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DNA hybridization analyses of a *Gossypium allotetraploid* and two closely related diploid species *

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Summary. The DNAs of two diploid species of *Gossypium, G. herbaceum* var. *africanum* (A_1 genome) and *G. raimondii* (D_5 genome), and the allotetraploid species, *G. hirsutum* (A_h and D_h genomes), were characterized by kinetic analyses of single copy and repetitive sequences. Estimated haploid genome sizes of A_1 and D_5 were 1.04 pg and 0.68 pg, respectively, in approximate agreement with cytological observations that A genome chromosomes are about twice the size of D genome chromosomes. This differences in genome size was accounted for entirely by differences in the major repetitive fraction (0.56 pg versus 0.20 pg), as single copy fractions of the two genomes were essentially identical (0.41 pg for A_1 and 0.43 pg for D_5). Kinetic analyses and thermal denaturation measurements of single copy duplexes from reciprocal intergenomic hybridizations showed considerable sequence similarity between A_1 and D_5 genomes (77% duplex formation with an average thermal depression of 6 °C). Moreover, little sequence divergence was detectable between diploid single copy sequences and their corresponding genomes in the allotetraploid, consistent with previous chromosome pairing observations in interspecific F_1 hybrids.

Key words: *Gossypium* – DNA hybridization – Single copy – Repetitive – Thermal stability.

Introduction

The genus *Gossypium* consists of 35 diploid species divided into 7 genomic groups (designated A through G) with a $2n$ chromosome number of 26, and 6 allotetraploid species with a $2n$ ($4x$) chromosome number of 52. The research of several investigators has established that the allotetraploids originated from interspecific hybridization between species of the A and D genomes, and that the allotetraploid genomes are most similar to the A_1 genome of *G. herbaceum* and the D_5 genome of *G. raimondii* (see review by Endrizzi et al. 1985).

One unusual feature of the genus is that different genomic groups exhibit characteristic chromosome sizes. This variation is particularly evident from cytological studies of natural and synthetic allopolyploids. Such studies have shown that the A genome chromosomes are about twice the size of the D genome chromosomes (Skovsted 1934; Endrizzi 1985). Biochemical estimates of nuclear DNA content are consistent with the relative distinctions measured cytologically, suggesting that DNA content is the primary determinant of chromosome size (Katterman and Ergle 1970; Edwards et al. 1974; Kadir 1976).

In the present paper, we present the analysis of kinetic components of genomic DNA from reassociation of total nuclear DNA to determine the nature of DNA variation accompanying change in genome size. We also present the analysis of DNA hybridization kinetics and thermal stability measurements of single-copy hybrids.

Materials and methods

Two diploid species and one allotetraploid species of *Gossypium* were analyzed. The diploids were *G. herbaceum* var. *africanum* (Watt) Mauer (G304) with the A_1 genome and *G. raimondii* Ulbr. with the D_5 genome. The allotetraploid species was a highly inbred line, TM1 (Kohel 1970), of Deltapine 14 of *G. hirisutum* L. containing the A_h and D_h genomes. Cytogenetic data of several investigators have shown that the genomes of the above two diploid species are most similar to the A and D genomes of the allotetraploid species (see review by Endrizzi et al. 1985).

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Preparation of DNA

Cotton species were cultivated in a greenhouse and mature leaves were periodically collected and stored at −70 °C. Frozen leaves were ground to a fine powder in a stainless steel Waring blender containing liquid nitrogen and nuclei isolated according to the method of Katterman and Shattuck (1983). Further purification of the DNA entailed using urea-phosphate and hydroxyapatite as described in Britten et al. (1974), followed by banding in CsCl gradients containing ethidium bromide.

Preparation of DNA fragments

DNAs were sheared in a Virtis 60 homogenizer as described in Davidson et al. (1973). For obtaining fragments with a mass average length of 250 nucleotides, DNAs were suspended in a medium containing 67% glycerol and 0.1 *M* sodium acetate, pH 6.0, chilled in an ethanol, dry-ice bath during a 30-min homogenization at 50,000 revs/min. Mass average fragment lengths for sheared DNA preparations were determined by alkaline band sedimentation in a Model E ultracentrifuge (Beckman) using the equations of Studier (1965), or from alkaline isokinetic sucrose gradients according to the method of McCarty et al. (1974).

Reassociation conditions

All reagents and DNAs were passed over Chelex 100 (BioRad) to remove heavy metal ions. Standard criterion for incubation and fractionation over hydroxypatite was a temperature of 60 °C and 0.12 *M* sodium phosphate buffer (PB), pH 6.8. This is approximately 24 °C below the T_m for native DNA of *G. hirsutum* (Walbot and Dure 1976). An equivalent criterion of 67 °C and 0.67 *M* PB was used to shorten the length of time required for reassociations at high Cot values (Britten et al. 1974).

Preparation of tracer DNAs

Tracers were made from either unfractionated or fractionated nuclear DNAs after shearing as described above. Unfractionated tracers, used to estimate the DNA components in total Cot curves, were terminally labeled with γ-^{32} P-ATP (New England Nuclear) and T_4 polynucleotide kinase (P-L Biochemicals) without prior treatment with alkaline phosphatase. Unincorporated label was separated by Gel filtration over Sephadex G-100 and the labeled DNA fraction concentrated by precipitation with ethanol. Fractionated tracers were prepared from sheared nuclear DNA by separating the kinetic components of the DNA over hydroxyapatite (HAP) at various Cot intervals. Single copy components for the three cotton species had the following fractionation histories: *G. hirsutum*, Cot 100 fraction unbound (FUB), then reassociation to Cot 10,000 and fraction bound (FB); *G. herbaceum*, Cot 100 FUB, then Cot 2,500 FB; and *G. raimondii*, Cot 75 FUB, then Cot 3,400 FB. After fractionation and concentration, tracers were terminally labeled with ^{32}P.

Thermal denaturations

Thermal stabilities of DNA duplexes formed at Cot 10,000 were determined by hydroxyapatite chromatography. Samples were diluted into 1 ml 0.12 *M* PB at 60 °C and applied to a water-jacketed column containing 0.4 g HP, previously equilibrated with 0.12 *M* PB. Single-stranded DNA was eluted with 3 ml 0.12 *M* PB at successive 3 °C increments from 60 °C to 105 °C *E. coli* DNA (type VIII, Sigma), mixed with each sample before loading, and used for internal standardization.

Computer programs

Cot curves and thermal denaturation curves were generated from discrete data points using non-linear, least-squares methods and the computer programs of Murphy et al. (1979).

Results

Reassociation kinetics of cotton DNAs

The cotton genome contains repetitive and single copy DNA sequences. The fraction of the genome represented by these distinct classes of DNA and their kinetic rate constants can be determined by analyzing the reassociation of randomly sheared DNA fragments. Quantitative determinations are made by reference to the reassociation kinetics of the well-characterized genome of *E. coli*.

Least-squares analyses of the reassociation kinetics of sheared DNAs from two diploid cottons, *G. herbaceum* and *G. raimondii*, and an allotetraploid, *G. hirsutum*, are shown in Fig. 1. Each curve represents a second-order kinetic solution fit by computer to discrete data points. Rate constants for single copy sequences were determined in separate experiments using tracer DNAs enriched for that class of sequences. Parameter estimates in reassociations involving total DNA were constrained by fixing single copy rate constants at values determined in the tracer enrichment experiments. Under these conditions, repetitive sequences constituted a single and distinct kinetic component. Additional repetitive components could not be resolved with accuracy in reassociations to total unfractionated DNA. For this reason, the two component solutions (Fig. 1) were chosen to best represent the kinetic components of the cotton genomes.

Parameters estimated from the reassociation kinetics of the three cotton species are evaluated in Table 1. Each has been normalized for complete reassociation. Complexity estimates of single copy DNAs for *G. herbaceum* (2.8×10^8 bp) and *G. raimondii* (3.9×10^8 bp) indicated that there was little, if any, difference in the amount of single copy DNA between the diploid A_1 (0.41 pg) and D_5 (0.43 pg) genomes. However, a comparison of the major repetitive components showed that *G. herbaceum* had 2.8 times more repetitive DNA than *G. raimondii*. Most of this (76.2%) could be accounted for by differences in average copy number (Table 1, column 6). The remainder was due to the greater, average, repetitive complexity of *G. herbaceum* DNA (2.1×10^6 bp) compared to *G. raimondii* DNA (1.6×10^6 bp).

Analysis of the reassociation kinetics of the allotetraploid, *G. hirsutum*, was complicated by potential cross hybridization between DNA sequences of A_h and D_h genomes. If complete sequence divergence had occurred between the two genomes, repetitive and single copy complexities of *G. hirsutum* should approximately equal

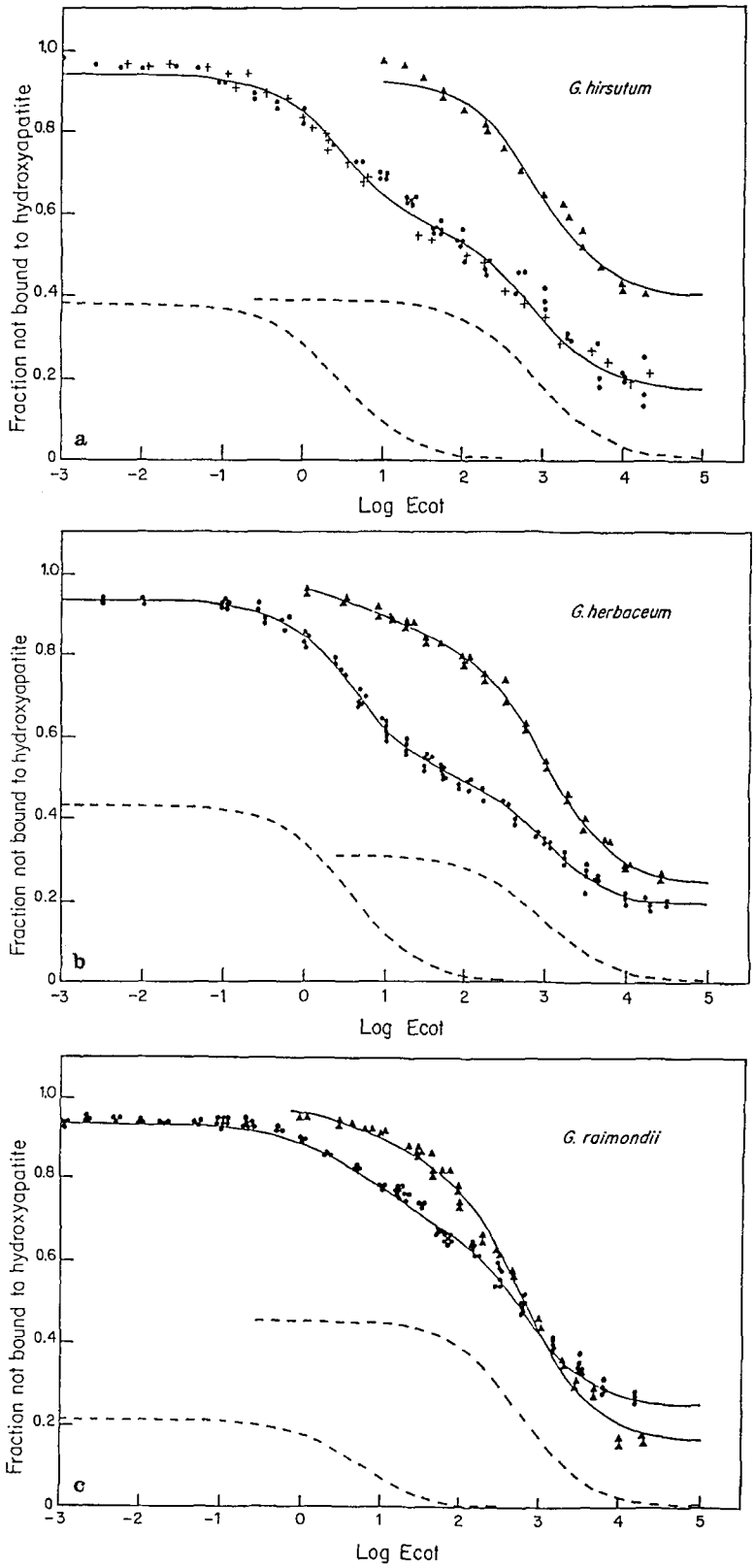

Fig. 1a–c. Reassociation of DNA from three cotton species. Solid curves represent components that have been fitted by computer, assuming ideal second-order kinetics. Fragments 250 nucleotides long from unfractionated (•) or fractionated (▲) DNAs were labeled as tracers and renatured with unlabeled, unfractionated DNAs, as drivers, in mass excess $\lesssim 5{,}000$. Single copy rate constants determined from reassociation curves with fractionated tracers were used to fix single copy rate constants for reassociation curves with unfractionated driver DNAs. Dashed lines represent a two-component computer fit to unfractionated data points. **a** *G. hirsutum* components are: 39% single copy DNA with a rate constant $k = 1.17 \times 10^{-3}\ M^{-1}\ s^{-1}$; 38% repetitive DNA with $k = 0.31\ M^{-1}\ s^{-1}$. Some data points were determined optically from reassociation of driver DNA alone (+). **b** *G. herbaceum* components are: 31% single copy DNA with $k = 1.05 \times 10^{-3}\ M^{-1}\ s^{-1}$; 43% repetitive DNA with $k = 0.26\ M^{-1}\ s^{-1}$. **c** *G. raimondii* components are: 46% single copy DNA with $k = 1.62 \times 10^{-3}\ M^{-1}\ s^{-1}$; 22% repetitive DNA with $k = 0.19\ M^{-1}\ s^{-1}$

Table 1. Kinetic analysis of DNA reassociations from cotton species

Species	Component	Fraction[a] (f_i)	Complexity[b] (bp)	Size[c] (pg)	Copy no.[d] (average)
G. hirsutum	zero time[e]	0.06	–	0.12	–
	repetitive	0.46	1.5×10^6	0.76	370
	single copy	0.48	4.1×10^8	0.84	2
				1.72	
G. herbaceum	zero time	0.07	–	0.07	–
	repetitive	0.54	2.1×10^6	0.56	250
	single copy	0.39	3.8×10^8	0.41	1
				1.04	
G. raimondii	zero time	0.07	–	0.05	–
	repetitive	0.30	1.6×10^6	0.20	120
	single copy	0.63	3.9×10^8	0.43	1
				0.68	

[a] Normalized for complete reassociation
[b] Estimated from the product of the rate constant and complexity of *E. coli* DNA (1.015×10^6 bp $M^{-1} s^{-1}$), the normalized fraction of the genome (f_i) and the component rate constant (k_i). Computed as $(1.015 \times 10^6)(f_k)/(k_i)$
[c] Estimated from the single copy rate constant and the fraction represented by each component. Values for the allotetraploid were derived from the sums of the diploids (see text)
[d] Average copy number, computed as $(k_i)/(k_{sc})$
[e] DNA reassociated at Cot 10^{-3}

Table 2. Fraction reassociated at terminal Cot and thermal stabilities of homologous and heterologous single copy DNA hybrids

Tracer	Driver	Fraction[a]	T_m[b]	Average	ΔT_m
G. herbaceum	*G. herbaceum*	100	79.3, 79.9	79.6	–
	G. hirsutum	97.2	77.2, 76.7	76.9	< 0.5[c]
	G. raimondii	78.7	74.2, 74.1	74.1	5.5
G. raimondii	*G. raimondii*	100	80.4, 80.8	80.6	–
	G. hirsutum	95.6	77.2, 77.3	77.2	0.8[c]
	G. herbaceum	76.4	74.5, 73.5	74.0	6.5

[a] Fraction renatured at terminal Cot was determined from the data in Fig. 2 and normalized to self-hybrid formation at terminal Cot
[b] Values determined by least-squares computer fit to data points collected by thermal elution on HAP at 3 °C increments. Each T_m value was corrected for minor variations in T_m of an *E. coli* internal standard. Average T_m s were derived from two independent hybridization experiments
[c] Estimates the extent of divergence between cytologically similar genomes after correction for the fraction of tracer hybridizing to the cytologically divergent genome

the sums of the diploid complexities (viz 3.7×10^6 bp and 7.7×10^8 bp).

Alternatively, if no sequence divergence had occurred between genomes, these values should approximately equal the average of the diploid complexities (viz 1.85×10^6 bp and 3.85×10^8 bp). From the data in Table 1, it was apparent that repetitive and single copy complexities for *G. hirsutum* resembled the average of the two diploid species, and suggested that a significant fraction of the stable duplexes formed during hybridization resulted from intergenomic hybrids. However, sequence divergence between intergenomic hybrids would retard the rate of duplex formation (Bonner et al. 1973), and the use of a second order equation for the determination of rate constants leads to an overestimation of component complexities. For this reason we have estimated the haploid genome size of *G. hirsutum* (Table 1, column 5) from the sums of those complexity values determined for the diploid genomes, assuming that no significant gain or loss of DNA sequences has occurred during allotetraploid evolution.

In a previous analysis of *G. hirsutum* (Walbot and Dure 1976), the rate constants for repetitive sequences ($0.156\ M^{-1}s^{-1}$) differed by two-fold from our analysis. Their single copy sequences ($0.00117\ M^{-1}s^{-1}$) however, are substantially in agreement with our findings. (For comparison between experiments, their values have been corrected to account for the use of a larger mass average fragment length). The minor discrepancy in the repetitive rate constant can, in part, be attributed to their fitting of a second highly repetitive component ($K = 147\ M^{-1}s^{-1}$ after size correction), which we have not observed. However, their values for the fractional representation of repetitive (0.27) and single copy (0.605) sequences differ greatly from our estimates (0.46 and 0.48, respectively).

Interspecific hybridizations with G. herbaceum and G. raimondii single copy DNAs

From the analyses of the allotetraploid, *G. hirsutum*, the suggestion that intergenomic duplexes represented a substantial fraction of the products formed during hybridization was examined by performing interspecific hybridization between diploid species. This analysis was confined to single copy sequences, because repetitive sequences reportedly exhibit considerable intragenomic divergence, potentially obscuring measurements of intergenomic relatedness (Britten et al. 1978).

Single copy tracers from *G. herbaceum* and *G. raimondii* were hybridized with excess total DNAs. Duplexes formed at Cot 10,000 were subjected to thermal chromatography on hydroxyapatite and sequence divergence estimated by determining the relative difference in the midpoint (T_m) of thermal elution between intraspecific and interspecific duplexes. From Table 2, interspecific hybrids from reciprocal experiments showed nearly iden-

tical T_ms (74.1 °C and 74.0 °C). This corresponds to thermal depressions of 5.5 °C and 6.5 °C below those of self hybrids and an average single copy divergence for duplexes formed between A_1 and D_5 genomes of 6%, assuming 1 °C depression in T_m equals 1% divergence (Britten et al. 1974). The difference between intraspecific T_ms of *G. herbaceum* (79.6 °C) and *G. raimondii* (80.6 °C) is likely caused by a disproportionate difference in the amount of repetitive DNA present in the preparations of single copy tracer and/or differences in GC content (Ergle et al. 1964). To compare reciprocal experiments, the extrapolated fractions of intergenomic duplexes formed at terminal Cot were normalized to values determined in intragenomic reassociations. From Table 2, both tracers renatured to comparable extents (76.4% and 78.7%) demonstrating that considerable sequence similarity exists between A_1 and D_5 genomes. The incubation criterion (0.18 *M* Na^+, 60 °C) would exclude the formation of intergenomic duplexes divergent beyond approximately 15% (Britten et al. 1974). Presumably, the fraction that fails to form stable duplexes (ca. 22%) contains these more divergent sequences. This was supported, in part, by hybridizations at a less stringent criterion (0.18 *M* Na^+, 50 °C), where approximately half of the unrenatured fraction formed stable duplexes (data not shown).

Interspecific hybridizations of single copy DNAs from G. herbaceum and G. raimondii with DNA from G. hirsutum

Single copy tracers from the two diploids were hybridized with excess total DNA from the allotetraploid, *G. hirsutum*, and T_ms determined from duplexes formed at Cot 10,000. Average interspecific T_ms observed were 76.9 °C and 77.2 °C, corresponding to thermal depressions of 2.7 °C and 3.4 °C below that of intraspecific hybrids (Table 2). Recalling that substantial sequence similarity was detected between A_1 and D_5 sequences, a fraction of each diploid tracer would be expected to form duplexes with allotetraploid sequences of the divergent genome. The average thermal depression observed for interspecific duplexes between diploid sequences was 6 °C, or about twice the thermal depressions observed for interspecific duplexes with allotetraploid sequences. Therefore, to ascertain the degree of sequence similarity between cytologically similar genomes (A_1 with A_h with D_5, A_h) the contribution to thermal depression caused by that fraction of duplexes formed with sequences of the divergent genome (A_1 with D_h, D_5 with A_h) had to be evaluated.

Combining equations derived by Bonner et al. (1973) and Britten et al. (1978) for $n=2$ genomes, the expected thermal midpoint (T_e) for hybridizations involving identical and divergent sequences is estimated by:

$$T_e = [T_i + T_d \times (2\exp - [T_i - T_d]/10)]/[1 + 2\exp - (T_i - T_d)/10].$$

Substituting the observed T_m values of intraspecific and interspecific diploid hybrids for T_i and T_d, respectively, provides the estimated maxima T_ms for duplexes formed between single copy diploid tracers and allotetraploid sequences. T_e values for *G. herbaceum* and *G. raimondii* tracers are 77.4 °C and 78.0 °C, respectively. The difference between these values and the observed T_ms from reassociations with allotetraploid sequences provides the corrected measure for the divergence of single copy sequences between cytologically similar genomes. These values are, respectively, ≤ 0.5 and 0.8 (Table 2).

The reassociation kinetics of interspecific hybridizations between diploid tracers and allotetraploid sequences were evaluated by adjusting the rate order parameter to account for retardation effects caused primarily by tracer duplexes formed with sequences of the divergent genome (Fig. 2). Normalized values for the fraction of each tracer renatured at terminal Cot (Table 2) showed nearly complete reassociations of both tracers with allotetraploid sequences (97.2% for A_1 and 95.6% for D_5). These observations are consistent with the small differences estimated from thermal denaturations and suggest extensive sequence conservation between cytologically similar genomes.

Discussion

Cytogenetic data have established that of all the diploid *Gossypium* species, the genomes of *G. herbaceum* var. *africanum* (A_1) and *G. raimondii* (D_5) are most closely related to the two genomes (A_h and D_h) of the allotetraploid *G. hirsutum*.

Analyses of the diploid species, *G. herbaceum* and *G. raimondii*, and the allotetraploid, *G. hirsutum*, were conducted at two levels. First, the kinetic component of genomic DNA were determined from reassociations of total nuclear DNA. Second, interspecific hybridizations of single copy sequences were analysed for the extent of reassociation and the degree of sequence divergence. The former was of interest with respect to cytological observations relating to the difference in size of the chromosome complement between A and D genomes. Length measurements taken from nuclei containing both genomes had shown that A chromosomes, on average, were about twice the size of the D chromosomes (Skovsted 1934). From our estimates based on kinetics analyses, the DNA ratio between A_1 and D_5 genomes was 1.53:1 (Table 1). The major repetitive fraction of the A_1 genome (0.56 pg) compared to the D_5 genome (0.20 pg) accounts

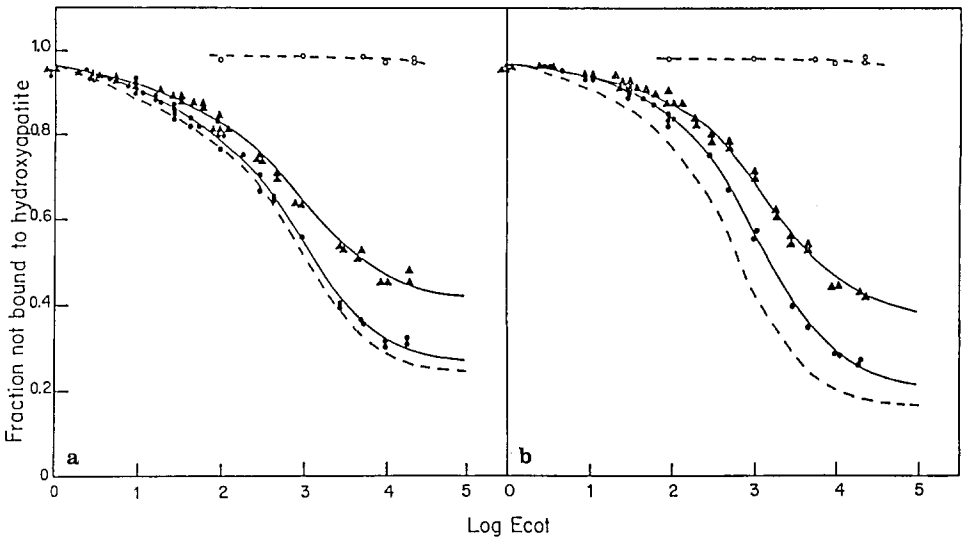

Fig. 2a and b. Interspecific hybridizations of single copy DNAs. Single copy tracers were prepared from each diploid species and hybridized with excess total DNA (driver) from the allotetraploid (•) or divergent diploid species (▲). Intraspecific hybridizations presented in Fig. 1 are indicated by the dashed curve. Data points were generated from the same labeled tracer preparations. Tracer self reassociations are indicated (o). Two components were fit to each curve, a major component representing the reassociation of single copy DNA and a minor component representing repetitive DNA. Rate constants for each were fixed at driver DNA values determined from the homologous reassociations in Fig. 1. The rate order of the reaction (n) was determined from the thermal denaturation values in Table 2 and using the equation: $n = 2 \exp -(T_i - T_d)/10$. **a** *G. herbaceum* tracer with *G. raimondii* driver: fraction reassociated (F) = 58%, $n = 0.64$. *G. herbaceum* tracer with *G. hirsutum* driver: F = 71%, $n = 0.83$. Homologous reassociation: F = 73%. **b** *G. raimondii* tracer with *G. herbaceum* driver: F = 61%, $n = 0.68$. *G. raimondii* tracer with *G. hirsutum* driver: F = 77%, $n = 0.79$. Homologous reassociation: F = 87%

for the difference in the total DNA content as there is little, if any, difference in the amount of single copy DNA (0.41 and 0.43 pg) (Table 1). In part, this is corroborated by data from interspecific single copy reassociations (Table 2), that showed that the relative fraction of duplexes formed with A_1 tracer and D_5 driver at terminal Cot (78.7%) approximately equals the fraction formed in the reciprocal hybridization with D_5 tracer and A_1 driver (76.7%). If a larger genome size was correlated with an increased single copy complexity, as has been reported among *Lathyrus* species (Hutchinson et al. 1980), then the fraction of interspecific hybrids formed with the A_1 tracer should be less than the fraction formed with the D_5 tracer.

Measurements of sequence similarity between diploid and allotetraploid DNAs showed extensive conservation between cytologically similar genomes. Under stringent criterion, A_1 and D_5 single copy tracers renatured to near completion with allotetraploid DNA (97.2% and 95.6% relative to homologous reassociations, respectively). Corrected estimates of thermal depression of duplexes formed between $A_1 \times A_h$, $D_5 \times D_h$ suggested near sequence identity ($\leqq 0.5$ °C and 0.8 °C relative to homologous duplexes, respectively). Differences in both measures of sequence similarity are small and probably not statistically significant. However, we note that this difference is consistent with previous chromosome pairing observations that indicated a closer relatedness of A_1 to A_h than D_5 to D_h (reviewed by Endrizzi et al. 1985).

A and D genomes are assumed to have diverged during the Cretaceous Period, but there is no fossil evidence to support this (Fryxell 1979). The assumption is primarily based on the observation that diploid species of different genomes occur on separate continents, suggesting that genomic divergence and geographic isolation was prior to or concurrent with continental drift. Consequently, we are surprised by the high degree of sequence similarity observed between A_1 and D_5 genomes, given their presumptive Cretaceous origin (65–135 MyBP). Whether this suggests that the rate of single copy divergence of tropical perennials is slower than what has been observed in other organismal lineages (Britten 1986), or that genomic divergence was post-Cretaceous, requires additional testing and independent confirmation.

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