Theor. Appl. Genet. 54, 219-224 (1979)

Characteristics of Nuclear DNA in the Genus *Oryza*

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Summary. DNAs have been isolated from various *Oryza* species and studied using physical techniques. The percent of guanine plus cytosine has been determined by thermal denaturation. While the base composition varied between the species, no heterogeneity in the base pair distribution was observed. Renaturation kinetics data of DNAs from different species show that the proportion of repeated DNA sequences vary considerably depending on the DNA content per cell, whereas the nonrepetitive DNA component remains relatively constant. These results suggest that in addition to a small range of DNA variation between the species, changes in the base composition and proportion of repeated sequences have accompanied divergence of the species within the genus.

Key words: DNA – Base pair distribution – Divergence – *Oryza*

Introduction

The DNAs of all the higher organisms investigated contain some nucleotide sequences present in many copies (Britten and Kohne 1968; Flamm 1972). Studies on protozoa, insects, amphibians and mammals indicated that the proportion of the genome containing repeated sequences may be between 15% in *Drosophila* (Laird 1971) and 90% in *Necturus* (Strauss 1971) when experiments were conducted under similar conditions. Higher plant DNAs may contain 45-90% repeated sequences (Flavell et al. 1974). Some families of repeated sequences consist of simple nucleotide sequences (Southern 1970; Fry et al. 1973) while others are more complex. Some sequences are clustered within the chromosome (Pardue and Gall 1970; Kramm et al. 1972; Macgregor et al. 1973) while others are interspersed with single copy sequences (Britten and Smith 1970; Britten 1972; Wu et al. 1972; Davidson et al. 1973). The functions, if any, of most of these repeated sequences are unknown, but the studies on a wide range of animal species indicate that mutations are more tolerated by these repeated sequences (McCarthy and Farquhar 1972). Analyses of the DNA of related animals have provided examples where repeated DNA sequences have been added to the genomes relatively recently in evolution (Rice 1972). Limited variations, often observed between repeated sequences, enable them to be recognised as members of the same family, and to be interpreted as an indication that the sequences could have arisen from a single ancestral sequence, most probably by errors in DNA replication. Such additional DNA possibly was stabilised without the loss of an equivalent amount of other DNA. A net increase in chromosomal DNA which is possibly without any function, would be a consequence of the addition of families of repeated sequences to chromosomes.

At the chromosomal level the genus *Oryza* has been extensively studied. Unlike most angiosperm genera, we have observed that the general range of genome size of the species in the genus *Oryza* is comparatively small, 1.03 to 2.02 $\times$ 10^{-12} g (Iyengar and Sen 1978), indicating that speciation within the genus could proceed without large scale changes in the nuclear DNA content. We have initiated a study in some species of *Oryza* in order to find whether there is any change in the overall base composition and how much of the DNA in different species is made up of repetitive and nonrepetitive base sequences. This kind of investigation is likely to provide direct and useful information on the changes in DNA which might have accompanied divergence of the species in this important genus.

Materials and Methods

The 8 diploid species of *Oryza* utilised in the present investigation have been collected from the Central Rice Research Institute, Cuttack, India. They have been identified according to Nayar (1973).

0040-5752/79/0054/0219/$ 1.20

They are *O. sativa* L. ssp. *indica* Var. 'I.R.8', *O. sativa* L. ssp. *japonica*, *O. rufipogon* (perennial) or *O. perennis* Moench, *O. rufipogon* (annual) or *O. rufipogon* Griff., *O. glaberrima* Steud., *O. longistaminata* Roschev., *O. stapfii* Roxchev. and *O. barthii* or *O. breviligulata* A. Chev. et Roehr.

Extraction of Nuclear DNA

Surface sterilised seeds (soaked in 1.5% sodium hypochlorite for 15 min) were washed in distilled water and soaked for 24 hours in distilled water. Imbibed seeds were grown on moist filter papers under axenic conditions. Shoot tissue of 8-10 days old seedlings was collected after washing in distilled water. Root tissue was not used for the extraction of DNA since it was not possible to eliminate bacterial contamination. The collected shoot tissue was wrapped in aluminum foil and stored at −20° C.

The method adopted for the extraction of DNA was essentially the same as that suggested by Smith and Flavell (1974). After repeated deproteinizations of the aqueous extract with chloroform-octanol, the DNA was precipitated in 2 volumes of 95% ethanol. The precipitated nucleic acids were collected by centrifugation at 5,000 g for 10 min. The collected precipitate, drained free of excess alcohol, was dissolved in 0.1 SSC (0.015 M sodium chloride plus 0.0015 M sodium citrate, pH 7.0). The DNA was further purified by treating with L-amylase (100/ug/ml) at 37° C for 45 min; with ribonuclease (previously heated at 90° C for 10 min.) at a concentration of 100/ug/ml at 37° C for 1 hour and finally with self-digested pronase (500/ug/ml) at 37° C for 1 hour. The solution was then deproteinized with chloroformoctanol until no visible protein layer at the interphase remained and the aqueous phase was finally precipitated in isopropyl alcohol in the presence of acetate-EDTA (3.0 M sodium acetate and 0.001 M EDTA, pH 7.0, Marmur 1961). The precipitate was redissolved in 0.1 SSC and then mixed with 50 ml of phosphate-buffered phenol (28 ml of 0.05 M sodium citrate sodium phosphate buffer, pH 5.0 plus 78 ml of redistilled phenol) and kept cold (4° C) for 4 hours. The aqueous phase collected from repeated phenol extractions was mixed with diethyl ether, shaken gently and the heavier phase was separated and precipitated with ethanol. The precipitated DNA was dissolved in 0.12 M phosphate buffer (PB = equimolar mixture of Na_2HPO_4 and NaH_2PO_4, pH 6.8) and also in 0.1 SSC.

Pigments in seedlings often coloured the DNA despite repeated precipitations. Hence, the DNA samples were passed over a column of Sepharose 4B (Pharmacia) which separated the pigments from the DNA. Purified DNA samples were dialysed overnight against respective buffers and stored at −10° C.

E. coli strain K_{12} was used as a standard reference and the DNA was extracted according to the procedure of Marmur (1961).

The DNA yield varied considerably depending on the stage of the development of the tissue. The DNA samples from all species utilized had the absorption ratio at 260/280 nm varying from 1.80 to 1.85. At least 90% of the UV absorbing material remained absorbed to hydroxyapatite at 60° C in 0.12 M PB. The DNA concentrations were determined by measuring the absorbance at 260 nm on UVChem spectrophotometer (Hilger and Watts).

Preparation of Sheared DNA

DNA solutions (5-10 ml) in 0.12 M PB were chilled and sonicated with an MSE sonicator by using a 3/4 inch probe for 1.5 minutes at full intensity. This did not cause any increase in the absorbance of the solution. Molecular weight of the sonicated DNA was determined by alkaline sucrose gradients in a preparative ultracentrifuge (VAC 601) using the method described by Van der Schans et al. (1969). The average molecular weight of the sonicated double stranded DNA was estimated as 2.7×10^5 daltons, corresponding approximately to 450 nucleotide pairs.

DNA Melting Experiments

All the DNA melting experiments were carried out by 2 methods a) by melting the DNA samples (in 0.1 SSC) in spectrophotometer cuvettes and b) by separation of denatured DNA from double-stranded DNA on hydroxypatite columns.

a) For melting DNA samples in a spectrophotometer approximately 20/ug/ml DNA samples were placed into stoppered UVChem absorption cells and paraffin oil was layered over these samples in order to prevent evaporation at high temperatures. Meltings were done by placing the cells in water jacketed cell holders of an UVChem H1650 spectrophotometer. The temperature of the cells was raised by circulating hot water through a universal thermostat NBE. Initial absorbance at 25° C 260 nm was recorded. The temperature of the cells was then quickly raised to 50° C and the temperature equilibrium had been obtained, the temperature thereafter was raised at about 2° C at a time. Five min was allowed for equilibration at each temperature and then the absorbance at 260 nm was recorded. This process was carried out until no further raise in absorbance was observed. The data were corrected for volume expansion using the table given by Mandel and Marmur (1968). The corrected absorbances were divided by the initial absorbance and the ratio, (relative hyperchromicity) converted to percent of relative hyperchromicity, was plotted against the temperature. The hyperchromicity values of different DNAs fell within the range of 30 to 38 percent. The GC content was calculated using the equation $GC = (T_m - 53.99)\ 2.44$ (Mandel and Marmur 1968).

b) Thermal fractionation of DNA was done on hydroxyapatite columns according to the method adopted by Flamm (1972). The DNA samples in 0.12 M PB were loaded onto a column previously equilibrated to 60° C and 0.12 M PB. Then the temperature of the column was raised in steps, 3-4° C at time, and at each temperature raise the column was washed twice with a total volume of 5 ml of 0.12 M PB. This process was continued till the column temperature reached close to 100° C. Two final washes with 0.5 M PB were then made to recover any double-stranded DNA still remaining. Rarely was DNA recovered at this temperature. The eluates at each temperature were measured by taking absorbances at 260 nm and the amount present was calculated. Total recovery from the column was greater than 95% in all cases. The melting temperature was taken at which half strands had separated. The GC content was estimated according to the formula: $GC = (T_m - 81.5 - 16.6 \log M)\ 2.44$ (Mandel and Marmur 1968).

C_0t Reassociation of the DNA

The reassociation kinetics of DNA samples were followed by measuring relative amounts of single-stranded DNA in a reaction mix on hydroxyapatite columns (Britten and Kohne 1968; Britten et al. 1974). The reaction mix contained different concentrations of sheared DNA (50/ug/ml − 2.0 mg/ml) in 0.12 M PB. The samples were denatured by heating in a water-bath at 100° C for 5-8 min and then rapidly cooled to 60° C. The denatured DNA samples in 0.12 M PB were incubated in screw-capped tubes at 60° C for reassociation to achieve desired C_0t values (C_0t = the product of the

concentration of DNA expressed as moles of nucleotides per liter and time of reassociation in seconds). The C_0t values were calculated in terms of absorbancy (C_0t = optical density at 260 nm × incubation time in hours/2). After incubation, the samples were either directly fractionated or they were chilled immediately and stored at −10°C. Frozen samples were brought to 60°C before fractionation.

Fractionation on Hydroxyapatite

Hydroxyapatite (Calbiochem) was pretreated by boiling 0.5 g of hydroxyapatite in 5 ml of 0.12 M PB plus 0.02% sodium dodecyl sulphate for 10 min. This eliminated the nonspecific binding of DNA. The water jacketed chromatographic column was similar to the one described by Miyazawa and Thomas (1965) except that the entire column was made up of glass. The column temperature was maintained by circulating hot water from a universal thermostat type NBE. The column temperature was determined by a temperature indicator. Fractionation of the reassociated DNA samples was done adopting the method described by Britten and Kohne (1968). DNA samples in 0.12 M PB were loaded onto the chromatographic column previously equilibrated to 60°C and 0.12 M PB. Three to five washes with 0.12 M PB were made to elute single-stranded DNA. Similar washes with o.5 M PB were subsequently made to elute reassociated DNA. The concentration of single-stranded and reassociated DNA was determined from the phosphate buffer elutes by their absorbances at 260 nm. The percent of reassociation was calculated as follows: percent of reassociation = $\frac{B}{A+B} \times 100$ where A and B are the amounts of DNA eluted by 0.12 M PB and 0.5 M PB respectively.

Results and Discussion

DNA Melting Profiles and the Base Ratio

The melting profile of the DNA expressed as the percent of hyperchromicity is a cumulative plot representing the DNA segments that differ in average base composition.

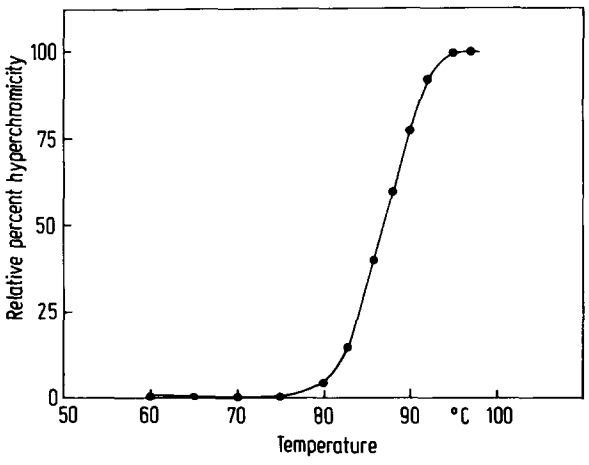

Fig. 1. The melting profile of the DNA of *O. sativa* ssp. *indica*

Comparisons between the melting profiles of DNA from different species give information about the proportion of the GC base sequences as well as about major heterogeneity of the base pairs within the DNA from different species. A melting profile of *O. sativa* ssp. *indica* in 0.12 M PB is given in Fig. 1. The curve is steep and smooth indicating that a large measure of homogeneity exists in the dispersion of AT and GC base pairs within the DNA. The results obtained for DNAs of other species were similar.

The GC contents estimated from the melting temperatures (T_m's) determined chromatographically and spectrophotometrically are presented in Table 1. The eight species studied show closely similar melting profiles with T_m's falling within the range 85.0°C to 88.0°C in 0.12 M PB and 70.0°C to 73.1°C in 0.1 SSC. The GC contents estimated from the T_m's in 0.12 M PB agree well with the corresponding values from the T_m's in 0.1 SSC. The differences in the GC content of the species from the two methods do not differ significantly. The maximum difference in the estimation of base composition is 1.07 mole percent of GC. The base ratios clustered around a mean value of 42.21%.

The Reassociation Kinetics of DNAs

The reassociation kinetics profiles of *Oryza* DNAs together with the DNA *E. colis* for comparison are shown in Figs. 2 and 3. The DNA samples from all plant species showed two phases in the reassociation process. A fast reassociating fraction represented the repeated nucleotide sequences and a slow reassociating fraction represented

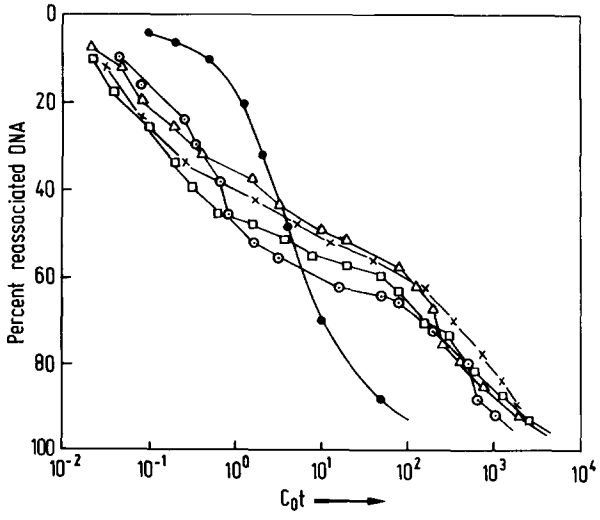

Fig. 2. The kinetics of reassociation of the DNAs of *Oryza* species and of *E. coli*; • *E. coli*; ○ *O. sativa* ssp. *indica*; ×, *O. sativa* ssp. *Japonica*: ■, *O. rufipogon* (perennial); △, *O. rufipogon* (annual)

Table 1. Melting temperatures base compositions and base compositional heterogeneity of eight *Oryza* species

Species	Melting temperature (o_c)		Percent of Guanine + Cytosine		Base compositional heterogenity as percent of GC
	in 0.12 M PB	in 0.1 SSC	From T_m's in 0.12 M PB	From T_m's in 0.1 SSC	
sativa ssp. *indica*	87.0	72.1	43.58	44.41	9.76
sativa ssp. *japonica*	86.0	71.0	41.14	41.72	8.54
rufipogon (perennial)	87.5	72.4	44.80	45.14	9.27
rufipogon (annual)	85.0	70.0	38.70	39.28	10.00
glaberrima	88.0	73.1	46.02	46.85	9.03
longistaminata	86.5	71.7	42.36	43.43	10.74
stapfii	86.0	70.8	41.14	41.24	8.05
barthii	85.5	70.4	39.92	40.26	10.25

Table 2. Repetive and nonrepetive DNA in eight *Oryza* species

Species	Total nuclear[a] DNA per genome (X 10^{-12} g.)	Repetive DNA		Nonrepetive DNA	
		As percent of total DNA	As absolute amount (X 10^{-12} g.)	As percent of total DNA	As absolute amount (X 10^{-12} g.)
sativa ssp. *indica*	1.67	66	1.10	34	0.57
sativa ssp. *japonica*	1.55	62	0.96	38	0.59
rufipogon (perennial)	1.35	60	0.81	40	0.54
rufipogon (annual)	1.28	59	0.76	41	0.52
glaberrima	1.37	63	0.86	37	0.51
longistaminata	1.25	59.5	0.74	40.5	0.51
stapfii	1.06	56	0.59	44	0.47
barthii	1.03	58	0.60	42	0.43

[a] Iyengar and Sen (1978)

Table 3. The complexities of nonrepetive DNA components in eight *Oryza* species

Species	Fraction	Observed	Estimated cot 1/2 (pure)[a]	Kinetic complexity (base pairs)[b]	Genome size (base pairs)[c]	Reiteration frequency
sativa ssp. *indica*	0.34	870	296	$2.22.10^8$	$2.59.10^8$	1.17
sativa ssp. *japonica*	0.38	915	348	$2.61.10^8$	$2.69.10^8$	1.03
rufipogon (perennial)	0.40	880	352	$2.64.10^8$	$2.45.10^8$	0.93
rufipogon (annual)	0.41	710	291	$2.18.10^8$	$2.40.10^8$	1.10
glaberrima	0.37	780	288	$2.16.10^8$	$2.31.10^8$	1.07
longistaminata	0.40	680	272	$2.04.10^8$	$2.28.10^8$	1.12
stapfii	0.44	675	297	$2.23.10^8$	$2.13.10^8$	0.96
barthii	0.42	685	288	$2.16.10^8$	$1.97.10^8$	0.91

[a] Estimated C_0t 1/2 (pure): fraction of DNA x observed C_0t 1/2;
[b] Kinetic complexities calculated relative to *E. coli* DNA whose genome size was 4.5×10^6 base pairs. The *E. coli* C_0t 1/2 was experimentally found to be 6;
[c] Genome size: fraction of DNA in that class x complexity of the organism. The haploid DNA amount of each species was converted to base pairs using the constant 0.913×10^9 base pairs per picogram (Britten and Davidson, 1971)

nonrepeated sequences. The fast reassociating fraction reassociated at a C_0t around 100 in all the species. The nonrepetitive DNA fraction which reassociated at C_0t 100 and beyond follows second order kinetics in its reassociation. The proportion of repetitive and nonrepetitive DNA fractions as percent of the total DNA and as absolute amount per genome, are presented in Table 2. The kinetic complexities of the nonrepetitive DNA from different species have been calculated using *E. coli* DNA (genome size 4.5×10^6 base pairs, Cairns 1963) as a standard. The C_0t 1/2 of *E. coli* DNA by the present procedure was found to be 6.0. The kinetic complexities of nonrepetitive DNA from different species appear in Table 3. The reiteration frequency of this fraction in the species studied approximates to unity thus showing that the nonrepetitive component measured by the present procedure is made up of a unique sequence of DNA.

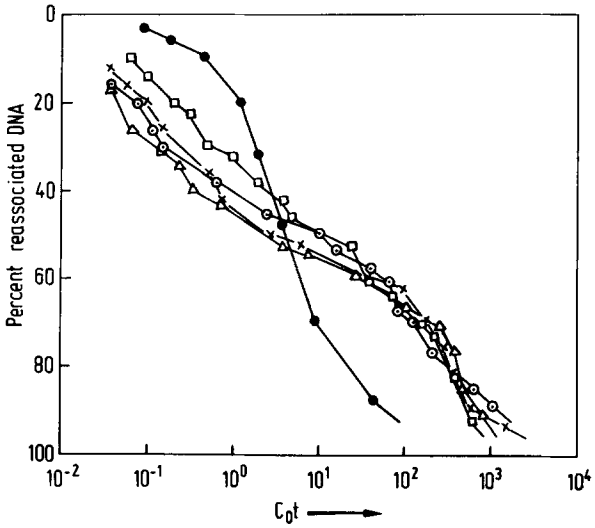

Fig. 3. The kinetics of reassociation of *Oryza* species and of *E. coli*; •, *E. coli*; ○, *O. glaberrima*; □, *O. stapfii*; △, *O. barthii*; ×, *O. longistaminata*

Variation Among the Species

The reassociation kinetics curves of different species show a general similarity. Nevertheless, there are well defined differences in the DNA composition from different species. As evident from Table 2, the amount of repetitive DNA varies between the species whereas the amount of nonrepetitive DNA in absolute terms remains relatively constant. It is also evident that the absolute amount and the proportion of repetitive DNA increased with the increase in the total nuclear DNA. The proportion of repetitive DNA in eight species was plotted against nuclear DNA amount in Fig. 4. The regression line drawn is the best fit for the data. It can be observed that as the nuclear DNA amount increased the proportion of repetitive sequences increased, indicating a positive correlation. The correlation (r = 0.93) is significant (P = less than 0.001). On the other hand, the proportion of nonrepetitive DNA showed no significant correlation with the nuclear DNA amount. Thus the variation in the DNA amount between the species of *Oryza* was largely due to the quantitative variation in the repetitive sequences. It seems clear that whatever the absolute values for the amount of repeated sequence DNA in any one of the species were, most of the changes in DNA content of the species have been due to the addition and/or loss of repeated sequences. Such additions could have taken place by saltatory amplification over a relatively short or long period. Additional information about the qualitative differences of repeated and nonrepeated sequences among the species will be obtained from the hybridization experiments of these fractions from different species. For the moment it will suffice to emphasize that the changes in DNA base composition and also the proportion of repeated sequences have accompanied divergence of *Oryza* species.

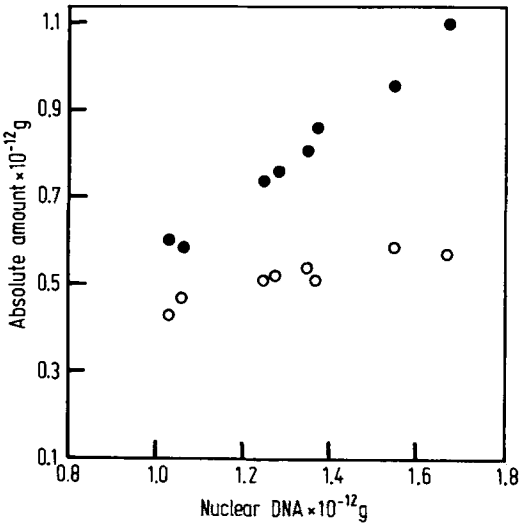

Fig. 4. The absolute amount of repetitive and non-repetitive DNA is shown against eight species of Oryza having varying amount of nuclear DNA: •, repetitive DNA and ○, non-repetitive DNA

Acknowledgement

This work was supported by financial grants from the Council of Scientific and Industrial Research, New Delhi, India. We thank Messrs S. Sampath, P.J. Jachuck and M.J.B.K. Rao of the Central Rice Research Institute, Cuttack, for help and cooperation. Helpful comments by Prof. W. Beermann are gratefully acknowledged.

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Received August 17, 1978
Accepted October 11, 1978
Communicated by D. von Wettstein

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