frac{\text{μg of DNA eluted with 0.4M phosphate buffer (pH 6.8)}}{\text{Total μg of DNA eluted from the HAP column}} \times 100
\]

As internal controls, reassociation kinetics of E.coli DNA, mouse liver DNA and wheat DNA were also studied under identical conditions.
3.2.4. Nonlinear least squares regression analysis of DNA reassociation kinetic data

An eukaryotic DNA normally reassociates over a range of Cot values spanning up to eight orders of magnitude or even more. Since the individual components each reassociating with second order kinetics are usually not well separated, a computer fitted programme is used to resolve the curves into two or more ideal second order components such that their sum fits the experimental data. Each individual curve represents a single kinetic component and is fitted to the second order equation by least square analysis of the data. The identified DNA components may not exist as discrete entities in the genome but are merely simplified mathematical descriptions of the data. The programme is usually run on the basis of finding the minimum number of curves required to fit the data [18].

Since DNA reassociation follows second order kinetics, it can be defined using the equation $\frac{dC}{dt} = -KC^2$ where $C$ is the concentration of single stranded sequences and 't' is the incubation time [5]. This yields the equation $C/Co = 1/1 + K \cdot Cot$ where $Co$ is the initial concentration of single stranded DNA in moles of nucleotides per liter e.g. $C = Co$ when $t = 0$. Least squares analysis of the experimental data was carried out by employing a standard optimization subroutine STEPIT (written by J.P. Chandler and distributed by QCPE, Indiana University, Bloomington, Indiana, USA). The computer fitted curves are drawn as solid lines through the data points. The RMS error is given by the equation:

$$\text{Error} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} \left( \frac{C_{\text{expt}}}{Co} - \frac{C_{\text{fit}}}{Co} \right)^2}$$

and is minimized by a direct search procedure. The form for $\frac{C}{Co}$ is
assumed to be
\[
\frac{C}{Co} = T + \frac{Fi}{1 + Ki \cdot Cot} \quad \text{... 2}
\]
where \(\frac{C}{Co}\) is the fraction denatured,

\(T\) the fraction of the genome that fails to reassociate.

\(Fi\) the fraction of genome reassociating in the \(i^{th}\) component.

\(Ki\) the reassociation rate for the \(i^{th}\) component. The parameters \(T, Fi,\) and \(Ki\) are all allowed to free float to minimize the error in equation 1.

The computer used for such curve fitting procedures was ICL 1900 series.

3.2.5. Isolation of SI nuclease resistant repetitive DNA duplexes

SI nuclease specifically digests single strand regions in reassociated DNA duplexes without digesting the duplex regions and hence is used to isolate repeat duplexes. These experiments were carried out according to the procedure of Murray et al. [15] with some modifications. Native, high molecular weight DNAs (>20 kbp) in 0.18 M NaCl containing 0.006M PIPES buffer, \(\text{pH} 6.8\) were reassociated unto a limit Cot value of each millet DNA either at 55°C or 75°C. Following reassociation, DNA samples were chilled on ice and adjusted to 25 mM sodium acetate (\(\text{pH} 4.5\)), 0.1 mM \(ZnSO_4\) and 25 mM 2-mercaptoethanol and SI nuclease digestion (0.1U/\(\mu\)g of DNA) was carried out at 37°C for 30 minutes. The reaction was terminated by chilling the solution and the DNA samples were deproteinised with chloroform - isoamylalcohol mixture, as described previously.
The SI nuclease resistant DNA was then precipitated and dissolved in 10 mM Tris-HCl (pH 7.4).

3.2.6. Thermal denaturation

The 1°C/min melting of SI nuclease resistant DNA duplexes was performed as described in Chapter 2.

3.2.7. High performance liquid chromatography (HPLC)

HPLC was used mainly to detect 5-methyl cytosine and other odd bases in the SI-nuclease resistant millet DNAs. DNAs were hydrolyzed with 60-70% perchloric acid (1 hr at 100°C) and the hydrolysates were neutralised with 10 N KOH. The resultant sparingly soluble potassium perchlorate precipitate was centrifuged out. Fresh DNA hydrolysate was used each time for determination of base composition by HPLC. An aliquot of the neutralised hydrolysate was applied to the reverse phase RP-18 column (10 μm, HP No.79916 B, 250 x 4.6 mm with a 2 μm frit pore) which was pre-equilibrated with 0.4 M ammonium phosphate buffer, pH 4.3, containing 5% v/v methanol. The chromatography was carried out in a Hewlett-Packard HPLC system (model No.1082B) interfaced with a Hewlett-Packard LC terminal (No. 79850B) and equipped with an automatic sample injection system. The UV detector was fixed at 254 nm. The chromatographic profiles were obtained at a chart speed of 1 cm/min and a flow rate of 0.5 ml/min. Peaks eluted from the column were identified by their retention time. For comparison, standard bases were run separately under identical conditions. The amount of 5-methyl cytosine was determined from the peak height and peak area of an authentic sample. At least three different DNA preparations were hydrolyzed and each hydrolysate was analysed in duplicate.
3.3. RESULTS

3.3.1. Optical reassociation studies of total nuclear DNAs at 55°C, 62°C, 69°C and 75°C

To determine the nature of repeat families, reassociations of 550 bp long DNAs were studied optically up to a Cot value of 10 M.S. at four different temperatures namely 55°C, 62°C, 69°C and 75°C (Fig. 3.2A and B). All the curves were evaluated using a computer aided non-linear regression method (Table 3.1). Though in a few cases, the reassociation curves could be modelled into two components, all the data were fitted as a single component to facilitate a better comparison of their rate constants. The Cot 1/2 of single copy DNA in all the reassociation experiments was considered to be the same as that at 62°C.

From Fig. 3.2, it is seen that all the millet DNAs contain rapidly reassociating DNA sequences which is typical of higher eukaryotes. However for determining the nature of repeat families, only those repetitive fractions which followed second order kinetics were analysed. The fraction recorded as repetitive is observed to decrease as the conditions of reassociation are made more stringent. This decrease is, for example, 4.3 fold in little millet, 2 fold in great millet and barn yard millet, 2.3 fold in fox tail millet and 2.6 fold in finger millet over the temperature range of 55°C to 75°C.

Apart from changes in the proportions of repetitive DNAs as a function of incubation temperature, differences are also observed in their kinetic complexity, copy number and rate of reassociation (Table 3.1). The kinetic complexity, for example, increases with an increase in incubation temperature.
in all cases except in fox tail millet. This increase is 45 fold in little millet, 7.4 fold in barn yard millet and only 1.1 fold in great millet and finger millet. The copy number on the other hand decreases 198 fold in little millet, 14 fold in barn yard millet, 2.2 fold in great millet and 2.4 fold in finger millet. Finally, the reassociation rates decrease with increase in the stringency of reassociation.

Thus in all the four millet DNAs, the fraction scored as repetitive decreases as the temperature of incubation is raised. This observation is consistent with the presence of either the homogeneous or heterogeneous model of repeat families proposed by Bendich and Anderson [2]. According to these authors, young and old families of repeats have the same copy number and hence an increase in the observed Cot 1/2 values would be expected if the repeat families in the genome were predominantly of the heterogeneous type and no significant change would be observed in the Cot 1/2 values if the genome contains predominantly homogeneous repeat families. In case of great millet, little millet, barn yard millet and finger millet, an increase in the Cot 1/2 values is observed suggesting that the repeat sequences in these plant genomes are predominantly heterogeneous in nature.

In case of fox tail millet, no definite trend is observed in the Cot 1/2 values over the temperature range of 55°C to 75°C. In order to assess whether the repeats in this genome belonged to homogeneous or heterogeneous type, total repeats were isolated at 55°C and 75°C and their kinetics were monitored at 62°C. It can be seen from Fig.3.3D and Table 3.3 that the 55°C fraction can be resolved into two distinct
FIG. 3.2(A): OPTICAL REASSOCIATION OF THE SONICATED

NUCLEAR DNAs (0.55 kbp) OF MILLETS. A- GREAT MILLET;
B- BARN YARD MILLET; C- LITTLE MILLET

DNA reassociation experiments were carried out as
detailed in the text. Each curve consisted of
500-1000 experimental points. The solid lines represent
the best least squares solutions allowing all parameters
to free float.
FIG 3.2 A

% REASSOCIATION

Cot (·MOL x SEC / LIT)
FIG. 3.2 (B): OPTICAL REASSOCIATION OF THE SONICATED NUCLEAR DNAs (0.55 kbp) OF MILLETS. D- FINGER MILLET; E- FOX TAIL MILLET

DNA reassociation experiments were carried out as detailed in the text. Each curve consisted of 500-1000 experimental points. The solid lines represent the best least squares solutions allowing all parameters to free float.
TABLE 3.1: KINETIC ANALYSIS OF 0.55 kbp LONG DNAs AT DIFFERENT CRITERIA OF TEMPERATURE STRINGENCY

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<th>Plant species</th>
<th>Fraction</th>
<th>K observed</th>
<th>Cot 1/2 observed</th>
<th>Cot 1/2 pure</th>
<th>K pure</th>
<th>Frequency of repetition per 1C genome</th>
<th>Kinetic complexity (nucleotide pairs)</th>
</tr>
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<td>Finger millet</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>55°C</td>
<td>0.24</td>
<td>4.34 x 10^9</td>
<td>2.2 x 10^-1</td>
<td>4.35 x 10^-2</td>
<td>2.29 x 10^1</td>
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<td>5.5 x 10^-1</td>
<td>4.9 x 10^-2</td>
<td>2.04 x 10^1</td>
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<td>3.41 × 10⁷</td>
<td>2.93 × 10⁰</td>
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<td>0.17</td>
<td>1.47 × 10⁰</td>
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<td>1.0 × 10⁰</td>
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<td>1.43 × 10¹</td>
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<td>5.94 × 10⁴</td>
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</table>

a,b = Values obtained from the optical reassociation curves in Fig. 3.2A and 3.2B.

c = Cot 1/2 observed = (k observed)⁻¹

d = Cot 1/2 pure = Cot 1/2 observed × fraction of the genome i.e. c × a

e = K pure = (Cot 1/2 pure)⁻¹

f = Cot 1/2 of unique DNA divided by Cot 1/2 values of the given fraction.

g = Values obtained from the standard relationship for E.coli DNA where Cot 1/2 is 5.3 (under our experimental conditions) and genome size is 4.5 × 10⁶ nucleotide pairs.
kinetic components having a Cot 1/2 of $2.35 \times 10^{-3}$ and $5.21 \times 10^{-2}$ respectively while the 75°C fraction is resolved into a single component with a Cot 1/2 of $5.32 \times 10^{-2}$. This indicates that the 75°C component entirely represents the second component of the 55°C fraction. Since the kinetics of this second component is unaffected when it is reassociated along with the first component (as in the 55°C repeat fraction), it can be suggested that these two repeat components are not interspersed with each other and also that the repeats in the genome of fox tail millet are largely homogeneous.

3.3.2. True repeats and fossil repeats

The kinetic data obtained by reassociating the DNAs at standard (62°C) and permissive (55°C) criteria were used to determine the percentage of true single copy and fossil repeats in the millet genomes, by applying the definitions of Murray et al. [16]. From the data in Table 3.2, it is seen that finger millet, fox tail millet and barnyard millet have the maximum percent of true single copy DNA of 98.48%, 96.42% and 95.43% respectively and this value decreases to 87.44% in little millet and 63.07% in great millet. Thus as compared to the other four millets, a greater amount of single copy DNA in great millet behaves as 'fossil' repeats at lower temperatures. A smaller fraction of fossil repeats and a larger fraction of true single copy indicates a relatively low rate of turnover of the genome while a larger fraction of fossil repeats and a lower fraction of true single copy indicates a relatively higher rate of turnover. Our data thus suggests that the genome of great millet is turning over at a rate faster than that of the other four millets.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Genome size(^a) in nucleotide pairs</th>
<th>% single(^b) copy at permissive criterion</th>
<th>% single(^c) copy at standard criterion</th>
<th>% fossil(^d) repeats</th>
<th>% true(^e) single copy</th>
<th>kinetic complexity(^f) of true single copy in nucleotide pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great millet</td>
<td>3.05 x 10(^8)</td>
<td>0.32</td>
<td>0.50</td>
<td>36.93</td>
<td>63.07</td>
<td>1.92 x 10(^8)</td>
</tr>
<tr>
<td>Barn yard millet</td>
<td>6.81 x 10(^8)</td>
<td>0.74</td>
<td>0.77</td>
<td>4.57</td>
<td>95.43</td>
<td>6.49 x 10(^8)</td>
</tr>
<tr>
<td>Little millet</td>
<td>7.99 x 10(^7)</td>
<td>0.52</td>
<td>0.60</td>
<td>12.56</td>
<td>87.44</td>
<td>6.98 x 10(^7)</td>
</tr>
<tr>
<td>Finger millet</td>
<td>1.6 x 10(^8)</td>
<td>0.65</td>
<td>0.66</td>
<td>0.01</td>
<td>98.48</td>
<td>1.57 x 10(^10)</td>
</tr>
<tr>
<td>Fox tail millet</td>
<td>3.82 x 10(^7)</td>
<td>0.67</td>
<td>0.70</td>
<td>3.57</td>
<td>96.42</td>
<td>3.68 x 10(^9)</td>
</tr>
</tbody>
</table>

\(a\) : Values obtained from the Cot 1/2 values of single copy and using the relationship for E. coli DNA where Cot 1/2 is 5.3 (under our experimental conditions) and the number of nucleotide pairs is 4.5 x 10\(^6\).

\(b, c\) : Values obtained from Table 3.1.

\(d\) % : Calculated as \(\frac{G-b}{C} \times 100\)

\(e\) % : calculated as \(\frac{b}{C} \times 100\)

\(f\) % : Calculated as genome size x fraction of true single copy i.e. \(\frac{a \times e}{100}\)
3.3.3. Self-reassociation at 62°C of the total repeat fractions isolated at permissive (55°C) and stringent (75°C) temperatures

The ratios of kinetic complexities of repeats formed recently in evolution to those that are relatively older also would give an idea about the relative rate of turnover of related genomes. In order to determine these ratios, the total repetitive (i.e. S1 nuclease resistant) DNA fractions were isolated in all the four millets at 55°C and 75°C and their optical reassociation was studied up to Cot 1.0 at 62°C to assess their kinetic heterogeneity (Fig. 3.3 and Table 3.3). The 75°C isolated repeat fraction is 2.2, 3.5 and 8.1 times more complex than the repeat fractions isolated at 55°C in case of little millet, finger millet and fox tail millet respectively. In great millet, the 75°C isolated repeat fraction is 0.3 fold complex than the corresponding 55°C repeat fraction.

Repeat sequences produced recently by saltatory replication would be present as blocks of repetitive DNA and would show less sequence divergence. However over the course of evolution, these sequences would tend to become interspersed and also would accumulate mutations and become increasingly diverged with time. The rate at which these processes occur would determine the turn over rate of the genome. Repeat sequences having functional significance would remain conserved. Thus, the sequences reassociating at 75°C would include some such conserved sequences as well as recently formed sequences. In a genome exhibiting a high turn over rate, the ratio of sequence complexities of the repeats isolated at 75°C to those isolated at 55°C would be less than 1 as seen in great millet, while higher ratios would be expected for a genome which is turning
FIG. 3.3: MINI COT CURVES OF THE TOTAL REPEATS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) TEMPERATURES, MONITORED AT 62°C.

Enriched total repeat fractions, isolated by S₁ nuclease treatment, were melted and their optical reassociation was followed in 0.12M sodium phosphate buffer, pH 6.8 up to a Cot value of 1.0 M.s. at 62°C. Each curve consists of 500-1000 experimental points. The solid lines represent the best least squares fit, allowing all parameters to free float.

$S^-_{55}$ and $S^-_{75}$: Total repeats isolated by S₁ treatment after performing the reassociations at 55°C and 75°C respectively.

A - Great millet  
B - Little millet  
C - Finger millet  
D - Fox tail millet
FIG 3.3
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Fraction $^a$</th>
<th>$k$ observed $^b$</th>
<th>Cot 1/2 $^c$ observed</th>
<th>Cot 1/2 $^d$ pure $(S_1-55)$</th>
<th>Cot 1/2 pure $(S_1-75)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great millet Cot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>0.35</td>
<td>1.16</td>
<td>$8.54 \times 10^{-1}$</td>
<td>$2.99 \times 10^{-1}$</td>
<td>0.29</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>0.31</td>
<td>3.45</td>
<td>$2.89 \times 10^{-1}$</td>
<td>$8.97 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>Little millet Cot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>0.22</td>
<td>7.59</td>
<td>$1.31 \times 10^{-1}$</td>
<td>$2.89 \times 10^{-2}$</td>
<td>2.23</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>0.41</td>
<td>6.32</td>
<td>$1.58 \times 10^{-1}$</td>
<td>$6.48 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>Finger millet ( Cot 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>0.26</td>
<td>42.74</td>
<td>$2.33 \times 10^{-2}$</td>
<td>$6.1 \times 10^{-3}$</td>
<td>3.49</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>0.29</td>
<td>13.61</td>
<td>$7.34 \times 10^{-2}$</td>
<td>$2.13 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>Fox tail millet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cot 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$ component 1</td>
<td>0.06</td>
<td>423.86</td>
<td>$2.35 \times 10^{-3}$</td>
<td>$1.34 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$ component 2</td>
<td>0.21</td>
<td>19.17</td>
<td>$5.21 \times 10^{-2}$</td>
<td>$1.09 \times 10^{-2}$</td>
<td>81.49</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>0.21</td>
<td>18.79</td>
<td>$5.32 \times 10^{-2}$</td>
<td>$1.11 \times 10^{-2}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$, $^b$ : Values obtained from Fig. 3.3.  
$^c$ : Cot 1/2 observed = $(k$ observed)$^{-1}$ 
$^d$ : Cot 1/2 pure = Cot 1/2 observed $\times$ fraction of the genome i.e. $c \times a$

$S_1$-55 and $S_1$-75: Total repeated DNA isolated by $S_1$ treatment after performing the reassociations at $55^\circ C$ and $75^\circ C$ respectively.
over at a slower rate as observed in the other three millets. Our data thus suggest that the genome of great millet is turning over at a faster rate than that of the other three millets.

3.3.4. Thermal stabilities of total repeats isolated at stringent and permissive temperatures

To determine whether the sequences reassociating at 75°C were GC rich, the total repeat fractions of these four millets DNAs isolated at 55°C and 75°C were denatured at a heat rate of 1°C/min. The data are depicted in Fig. 3.4 and summarised in Table 3.4. It is seen that the melting profiles of great millet and finger millet repeat fractions are monophasic while those of little millet and foxtail millet are biphasic. The high melting component in little millet and foxtail millet increases almost 1.6 fold in the 75°C isolated fraction. The 75°C isolated fraction of finger millet has a Tm of 87°C while the corresponding 55°C isolated fraction has a Tm of 76.6°C. Thus if the repetitive sequences isolated at 75°C are capable of reassociation at high temperature due to a high G + C content, their reassociated Tm would be expected to be similar to or greater than that of the native DNA. From our data, it is very clear that sequences that reassociate at 75°C in case of little millet, foxtail millet and finger millet are highly thermostable due to the abundance of G+C rich sequences. In case of great millet, the Tm of the 75°C isolated repeats is less than that of the native DNA and is also not significantly different from the Tm of the 55°C isolated fraction. Moreover no high melting component is evident from its melting curves. It is thus likely that in case of great millet, some phenome-
FIG. 3.4: THERMAL DENATURATION PROFILES OF THE TOTAL REPEATS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) TEMPERATURES.

A - Great millet ($S_1$ -55°C)
B - Great millet ($S_1$ -75°C)
C - Little millet ($S_1$ -55°C)
D - Little millet ($S_1$ -75°C)
E - Finger millet ($S_1$ -55°C)
F - Finger millet ($S_1$ -75°C)
G - Fox tail millet ($S_1$ -55°C)
H - Fox tail millet ($S_1$ -75°C)

$S_1$-55°C and $S_1$-75°C represent total repeat DNA fractions isolated by $S_1$ nuclease treatment after performing the reassociation at 55°C and 75°C respectively.

The melting of DNA was carried out in 0.12 M sodium phosphate buffer, pH 6.8 at a heat rate of 1°C/min.
FIG 3.4

TEMPERATURE (°C)

% HYPERCHROMICITY

A

B

C

D

E

F

G

H
### TABLE 3.4: MELTING ANALYSIS OF THE TOTAL REPEAT FRACTIONS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) CRITERIA OF TEMPERATURES

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Nature of the melting curve</th>
<th>Low melting component</th>
<th></th>
<th>High melting component</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proportion (%), Tm (°C)</td>
<td>Proportion (%), Tm (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Little millet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-55) Biphasic</td>
<td>72.5 ± 1.0, 78.8 ± 1.3</td>
<td>27.5 ± 1.2, 96.8 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-75) Biphasic</td>
<td>55.0 ± 0.8, 78.4 ± 0.7</td>
<td>45.0 ± 0.9, 95.8 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fox tail millet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-55) Biphasic</td>
<td>79.0 ± 0.7, 78.4 ± 0.9</td>
<td>21.0 ± 0.9, 98.2 ± 1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-75) Biphasic</td>
<td>63.0 ± 1.2, 84.2 ± 1.1</td>
<td>37.0 ± 0.8, 96.6 ± 1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great millet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-55) Monophasic</td>
<td>81.5 ± 0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-75) Monophasic</td>
<td>82.2 ± 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finger millet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-55) Monophasic</td>
<td>76.6 ± 1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S₁-75) Monophasic</td>
<td>87.0 ± 1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values in the table are an average of the melting data of six DNA samples melted in duplicate.
non other than that of G+C content is responsible for its ability to reassociate at the elevated temperature.

Since 5 methyl cytosine is known to increase the Tm of DNA, we were interested in determining the 5 methyl cytosine content of the repeat fractions isolated at 55°C and 75°C (Fig. 3.5A and B and Table 3.5). In case of little millet and fox tail millet, the 5 methyl cytosine content of the 75°C isolated fraction is higher than that of the fraction isolated at 55°C. This odd base may partly contribute to the high Tms of the 75°C isolated fraction in these two plant DNAs and may also allow these sequences to reassociate at higher temperatures. In case of the other two millets, no significant difference is observed in the 5 methyl cytosine content of the repeat fractions isolated at the two temperatures.

3.3.5. Interspersion studies in great millet

Sivaraman et al [20] from our laboratory have reported that approximately 90% of the great millet genome consists of repeated sequences interspersed amongst themselves and with single copy sequences in a mixed type of interspersion pattern. Since these studies were conducted at the standard criterion of 62°C, we conducted reassociation experiments using 550 bp and 5 kbp great millet DNA at 55°C, 69°C and 75°C in order to determine if a similar trend in interspersion was observed at these temperatures. From Table 3.6, it is seen that at each temperature and at each Cot value, the proportion of DNA bound to hydroxyapatite increases on increasing the fragment length from 550 bp to 5 kbp indicating an interspersion of repeat sequences among themselves and with single copy DNA sequences at all the above three incubation temperatures.
FIG. 3.5(A): HPLC PROFILES OF THE TOTAL REPEATS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) TEMPERATURES.

a - Great millet (S1 -55°C)
b - Great millet (S1 -75°C)
c - Finger millet (S1 -55°C)
d - Finger millet (S1 -75°C)

C, G, T and A indicate peaks for cytosine, guanine, thymine and adenine respectively. The arrows represent 5-methyl cytosine.

Standard bases were also run but are not shown in the figure.
FIG 3-5 A

ABSORBANCE (254 nm)

RETENTION TIME (mins.)

C G T A

10 15 20 25

FIG 3-5 A
FIG. 3.5(B): HPLC PROFILES OF THE TOTAL REPEATS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) TEMPERATURE.

\( e \) - Little millet (S\(_1\) -55°C)
\( f \) - Little millet (S\(_1\) -75°C)
\( g \) - Fox tail millet (S\(_1\) -55°C)
\( h \) - Fox tail millet (S\(_1\) -75°C)

C, G, T and A indicate peaks for cytosine, guanine, thymine and adenine respectively.

The arrows represent 5-methyl cytosine.

Standard bases were also run but are not shown in the figure.
FIG 3.5 B

RETENTION TIME (mins.)

ABSORBANCE (254 nm)
TABLE 3.5: HPLC ANALYSES OF THE 5-METHYL CYTOSINE CONTENT OF THE TOTAL REPEATS ISOLATED AT PERMISSIVE (55°C) AND STRINGENT (75°C) CRITERIA OF TEMPERATURES

<table>
<thead>
<tr>
<th>Plant species</th>
<th>5-methyl cytosine content in the fraction (Mole %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great millet</td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>14.65 ± 1.59</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>5.56 ± 0.93</td>
</tr>
<tr>
<td>Little millet</td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>8.75 ± 1.23</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>11.15 ± 0.96</td>
</tr>
<tr>
<td>Fox tail millet</td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>11.72 ± 1.12</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>18.51 ± 0.83</td>
</tr>
<tr>
<td>Finger millet</td>
<td></td>
</tr>
<tr>
<td>$(S_1-55)$</td>
<td>19.44 ± 0.98</td>
</tr>
<tr>
<td>$(S_1-75)$</td>
<td>19.79 ± 1.33</td>
</tr>
</tbody>
</table>

The values in the above table are an average of three experiments each done in duplicate.
At the standard temperature of 62°C and fragment length of 550 bps, 52% of the total DNA reassociates by the limit Col value of 20 M.s. Since at the same temperature but with a fragment length of 5 kbp, 77% reassociation is observed, 77%-48% i.e. 25% reassociation is due to single copy sequences which are interspersed with repeat sequences on the longer fragment. At a higher temperature of 69°C, an increase of 73% - 43% i.e. 30% is observed when the fragment length is increased from 550 bps to 5 kbp. But since at a fragment length of 5 kbp, at standard conditions, 25% of the increase is due to single copy sequences at least 30%-25% i.e. 5% increase may be due to other thermal stability classes of repeat sequences which are not able to reassociate at 69°C but are present as single stranded regions on the 5 kbp fragments. Similar calculations done for the experiments at 75°C give a 9% increase due to other thermal stability classes which may be interspersed with single copy and with the class of repeat sequences able to reassociate at 75°C.
TABLE 3.6: THE PERCENTAGE BINDING OF REPETITIVE DUPLEXES TO HYDROXYAPATITE AT FRAGMENT LENGTHS OF 0.55 kbp AND 5 kbp IN GREAT MILLET

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Cot 1.0 M.S.</th>
<th>Cot 5.0 M.S.</th>
<th>Cot 10.0 M.S.</th>
<th>Cot 20.0 M.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.55 kbp</td>
<td>47.00</td>
<td>64.00</td>
<td>68.00</td>
<td>70.00</td>
</tr>
<tr>
<td>5 kbp</td>
<td>68.82</td>
<td>84.51</td>
<td>83.10</td>
<td>85.71</td>
</tr>
<tr>
<td>62°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.55 kbp</td>
<td>37.00</td>
<td>48.00</td>
<td>50.00</td>
<td>52.00</td>
</tr>
<tr>
<td>5 kbp</td>
<td>64.53</td>
<td>75.05</td>
<td>78.40</td>
<td>77.09</td>
</tr>
<tr>
<td>69°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.55 kbp</td>
<td>30.00</td>
<td>41.00</td>
<td>42.00</td>
<td>43.00</td>
</tr>
<tr>
<td>5 kbp</td>
<td>59.27</td>
<td>67.86</td>
<td>79.20</td>
<td>73.00</td>
</tr>
<tr>
<td>75°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.55 kbp</td>
<td>20.00</td>
<td>34.00</td>
<td>35.00</td>
<td>41.00</td>
</tr>
<tr>
<td>5 kbp</td>
<td>60.47</td>
<td>61.08</td>
<td>59.44</td>
<td>71.58</td>
</tr>
</tbody>
</table>
3.4. DISCUSSION

According to Britten and Kohne [4] and Kohne [10], most of the repetitive sequences in eukaryotic DNA are considered to originate from periodic amplification events, with members of the resulting sequence families being subject to translocation, base substitution and deletion events during evolution. This model has been further extended by Flavell [6] to include repeated cycles of amplification within a single family and compound amplification events in which adjoining portions of unrelated sequences are amplified together. When both amplifications and deletions occur during evolution, the portion of the genome subject to these processes can be viewed as turning over on a geological time scale.

According to Murray et al [16] since base substitutions accumulate with time, the fraction of previously amplified DNA to be recognised as repetitive at a given criterion will depend upon the rate of amplification or turnover of the genome. A genome which is undergoing rapid amplification or turnover would exhibit a small fraction of 'true' single copy DNA while the fraction of 'fossil' repeats will be prominent. On the other hand, a slow rate of amplification or turnover will be characterised by a larger fraction of 'true' single copy and smaller proportion of fossil repeats. High rates of amplification and turnover would result in increased secondary amplification events which would affect one or more members of a previously amplified family. Secondary amplification will result in repeat families which are heterogeneous while lower amplification rates will result in more homogeneous families.
In the present work, the most important conclusion is that among the five millets under consideration, the turnover rate of the great millet genome is the fastest while that of fox tail millet is the slowest. This conclusion has been arrived at by two experimental approaches. In the first approach, the kinetics of the total DNAs have been studied at four different temperatures namely 55°C, 62°C, 69°C and 75°C. The data (Table 3.1) obtained from such studies indicate the presence of homogeneous families in fox tail millet and of heterogeneous families in the other four millets. The data obtained from the 55°C and 62°C optical reassociation curves indicate that in great millet, of the total DNA reassociating as single copy at standard criterion, almost one third behaves as fossil repeats at permissive criterion (Table 3.2). In case of the other four millets, most of the single copy sequences are 'true' single copy. In the second approach, the self-reassociation of the repeats isolated at 55°C and 75°C was studied at the standard criterion of 62°C in order to determine the kinetic complexities. Such studies have shown that the fraction isolated at 75°C is 81.3.5 and 2.2 times more complex than the 55°C fraction in case of fox tail millet, finger millet and little millet respectively, while it is only 0.3 fold complex in case of great millet. Both the approaches suggest that among these millets, the rate of turnover of the genome of fox tail millet is the slowest. The presence of a relatively higher proportion of fossil repeats in great millet than in the other four millets indicates that the rate of turn over of the great millet genome is comparatively the fastest.

Murray et al [16], have extended their model to explain the interspersion patterns. They predict that rapid amplifications or high
turnover would produce highly repetitive genomes with extensive short period interspersion, while slower turnover should result in a smaller fraction of repetitive DNA and an increase in the length of interspersed single copy and repeat DNA. Data reported from our laboratory have shown that fox tail millet exhibits only a long period interspersion pattern, while the remaining four millets exhibit a mixed type of interspersion pattern [8,20,21]. Thus the present studies on the rate of turnover and nature of repetitive families in the five millets further support Murray et al's views on the relationship between turnover rate, type of repeat families and interspersion pattern observed in the genome.
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