



Figure A Overview of Steps Involved in a Cot Analysis. (A) Nuclear DNA is sheared into 200-500 bp fragments by high-speed blending, and (B) fragment size is checked by gel electrophoresis. (C) Sheared DNA is precipitated, dissolved in 0.03, 0.12, or 0.5 M sodium phosphate buffer (SPB), and aliquots are loaded/sealed into glass microcapillary tubes/ampoules. (D) One tube/ampoule is (E) placed in boiling water to denature DNA duplexes, and then transferred (F) into a water bath set at the DNA sample's "criterion", *i.e.*, $T_m - 25^\circ\text{C}$ for genomic DNA in the same SPB buffer as the sample. Renaturation is allowed to proceed to a specific Cot value (Cot = the product of nucleotide concentration, reassociation time, and an appropriate factor based upon the cation concentration of the buffer). (G) Once the sample has reached the desired Cot value, it is quickly diluted in a 100-fold excess of 0.03 M SPB, and (H) loaded onto a hydroxyapatite (HAP) column equilibrated with 0.03 M SPB. At this buffer concentration all DNA binds to the HAP. (I) 0.12 M SPB is added causing single-stranded DNA (ssDNA) to elute. (J) 0.50 M SPB is added to the column to elute double-stranded DNA (dsDNA). (K) The volumes and (L) A_{260} values (adjusted for light scatter at 320 nm) of the ssDNA eluant and the dsDNA eluant are used to determine the percentage of ssDNA (% ssDNA) for the Cot value (see Peterson et al. 1998 for details). Steps D-L are repeated for all the DNA samples with each sample being renatured to a different Cot value. (M) The logarithms of Cot values ranging from essentially no renaturation to nearly complete renaturation are plotted against corresponding % ssDNA values to yield a Cot curve. Because a DNA sequence reassociates at a rate that is directly proportional to the number of times it occurs in the genome, repetitive sequences reassociate at lower Cot values than single/low-copy sequences. Analysis of a Cot curve permits estimation of genome size, the number and relative proportion of the repetitive and single/low-copy components comprising the genome, and each component's 'kinetic complexity' (*i.e.*, its estimated sequence complexity).

Limitations of the EST Data

Approximately 41.9% of the HRCot, 50.0% of the MRCot, and 11.8% of the SLCot clones with significant hits to EST sequences also show primary GenBank Nr hits to known repeat sequences (see Table A below). While certain repetitive DNAs in the EST databases are contaminants (e.g., rDNA, chloroplast DNA), others represent transcribed portions of repetitive elements (e.g., retrotransposon genes). As there are many repetitive DNA sequences not in the GenBank Nr Database, it seems likely that some of the “EST-hit clones” (ECs) without significant homology to Nr database repeats (i.e., ^{Nr}ECs) might also represent repetitive DNA. Recognition of the same database entry by multiple clones is one means by which clone repetitiveness has been estimated (e.g., Bureau et al. 1996; Rabinowicz et al. 1999). Consequently, the significant EST hits for each of the 308 ^{Nr}ECs were compared to the significant EST hits of all other ECs (including other ^{Nr}ECs). If sorghum has roughly 25,000 non-repetitive gene sequences like *Arabidopsis* (*Arabidopsis* Genome Initiative 2000) and the 308 ^{Nr}ECs are single-copy sequences, the average expected number of hits by an ^{Nr}EC to any one of the hypothetical sorghum genes is $(308 \div 25,000 =) 0.0123$. The probability of multiple ^{Nr}ECs recognizing a particular “single-copy EST” (\approx gene) sequence by chance can be roughly estimated using the Poisson probability distribution function

[Equation A]
$$P(X) = \mu^X \div (e^\mu X!)$$

where P = probability, X = number of occurrences, and μ = is the population mean number of occurrences in a unit of space or time (Zar 1996). If $\mu = 0.0123$ (see above), the probabilities of two, three, four, and five ^{Nr}ECs recognizing the same single-copy EST by chance are 7.5×10^{-5} , 3.1×10^{-7} , 9.5×10^{-10} , and 2.3×10^{-12} , respectively. If sorghum has more than 25,000 genes, the likelihood of multiple clones having single-copy EST hits in common would be even lower. However, as shown in Table A (below), 140 of the 308 ^{Nr}ECs (i.e., 45.5%) show significant homology to at least one EST(s) identified by other Cot clones. This finding clearly indicates that many of the ^{Nr}ECs clones contain repetitive sequences. Interestingly, 88.1% of the ^{Nr}ECs in the SLCot library share no significant EST hits with other ECs while only 30.4% of the HRCot and 24.2% of the MRCot ^{Nr}ECs possess EST hits not shared by other ECs.

Probability of Significant BAC End/Cot Clone Homology

For a genomic library containing random pieces of genomic DNA, the probability (p) that the library contains a particular sequence of interest can be estimated using the formula

[Equation B]
$$p = 1 - e^{-n \cdot \ln[1 - (Z \div G)]}$$

where n = the number of clones in the library, Z = mean insert size in bp, and G = 1C genome size in bp (Paterson 1996). However, for a library containing DNA from a particular Cot component, the probability of finding a given sequence from that component can be estimated by replacing genome size in Equation B with the component's kinetic complexity. Assuming that the sorghum Cot libraries (mean insert size = 200 bp) are representative of the components from which they were derived, the likelihood of finding a particular HR component sequence among the 253 HRCot clones would be $p = 1 - e^{-253 \cdot \ln[1 - (200 \div 18,800)]} = 0.93$ or 93%. Likewise, the probability of finding a given MR sequence in the 409 MRCot clones would be 0.02 (2.0%) while the probability of finding a particular SL sequence in the 499 SLCot clones would be 6.1×10^{-4} (0.06%). Based on the Cot curve, the HR, MR, and SL components constitute 15, 41, and 24% of the sorghum genome, respectively. Thus the probability of a randomly selected sorghum sequence sharing significant sequence identity with a clone in one of the BAC libraries would be $[(0.93 \times 0.15) + (0.02 \times 0.41) + (0.0006 \times 0.24) =] 0.15$ or 15%.

As described in the *RESULTS* section *Molecular Genetic Markers, BAC End Sequences, and Cot Clones*, 17% of 116 random sorghum BAC end sequences possess significant homology to one or more of the 1161 sorghum Cot clones. The expected (15%) and observed (17%) numbers of BAC ends containing Cot clone sequences are statistically indistinguishable (see *Test of Significance of a Binomial Proportion* below).

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Test of Significance of a Binomial Proportion

The following statistical test was performed according to Snedecor and Cochran (1980).

H_0 : The expected number of BAC ends sharing sequence identity with Cot clones (np) is not significantly different from the observed number (r); 95% confidence level, i.e., if $z_c \geq 1.96$, reject the null hypothesis

n = 116 BAC ends

r = Observed number of successes, i.e., BLAST hits to Cot clones = 20

n – r = Observed number of failures = 96

p = Expected probability of successes = 0.15

q = Expected probability of failures = 0.85

np = Expected number of successes = (116 x 0.15) = 17.4

nq = Expected number of failures = (116 – 17.4) = 98.6

$z_c = (|r - np| - 0.5) \div \sqrt{npq}$

$z_c = 0.55$

0.55 is not ≥ 1.96 . Thus the expected and observed values are not significantly different.

Table A. Intra- and Interlibrary Comparison of Cot Clones With Significant Hits to ESTs.

Category	HRCot n = 253		MRCot n = 409		SLCot n = 499		HRCot + MRCot + SLCot n = 1161	
	#	%	#	%	#	%	Total #	Total %
ECs ^a	136	53.8	190	46.5	152	30.5	478	41.2
Repetitive ECs ^b	57	22.5	95	23.2	18	3.6	170	14.6
^{Nr} ECs ^c	79	31.2	95	23.2	134	26.9	308	26.5
^{Nr} EC Ψ ^d	HRCot n = 79		MRCot n = 95		SLCot n = 134		HRCot + MRCot + SLCot n = 308	
	#	%	#	%	#	%	Total #	Total %
0	24	30.4	23	24.2	118	88.1	165	53.6
1	8	10.1	13	13.7	4	3.0	25	8.1
2-5	17	21.5	16	16.8	3	2.2	36	11.7
6-10	24	30.4	14	14.7	0	0	38	12.3
> 10	6	7.6	29	30.5	9	6.7	44	14.3

^a ECs (EST-hit clones) are Cot clones with at least one significant hit to an EST sequence(s) in the GenBank EST and/or the SUCEST EST Databases.

^b Repetitive ECs are ECs with significant homology to known repeat sequences (e.g., rDNA, chloroplast DNA, retroelements, MITEs, etc.) in the GenBank ^{Nr} Database.

^c ^{Nr}ECs are ECs without significant homology to known repeat sequences in the GenBank ^{Nr} Database.

^d ^{Nr}ECs with a Ψ of “0” have no significant EST hits in common with other ECs, ^{Nr}ECs with a Ψ of “1” share an EST hit(s) with one other EC, etc. At the extreme, six ^{Nr}ECs in the HRCot library and 29 ^{Nr}ECs in the MRCot library possess significant homology to a SUCEST EST recognized by a total of 48 ECs (the remaining 13 clones with hits to this EST sequence show primary GenBank ^{Nr} hits to rDNA or chloroplast DNA).

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Table B. Densitometric Analysis of BAC Grids Probed With *Retrosor-6*.

Row	Description	<i>S. bicolor</i>	<i>S. propinquum</i>
A	Section IDV ^a	3,743,625	3,281,760
B	LowIDV ^b	1230	1002
C	HighIDV ^b	4760	3912
D	Range (C ÷ B)	3.87	3.90
E	Copies of <i>Retrosor-6</i> per section (A ÷ B)	3043.6	3275.2
F	BAC clones per section	3072	3072
G	Mean BAC insert size (bp) ^c	120,000	126,000
H	BAC insert DNA per section in bp (F x G)	368,640,000	387,072,000
I	Genome size in bp ^d	760,000,000	772,000,000
J	Fraction of genome in a section (H ÷ I)	0.49	0.50
K	Copies of <i>Retrosor-6</i> per genome (E ÷ J)	6211.4	6550.4
L	Size of <i>Retrosor-6</i> in bp	7377	7377
M	Bp of <i>Retrosor-6</i> in genome (K x L)	45,821,498	48,322,301
N	Genome fraction in <i>Retrosor-6</i> (M ÷ I)	0.060	0.063

^a IDV or “Integrated Density Value” is the sum of all pixel values after background correction. Section IDV is the IDV of an entire grid section, i.e., one-sixth of a BAC grid (see Figure 4E), divided by two to correct for double-spotting of clones.

^b LowIDV is the mean of the five colonies with the lowest IDVs while HighIDV is the mean of the five colonies with the highest IDVs.

^c Mean insert size for *S. bicolor* BTx623 BAC clones is from D. Begum., unpublished results (see www.clemson.genome.edu). Mean insert size for *S. propinquum* BAC clones is from Lin et al. (1999).

^d Genome size (1C) for *S. bicolor* is from Arumuganathan and Earle (1991). The genome size for *S. propinquum* is from K. Arumuganathan, personal communication as cited in Lin et al. (1999).

Table C. Cot Clones Corresponding to Sorghum RFLP Markers.

Marker ^a	Map position(s) ^b	Cot clones ^c	BLAST category ^d
AEST602	C:46.2	HRCot1C02	rDNA
CO152	C:46.2	HRCot2E08, MRCot4G11, HRCot2E03	rDNA
PRC0015	C:46.0	HRCot2A02	rDNA
PRC1151	A:59.3	SLCot6G11	Characterized gene ^e
pSB0415	H:40	HRCot3F09, HRCot3F06, HRCot3B12, MRCot1F11	No significant hits
pSB0986	B:69.3	SLCot1C09	No significant hits
pSB1021	C:91.6	SLCot4H08	No significant hits
pSB1524	F:75.4	MRCot1F03	No significant hits
RZ014	A:112.4	SLCot4B11	Unique EST

^a The molecular genetic marker to which one or more Cot clones shares significant homology ($S' \geq 76.28$).

^b *Sorghum bicolor* linkage group (traditionally represented by letters A through J) and map position (cM) are separated by a colon (e.g., B:33.5).

^c Cot clones producing a significant hit when BLASTed against all the marker sequences on the sorghum molecular genetic map

^d BLAST category is the group into which a Cot clone(s) was placed based upon the scheme shown in Figure 2.

^e The characterized gene recognized by SLCot6G11 is the *Zea mays peroxidase-3* (*pox3*) gene (GenBank AJ401276).

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Molecular Markers

The sorghum Cot clone sequences were compared to molecular markers on the sorghum molecular map. The GenBank accession numbers for these molecular markers are listed below:

AA011857, AA011861, AA011862, AA011863, AA011865, AA011867, AA011873, AA011877, AA011880, AA011883, AA030706, AA030709, AA030712, AA030714, AA030715, AA030716, AA030717, AA030718, AA030721, AA051875, AA051881, AA051886, AA051888, AA051890, AA051892, AA051895, AA051898, AA051899, AA051902, AA054786, AA054787, AA054789, AA054794, AA054796, AA054801, AA054818, AA054821, AA072429, AA072430, AA072431, AA072432, AA072434, AA072437, AA072438, AA072439, AA072440, AA072443, AA072444, AA072446, AA072447, AA072449, AA072453, AA072454, AA072455, AA072460, AA072462, AA072463, AA072466, AA143899, AA143901, AA143905, AA143910, AA143914, AA143915, AA143917, AA143918, AA143919, AA143920, AA143921, AA143922, AA143923, AA143925, AA143928, AA143929, AA143931, AA143932, AA231639, AA231640, AA231642, AA231646, AA231649, AA231651, AA231652, AA231654, AA231655, AA231660, AA231661, AA231674, AA231679, AA231687, AA231695, AA231697, AA231699, AA231703, AA231718, AA231728, AA231732, AA231733, AA231738, AA231741, AA231743, AA231745, AA231746, AA231749, AA231752, AA231754, AA231758, AA231761, AA231763, AA231764, AA231774, AA231776, AA231779, AA231781, AA231782, AA231784, AA231788, AA231797, AA231805, AA231807, AA231810, AA231815, AA231822, AA231827, AA231828, AA231834, AA231835, AA231836, AA231837, AA231838, AA231839, AA231850, AA231852, AA231855, AA231858, AA231859, AA231860, AA231861, AA231864, AA231871, AA231872, AA231874, AA231881, AA231883, AA231885, AA231886, AA231888,

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