

Organization of the ribosomal ribonucleic acid genes in various wild-type strains and wild-collected strains of *Neurospora*

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Summary. The organization of the ribosomal DNA (rDNA) repeat unit in the standard wild-type strain of *Neurospora crassa*, 74-OR23-1A, and in 30 other wild-type strains and wild-collected strains of *N. crassa*, *N. tetrasperma*, *N. sitophila*, *N. intermedia*, and *N. discreta* isolated from nature, was investigated by restriction enzyme digestion of genomic DNA, and probing of the Southern-blotted DNA fragments with specific cloned pieces of the rDNA unit from 74-OR23-1A. The size of the rDNA unit in 74-OR23-1A was shown to be 9.20 kilobase pairs (kb) from blotting data, and the average for all strains was 9.11 ± 0.21 kb; standard error = 0.038; coefficient of variation (C.V.) = 2.34%. These data indicate that the rDNA repeat unit size has been highly conserved among the *Neurospora* strains investigated. However, while all strains have a conserved HindIII site near the 5' end of the 25 S rDNA coding sequence, a polymorphism in the number and/or position of HindIII sites in the nontranscribed spacer region was found between strains. The 74-OR23-1A strain has two HindIII sites in the spacer, while others have from 0 to at least 3. This restriction site polymorphism is strain-specific and not species-specific. It was confirmed for some strains by restriction analysis of clones containing most of the rDNA repeat unit. The current restriction map of the 74-OR23-1A rDNA repeat unit is presented.

Introduction

The rRNA genes in eukaryotes are generally arranged in a tandemly-repeated head-to-tail array. These genes are of interest, for example, because: (a) the number of repeat units in the array is maintained approximately constant from generation to generation; (b) at least the rRNA coding sequences within the tandem repeats are homogeneous; and (c) meiotic recombination within the array is much lower than that found elsewhere in the genome, at least in some systems (Petes and Botstein 1977; Petes 1979). Free et al. (1979) presented some evidence that the standard St. Law-

rence wild-type strain of *Neurospora crassa* contains rDNA coding for 17 S, 5.8 S and 25 S rRNAs organized as a tandemly-repeated 9.23 kb (6.0 MDa) sequence. About 200–220 copies of the repeat unit are present in the *N. crassa* 74-OR23-1A standard wild-type strain (Krumlauf and Marzluf 1979, 1980; Rodland and Russell 1982). A 5.9 kb segment of the repeat unit containing the three rRNA coding sequences was cloned and a restriction map constructed of the clone (Free et al. 1979). The 5S rRNA genes are not found within the cluster but are scattered through the genome (Selker et al. 1981; Metzenberg et al. 1983).

The organization of *N. crassa* rDNA was also studied by Cox and Peden (1979) using gene cloning and restriction enzyme analysis. They reported a repeat unit length of 8.6 kb in the strain *rec-1; cog⁺; cot-1; his-3* (R.A. Cox, personal communication) which has a different genetic background from the wild-type strain used by Free et al. (1979).

In this paper, data for the size and restriction enzyme sites of the complete rDNA repeat unit of the 74-OR23-1A strain of *N. crassa* are presented. In addition, the extent of variation between the rDNA of wild-type and wild-collected strains of *Neurospora* representing five species is reported. Our data indicate a restriction enzyme site polymorphism between strains, and show that the interstrain length variation in rDNA repeat units is relatively small.

Materials and methods

Neurospora strains, media and culture conditions. The growth media (Vogel's minimal and Vogel's complete) and culture techniques used were described previously (Russell et al. 1976; Schlitt and Russell 1974). The wild-type and geographically-isolated wild-collected strains of *N. crassa*, *N. intermedia*, *N. sitophila*, *N. tetrasperma*, and *N. discreta* used in this study are listed in Table 1. The wild-type strain of *N. crassa* used as a "reference" or typestrain is the Oak Ridge-derived St. Lawrence strain 74-OR23-1A. All strains were obtained from the Fungal Genetics Stock Center (FGSC), Humboldt State University Foundation, Arcata, California, except strain 74A, which was from A.M. Srb, Cornell University, Ithaca, N.Y.

DNA extraction from *Neurospora*. Mycelia were grown to stationary phase in 20 ml of liquid Vogel's minimal. Typically, this took 3 days at 25° C. Mycelial pads were har-

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Table 1. *Neurospora* strains used in the investigation

Strain	Mat- ing type	Spe- cies ^a	FGSC ^b no.	Origin
Wild-type strains ^c				
74-OR23-1A ^d (St. Lawrence)	A	cra	—	Marrero, Louisiana
74-OR23-1VA ^d (St. Lawrence)	A	cra	2489	Marrero, Louisiana
74A ^d (St. Lawrence)	A	cra	—	Marrero, Louisiana
STA ^d (St. Lawrence)	A	cra	262	Marrero, Louisiana
ORSa ^d (St. Lawrence)	a	cra	2490	Marrero, Louisiana
3.1a ^d (St. Lawrence)	a	cra	935	Marrero, Louisiana
1A ^d (Beadle and Tatum)	A	cra	354	Marrero, Louisiana
25a ^d (Beadle and Tatum)	a	cra	353	Marrero, Louisiana
EM 5256 (Emerson)	A	cra	424	Marrero, Louisiana
EM 5297 (Emerson)	a	cra	627	Marrero, Louisiana
<i>rec-1</i> ; <i>cog</i> ⁺ ; <i>cot-1</i> ; <i>his-3</i> ^e (Emerson)	A	cra	2564	Laboratory derived
85	A	tet	1270	
Wild-collected strains				
Aarey-1e Costa Rica	A	cra	2499	Bombay, India
Houma-1n	A	cra	851	Coto, Costa Rica
North Africa I	A	cra	2220	Houma, Louisiana
Panama CZ30.6	A	cra	430	Adiopodoume, Ivory Coast
Puerto Rico 18	a	cra	1131	Canal Zone
2	a	sit	429	Puerto Rico
Obama-1b	a	sit	1779	Chichester, U.K.
Panama 4NHB6B	A	sit	1765	Obama, Kyushu
Panama UP203	A	sit	1135	Jaques, Panama
Bodjongloa Djalan	a	sit	1134	Canal Zone
Hanalei	A	int	2560	Standard ontjom (peanut waste) strain; Bandung
Honduras	a	int	3722	Hanalei, Kauai
Liberia 4	a	int	1300	LaLima, Honduras
Philippine Islands 4	a	int	434	Liberia
Singapore-1b	a	int	433	Philippines
Singapore-2	a	int	1812	Singapore
Kirbyville-6	A	dis	436	Singapore
Santa Maria	a	dis	3228	Kirbyville, Texas
			3319	Santa Maria, Guatemala

^a cra = *N. crassa*; tet = *N. tetrasperma*; sit = *N. sitophila*; int = *N. intermedia*; dis = *N. discreta*

^b FGSC = Fungal Genetics Stock Center

^c In parentheses is the designated genetic background for each strain

^d "Laboratory wild-type" strains. All others are natural isolates that have been through relatively few transfers under laboratory conditions

^e Strain from which the rDNA repeat unit was cloned by Cox and Peden (1979)

vested onto Whatman no. 2 filters by vacuum filtration, dried by vacuum desiccation, and then pulverized in glass centrifuge tubes by vortexing with three 4 mm glass beads for 30 to 60 s. The powder was suspended by gentle agitation in 1.8 ml of an extraction buffer consisting of 250 mM

triethylamine-ethylene diaminetetraacetate (TEA-EDTA), pH 8.0, 0.5% (v/v) Triton X-100, 50 mM NaCl, and 0.25 mg/ml Pronase (Sigma Chemical Co., protease type XIV). The suspension was incubated at 37° C for 2 to 3 days with gentle agitation. The beads and cell debris were removed by centrifugation, and the supernatant was treated with 5 ml of cold 100% ethanol. The remainder of the DNA purification procedure was essentially that described in Rodland and Russell (1982), with proportionately smaller volumes. When larger amounts of DNA were required, larger cultures consisting of germinated conidia were harvested, and the cells were disrupted by a French pressure cell as described in Rodland and Russell (1982).

Bacterial strains, plasmids, and media. Plasmid pBR322 was used as the cloning vector. pMF2 (Free et al. 1979), pKD002, and pKD003 are pBR322-based recombinant plasmids containing various segments of the rDNA repeat unit of *N. crassa* strain 74-OR23-1A (see Fig. 1). Bacteria were grown in Luria-Bertani medium (L broth) supplemented with 10 µg/ml cycloheximide to guard against yeast or mold contaminants, and 20 µg/ml ampicillin or 2 µg/ml tetracycline (as appropriate) for growth of strains containing recombinant plasmids. Ampicillin at 200 µg/ml and tetracycline at 12.5 µg/ml were present in L agar (1.5%) plates when needed for selection. Plasmids were isolated from transformed liquid cultures of *Escherichia coli* RR1 (a *recA*⁺ derivative of strain HB101) using standard procedures (see, e.g. Maniatis et al. 1982).

Restriction enzyme digestion and electrophoresis. Restriction enzymes were obtained from Bethesda Research Laboratories, Gaithersburg, Maryland, and were used according to the supplier's instructions. DNA fragments resulting from the restriction enzyme digestion were analyzed in 1.0% agarose gels made up in a Tris-acetate buffer. *Hind*III + *Eco*RI cut lambda DNA was used as the fragment size marker. After staining in ethidium bromide, gels were photographed with Polaroid Type 57 film using long-wave UV subillumination.

Hybridizations. The DNA fragments on agarose gels were transferred to the nylon-based GeneScreen or GeneScreen Plus hybridization membranes (New England Nuclear, Boston, Massachusetts) by capillary blotting using 20 × SSC (3.0 M NaCl, 0.3 M sodium citrate) essentially as described by Southern (1975). The membranes were dried for 2 h in air; baking at 80° C was not necessary to bind DNA to the membranes. With the substitution of GeneScreen for nitrocellulose, the methods of Thayer (1979) were followed in fixing *E. coli* colonies containing recombinant DNA plasmids to the hybridization membrane. Colonies were replicated to the membrane from cultures in microtiter dishes.

Nick translation of probe DNA in the presence of either ³²P-dCTP or ³⁵S-dATPαS used the procedure of Rigby et al. (1977) as modified by Rodland and Russell (1982). Gel blots were hybridized with ³²P-labeled probes, and colony blots with ³⁵S-labeled probes. Hybridizations were done at 65° C using standard procedures.

Molecular cloning. Standard procedures were used (e.g. Maniatis et al. 1982). All enzymes used were from Bethesda Research Laboratories, Gaithersburg, Maryland. Since the inserts had identical ends, the cut pBR322 vector was incubated with alkaline phosphatase before ligation. Plasmids

containing the desired rDNA recombinant fragments were identified by transferring bacterial colonies exhibiting the desired antibiotic resistance to microtiter wells containing Luria broth supplemented with cycloheximide and the selective antibiotic. The colonies were amplified by overnight incubation at 37° C, then transferred to GeneScreen or GeneScreen Plus placed directly on nutrient agar plates supplemented with appropriate antibiotics. A replica plating device consisting of a grid of stainless steel bolts was used to inoculate the membranes, producing reproducible patterns of colonies on various selective media. The colonies were incubated overnight at 37° C, and grew vigorously on the nylon membranes. The bacterial cells were lysed, and the DNA denatured and neutralized following the procedure of Thayer (1980). The membranes were pre-hybridized and hybridized with appropriate probes after air-drying alone. The rDNA clones were also confirmed by restriction enzyme analysis.

Results and discussion

Probe construction

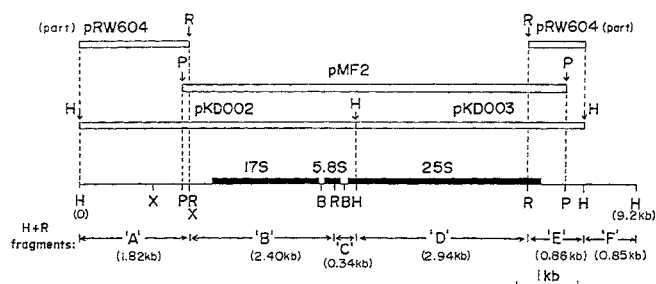
Figure 1 shows the *N. crassa* rDNA repeat unit probes used in the study, and shows a skeleton map of the whole repeat unit deduced from these experiments. In all cases the DNA fragment was from strain 74-OR23-1A and the plasmid vector was pBR322. Clone pMF2 has a *PstI*–*PstI* insert containing the 17 S, 5.8 S, and 25 S rRNA coding sequences plus spacer material (Free et al. 1979). Clones pKD002 and pKD003 have *HindIII*–*HindIII* inserts and were obtained using pMF2 as a probe for overlapping material. pKD002 contains the complete 17 S and 5.8 S rRNA coding sequences, a very small portion of the 5' end of the 25 S rRNA sequence, and spacer material to the 5' end of the 17 S sequence. pKD003 contains the remainder of the 25 S rRNA coding sequence plus spacer material 3' to it. Clone pRW604 contains two *HindIII*–*EcoRI* fragments; one in common with pKD002 and one in common with pKD003 (fragments 'A' and 'E', respectively, which were pieced together in vitro: see Fig. 1) inserted into the *EcoRI* site of pBR322. Thus, pRW604 contains mostly spacer material. In genomic DNA, 'A' and 'E' are separated by 'F'.

Variation in the rDNA repeat unit between strains?

A study was made of the available large array of wild-type and wild-collected strains of several *Neurospora* species to assess the degree to which the rDNA repeat unit varies, particularly with regard to size.

The wild-type strain 74-OR23-1A is the standard reference strain in these studies. Restriction enzyme mapping experiments were done with pKD002, pKD003, and pMF2 using *HindIII* and *EcoRI*. Five fragments, 'A' to 'E' (Fig. 1), collectively constitute the rDNA segment spanned by these clones. The sizes of these fragments shown in Fig. 1 are the mean values of measurements obtained from restriction mapping experiments of the clones, and from experiments in which blots of *HindIII*+*EcoRI*-cut genomic DNA were probed with rDNA clones shown in Fig. 1. The sum of fragments 'A' through 'E' for the 74-OR23-1A type-strain is 8.36 kb.

Significant variation in the size of the non-transcribed spacer within or between organisms has been observed, for



Enzyme key: H = *HindIII*; R = *EcoRI*; X = *XhoI*; P = *PstI*; B = *BamHI*

Fig. 1. Organization of the rDNA repeat unit in *N. crassa* 74-OR23-1A as determined by mapping the known plasmid clones pMF2, pKD002, pKD003 and pRW604 (the open rectangles in the figure). The coding regions for 17 S, 5.8 S and 25 S rRNAs are shown as solid rectangles. The five fragments produced by digestion with *HindIII*+*EcoRI* are designated 'A' through 'E'. The sixth fragment, 'F', has been detected by the experiments described in this paper.

example in *Xenopus laevis* (Botchan et al. 1977), in mice and humans (Arnheim and Southern 1977), and in *Drosophila* (Rae et al. 1981; Wellauer and Dawid 1977, 1978). We wished to determine if significant rDNA size variation exists within or between strains of *Neurospora*. Blots of *HindIII*+*EcoRI*-cut genomic DNA were hybridized with nick-translated pRW604 and pMF2, separately and simultaneously. Because we did not at the time appreciate the existence of fragment 'F' nor detect it with the probes shown in Fig. 1, we measured the fragments which did have homology to those probes. Substantial variation within strains would have appeared as additional bands in the pattern, or as broadening of the major bands. While some very minor bands with rDNA homology could be observed on heavily exposed autoradiograms, these were of an intensity which suggested that they could be single-copy DNA, perhaps junctions between rDNA and other DNA species (Zamb and Petes 1982). The sharpness of the major bands was consistent with there being no size-microheterogeneity.

Variation between strains is shown in Table 2, which gives the mean sizes of the presumptive 'A', 'B', 'D', and 'E' fragments in the strains tested. (The very small 'C' fragment was omitted from the table since it is constant within experimental error in all strains, the best size estimate being 0.34 kb.) The data also indicate that fragments 'B', 'D', and 'E' are constant between strains, the only exceptions being the Aarey-1e and Santa Maria strains, in which nothing corresponding to fragment 'E' is seen. In contrast, there is an obvious size variation in the fragment which corresponds to 'A' in the 74-OR23-1A type-strain. The range was from 1.11 kb in Liberia 4 to 3.81 kb in Aarey-1e. Less variation is seen in the sum of the fragment sizes 'A'–'E' if the size of 'E' is taken as zero when it is absent.

The fragment corresponding to 'A' which exhibits size variation is a *HindIII*–*EcoRI* fragment containing no mature rRNA-coding sequences, only spacer, most of which is probably nontranscribed.

To test the apparent spacer size variability more directly, *EcoRI*-cut genomic DNA was hybridized with the same probes as before. There are three *EcoRI* cleavage sites within the rDNA of 74-OR23-1A and *EcoRI* digestion produces

Table 2. Sizes (in kb) of the presumptive 'A', 'B', 'D', and 'E' fragments produced by cutting the rDNA of *Neurospora* strains with *Hind*III + *Eco*RI^a

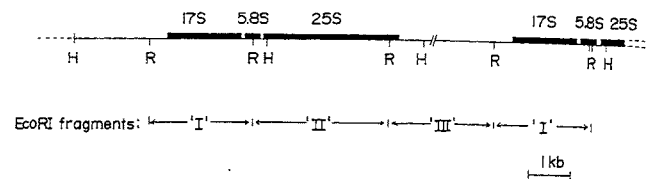
Strain	Species ^b	Size of fragment				Total ^c
		'A'	'B'	'D'	'E'	
Wild-type strains						
74-OR23-1A	cra	1.82	2.40	2.94	0.86	8.36
74-OR23-1VA	cra	1.90	2.38	2.90	0.92	8.44
74A	cra	1.82	2.33	2.80	0.85	8.14
STA	cra	1.90	2.36	2.93	0.91	8.44
ORSa	cra	1.85	2.29	2.84	0.88	8.20
3.1a	cra	1.83	2.34	2.85	0.88	8.24
1A	cra	1.83	2.29	2.85	0.90	8.21
25a	cra	2.90	2.34	2.89	0.90	9.37
Em 5256	cra	1.74	2.36	2.96	0.95	8.01
Em 5297	cra	2.85	2.33	2.79	0.85	9.14
<i>rec-1</i> ; <i>cog</i> ⁺ ; <i>cot-1</i> ; <i>his-3</i>	cra	2.82	2.30	2.78	0.93	9.17
85	tet	2.75	2.36	2.87	0.86	9.18
Wild-collected strains						
Aarey-1e	cra	3.92	2.35	2.85	-	9.46
Costa Rica	cra	2.87	2.37	2.86	0.99	9.43
Houma-1n	cra	2.91	2.33	2.83	0.84	9.25
North Africa I	cra	2.84	2.33	2.87	0.87	9.25
Panama CZ30.6	cra	1.90	2.35	2.88	0.93	8.40
Puerto Rico 18	cra	2.96	2.35	2.86	0.93	9.44
2	sit	1.69	2.32	2.87	0.89	8.11
Obama-1b	sit	1.77	2.37	2.90	0.88	8.26
Panama 4NHB6B	sit	2.41	2.32	2.85	0.90	8.82
Panama UP203	sit	1.72	2.33	2.87	0.91	8.17
Bodjongloa Djalan	int	1.41	2.41	2.91	0.95	8.02
Hanalei	int	1.72	2.39	2.90	0.92	8.27
Honduras	int	1.18	2.35	2.87	0.97	7.71
Liberia 4	int	1.14	2.27	2.85	0.88	7.48
Philippine Islands 4	int	2.34	2.35	2.89	0.80	8.88
Singapore-1b	int	2.92	2.36	2.89	0.86	9.37
Singapore-2	int	1.80	2.36	2.94	0.95	8.39
Kirbyville-6	dis	2.98	2.35	2.92	0.95	9.54
Santa Maria	dis	3.58	2.31	2.79	-	9.02
Statistics:						
	<i>n</i> = 31	31	31	29	31	
Mean (\bar{x}) =	2.26	2.34	2.87	0.903	8.65	
Std. dev. (<i>s</i>) =	0.695	0.032	0.045	0.040	0.589	
Std. error (S.E.) =	0.125	0.006	0.0080	0.0074	0.106	
Coefficient of variation	(C.V.) = 30.8%	1.36%	1.56%	4.39%	6.82%	

^a The sizes presented are the mean values of at least three independent determinations. The C.V. was computed for each replicate sample set: the mean was 4.8% and the maximum was 13.0%. The fragment nomenclature is from Fig. 1

^b Species abbreviations are as in Table 1

^c The total value given is that for all fragments identified by the hybridization experiments and hence includes the 'C' fragment value of 0.34 kb in each case

three fragments, designated 'I', 'II', and 'III' in Fig. 2. Fragment 'I' is the same as fragment 'B' in the *Hind*III + *Eco*RI experiment. Fragment 'II' contains the 3' "half" of the 5.8 S sequence, the spacer between the 5.8 S and 25 S, and all but a small 3' piece of the 25 S coding sequence. Fragment 'III' contains the rest of the 25 S sequence, and most of the spacer material of the rDNA repeat unit; this fragment spans adjacent rDNA repeat units. The initial (incorrect) hypothesis was that fragment 'III' con-



Enzyme key: H = *Hind*III; R = *Eco*RI

Fig. 2. Locations of the *Eco*RI restriction enzyme sites in the rDNA repeat unit of 74-OR23-1A. *Eco*RI digestion produces three fragments, designated 'I' through 'III'

tains only sequences of the variable-sized fragment 'A' plus the usually-constant-sized fragment 'E'; therefore it should show the same extent of size variation as does fragment 'A' alone. The results in Table 3 show that there is relatively little size variation in fragment 'III' – certainly less than the size variation in the fragment corresponding to 'A'. Fragment 'III' does show more variation in size than do fragments 'I' or 'II' (see statistics in Table 3).

The data in Table 3 indicate that all but one of the strains have three *Eco*RI sites like the standard 74-OR23-1A wild-type strain. In view of the relative constancy of the size of the *Eco*RI fragments produced in genomic digests, it may be concluded that the *Eco*RI cleavage sites have been conserved during the evolution of the *Neurospora* strains represented in this study. The exceptional strain is Bodjongloa Djalan in which four fragments were produced by *Eco*RI digestion of the rDNA. Two of the fragments correspond to 'I' and 'II' of the other strains. The other two fragments, of 1.92 and 1.35 kb, both hybridize with the pRW604 probe, and hence consist of spacer material. The simplest hypothesis is that there is an extra *Eco*RI site in the spacer region of this strain.

Comparison of the data in Tables 2 and 3 indicates that, in many cases, the total size of the recognized rDNA unit is significantly greater in the *Eco*RI experiment (Table 3) than in the *Hind*III + *Eco*RI experiment (Table 2). For example, the size of the rDNA unit in 74-OR23-1A was calculated to be 8.36 kb both from mapping the known clones of this region, and analysis of the *Hind*III + *Eco*RI genomic blot data, while the *Eco*RI blot data indicate a size of 9.20 kb. The data are best explained by polymorphism in the position and/or number of *Hind*III sites in the spacer region. In 74-OR23-1A, for example, if there were two *Hind*III sites in the spacer, then a *Hind*III to *Hind*III fragment would not be detected in *Hind*III + *Eco*RI cuts since neither the pKD002 or pKD003 probes (which were "walked out" from a *Pst*I – *Pst*I fragment to the *Hind*III sites: see Fig. 1) would contain that sequence. The *Hind*III – *Hind*III piece would be present in the *Eco*RI – *Eco*RI fragment 'III' and, by subtracting the Table 2 total from the Table 3 total, it would be about 0.85 kb.

Among wild-type *N. crassa* strains (and their derivatives), Em 5297, and *rec-1*; *cog*⁺; *cot-1*; *his-3* apparently have only one *Hind*III site in the non-transcribed spacer, the one equivalent to the left one of the pair in the 74-OR23-1A typestrain. As a result, the fragment corresponding to 'A' of Table 2 appears substantially larger in these strains. Cox and Peden (1979) cloned rDNA from *rec-1*; *cog*⁺; *cot-1*; *his-3* (our reason for including it in this study), and they reported a repeat unit of 8.6 kb. Comparison of their restriction map with that of 74-OR23-1A (Fig. 1, and personal communication with R.A. Cox) indicated some size

Table 3. Sizes (in kb) of the presumptive 'I', 'II', and 'III' fragments produced by cutting the rDNA of *Neurospora* strains with *EcoRI*^a

Strain	Species ^b	Size of fragment			Total
		'I'	'II'	'III'	
Wild-type strains					
74-OR23-1A	cra	2.32	3.22	3.66	9.20
74-OR23-1VA	cra	2.29	3.27	3.77	9.33
74A	cra	2.35	3.08	3.59	9.02
STA	cra	2.35	3.23	3.88	9.46
ORSa	cra	2.25	3.19	3.82	9.26
3.1a	cra	2.23	3.15	3.68	9.06
1A	cra	2.25	3.17	3.69	9.11
25a	cra	2.30	3.25	3.74	9.29
Em 5256	cra	2.28	3.23	3.62	9.13
Em 5297	cra	2.22	3.06	3.57	8.85
<i>rec-1</i> ; <i>cog</i> ⁺ ; <i>cot-1</i> ; <i>his-3</i>	cra	2.24	3.12	3.63	8.99
85	tet	2.30	3.13	3.55	8.98
Wild-collected strains					
Aarey-1e	cra	2.35	3.29	3.80	9.44
Costa Rica	cra	2.32	3.23	3.75	9.30
Houma-1n	cra	2.32	3.22	3.79	9.33
North Africa I	cra	2.37	3.25	3.67	9.29
Panama CZ30.6	cra	2.30	3.28	3.71	9.29
Puerto Rico 18	cra	2.29	3.23	3.64	9.16
2	sit	2.27	3.20	3.54	9.01
Obama-1b	sit	2.23	3.15	3.60	8.98
Panama 4NHB6B	sit	2.33	3.25	3.48	9.06
Panama UP203	sit	2.30	3.22	3.59	9.11
Bodjongloa Djalan	int	2.25	3.19	1.92	8.72
				+1.36	
Hanalei	int	2.30	3.20	3.54	9.04
Honduras	int	2.32	3.25	3.32	8.89
Liberia 4	int	2.28	3.20	3.40	8.88
Philippine Islands 4	int	2.33	3.26	3.26	8.85
Singapore-1b	int	2.26	3.20	3.66	9.12
Singapore-2	int	2.32	3.24	3.75	9.31
Kirbyville-6	dis	2.32	3.34	3.73	9.29
Santa Maria	dis	2.16	3.03	3.35	8.54
Statistics:		n=	31	31	31
Mean (\bar{x})=			2.29	3.20	3.61
Std. dev. (s)=			0.046	0.068	0.160
Std. error (S.E.)=			0.008	0.012	0.029
Coefficient of variation					
(C.V.)=			2.02%	2.12%	4.44%
					2.34%

^a The sizes presented are the mean values of at least three independent determinations. The C.V. was computed for each replicate sample set: the mean was 2.3% and the maximum was 5.5%. The fragment nomenclature derives from Fig. 2

^b Species abbreviations are as in Table 1

difference in the fragment corresponding to 'A'. That difference may now be explained on the basis of *HindIII* site polymorphism, especially since their strain has an Emerson genetic background and is closely related to Em 5297. The one strain of *N. tetrasperma* included in our study, 85A, has a single *HindIII* site located very similarly, perhaps identically, to that in the *N. crassa* 25a and Em 5297. The inheritance of restriction site polymorphism can be checked against the known pedigree of these strains. According to Barratt (1962) the Emerson strains were derived many years

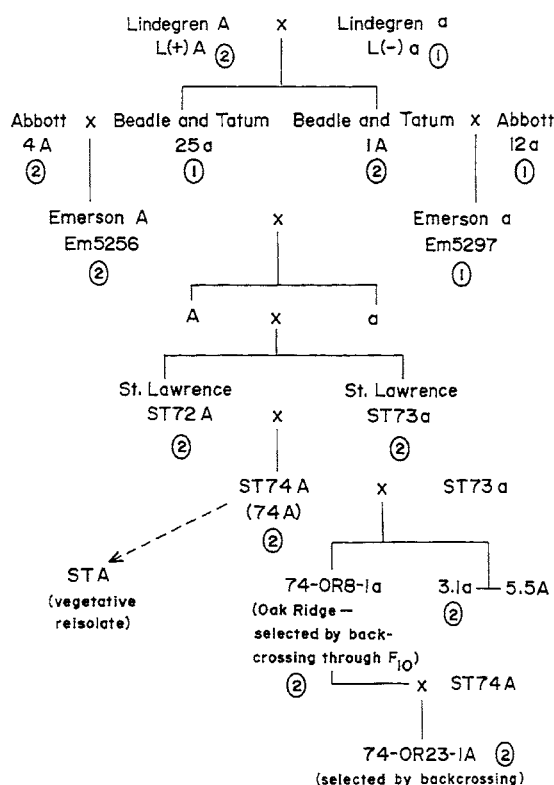
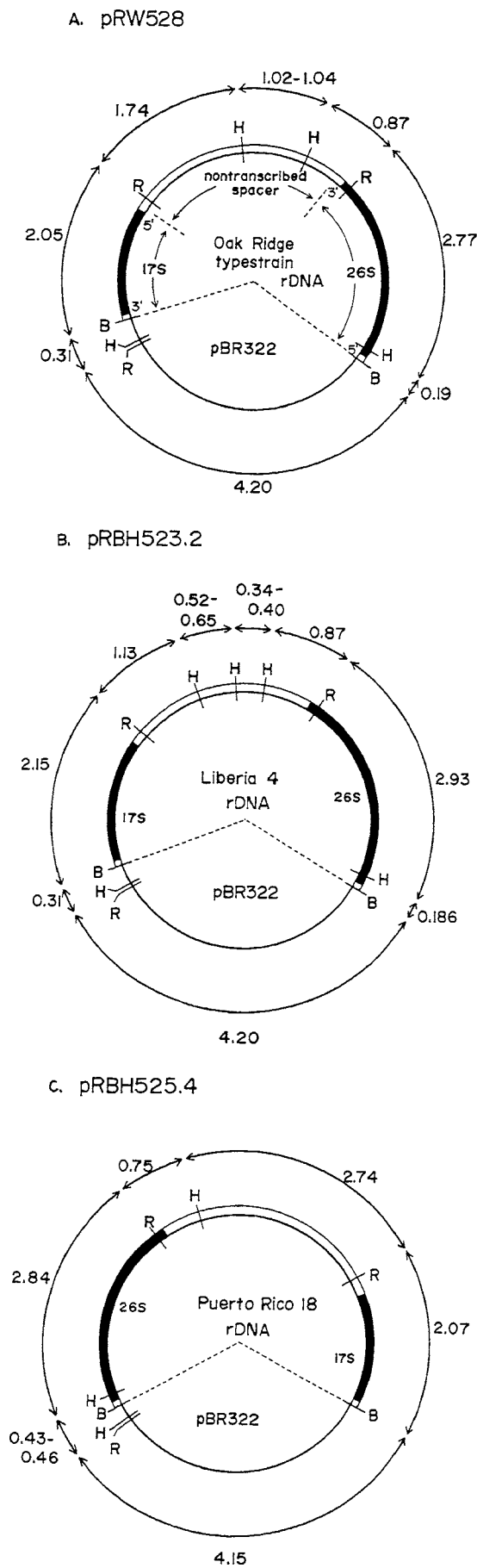


Fig. 3. Origin of some of the laboratory wild-type strains of *N. crassa* used in the study (adapted from Barratt 1962). The number of *HindIII* sites in the nontranscribed spacer region is indicated by the circled, bold-face number 1 or 2, and is based on this work and on other experiments not reported here. The Lindegren L(-)a is no longer available, and its genotype is inferred from its progeny

ago from crosses of strains which included 25a, and the two Emerson strains, Em 5297 and Em 5256, which have, respectively, one and two *HindIII* sites in their non-transcribed spacer, and are ancestors of the Oak Ridge strains. The inheritance of rDNA in commonly-used laboratory wild-type strains can be followed in the pedigree (Fig. 3).

The rDNA differences among wild-collected strains may also be understood on the basis of *HindIII* site polymorphism. The *N. crassa* strains Costa Rica, Houma-1n, North Africa I, Puerto Rico 18, the *N. intermedia* strains Philippine Islands 4, Singapore-1b, and the *N. discreta* strain Kirbyville-6 appear to have one *HindIII* site in the spacer like 25a etc., while *N. crassa* strain Panama CZ30.6, *N. sitophila* strains 2, Obama-1b, and Panama UP203, and *N. intermedia* strains Hanalei and Singapore-2 appear to have the 74-OR23-1A two-site pattern. The situation regarding the *N. sitophila* strain Panama 4NHB6B is not clear from the data since, while the *HindIII*-*EcoRI* fragment corresponding to 'A' is 0.50 kb larger than in 74-OR23-1A, the difference in total rDNA size for the two sets of cuts is only 0.20 kb. The fragment corresponding to 'A' in *N. intermedia* strains Honduras and Liberia 4 is significantly smaller than either that in 74-OR23-1A or the average size of that fragment for all strains. This means that there is a *HindIII* site in the spacer located closer to the *EcoRI* site near the start of the 17 S sequence than is the case in 74-OR23-1A. At least one other *HindIII* site must be present in each strain in view of the difference in 'total' rDNA size for



these strains in Tables 2 and 3. A similar situation appears to be the case in the Bodjongloa Djalan strain.

The fragment corresponding to 'A' in both the *N. crassa* Aarey-1e strain and the *N. discreta* Santa Maria strain is much larger than that seen in any other strain. However, given: (1) the absence of fragment 'E'; (2) the similarities in size of the presumptive fragment 'A' and the *EcoRI*—*EcoRI* fragment 'III' in Tables 2 and 3, respectively; and (3) the fact that a *HindIII* digest of genomic DNA produces one and not two fragments detectable by the available probes (data not shown), we conclude that there is only one *HindIII* site in the rDNA of these strains. That site is in the beginning of the 25 S sequence; no *HindIII* site is present in the spacer.

Cloning the rDNA

The *HindIII* site polymorphism hypothesis was proved for at least some of the strains by analyzing clones containing most of the rDNA repeat unit, including all of the spacer region showing the variation. The clones were made by inserting a large *BamHI*—*BamHI* fragment into the *BamHI* site of pBR322. Cutting the tandem set of rDNA units with *BamHI* produces two fragments: one is a very small fragment of about 0.3 kb containing the 5.8 S rRNA sequence and transcribed spacer material on either side; the other is a very large fragment spanning adjacent rDNA units and containing almost all of a repeat unit. Clearly the *BamHI* fragment can insert into the pBR322 in either of two possible orientations. Clones were obtained from strains 74-OR23-1A, Panama CZ30.6, Panama UP203, Liberia 4, and Puerto Rico 18. In some cases a number of replicate clones were obtained.

A restriction map was made for each clone using the enzymes *HindIII*, *EcoRI*, and *BamHI*. Each agarose gel was blotted to GeneScreen and the blots were hybridized with the probes described before in order to confirm the nature of each restriction fragment. Restriction maps for a number of the clones are shown in Fig. 4.

The map for clone pRW528 (Fig. 4A), containing the rDNA of the 74-OR23-1A type strain, clearly indicates the presence of two *HindIII* sites within the spacer. Thus, any cuts with *HindIII* would produce an approximately 1 kb piece that is not represented in any of the hybridization probes. The map for pRBH526 (from Panama CZ30.6) is essentially identical to that of pRW528, as expected from the data presented in Tables 2 and 3 (maps not shown). The rDNA insert in pRBH527 (from Panama UP203) is in the opposite orientation from the previous two, but the map is otherwise indistinguishable from them (map not shown).

The map for pRBH523.2 (Liberia 4; Fig. 4B) indicates at least three *HindIII* sites in the spacer region. The central *HindIII* site could be in either one of two possible locations relative to the other two sites. This explains the small size

Fig. 4A–C. Restriction maps of rDNA clones from several *Neurospora* wild-type strains. In each case, the insert is a *BamHI* to *BamHI* fragment. The distances shown are in kb, and the maps were drawn based on the sizes of the smallest fragments. A Map of clone pRW528: *N. crassa* 74-OR23-1A; B Map of clone pRBH523.2: *N. intermedia* Liberia 4; C Map of clone pRBH525.4: *N. crassa* Puerto Rico 18

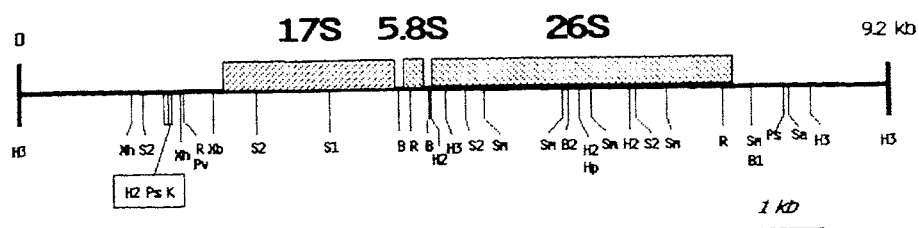


Fig. 5. Restriction map of the rDNA repeat unit of *N. crassa* strain 74-OR23-1A

Enzyme Key: B = BamHI ; H3 = HindIII ; Pv = PvuII ; S1 = SstI ;
 B1 = BglI ; Hp = HpaI ; R = EcoRI ; S2 = SstII ;
 B2 = BglII ; K = KpnI ; Sa = Sall ; Xb = XbaI ;
 H2 = HincII ; Ps = PstI ; Sm = SmaI ; Xh = XhoI

of the fragment corresponding to 'A' (Table 2) in this strain relative to that in 74-OR23-1A. However, the total length of the rDNA insert in the plasmid is significantly less than the repeat unit length calculated from the *EcoRI* genomic blots, even allowing for the small *BamHI* piece missing in the clone. Moreover, the *EcoRI* to *EcoRI* distance for the spacer region in the clone is 3.23 kb which is significantly larger than the sum of the small fragments represented in the Figure. We presume, therefore, that one or more *HindIII* sites were undetected in these experiments.

The map for pRBH525.4 (Puerto Rico 18: Fig. 4C) indicates only one *HindIII* site in the spacer, thus giving a much larger fragment 'A' than in 74-OR23-1A. Nonetheless, the overall length of the region between the *EcoRI* sites encompassing the spacer is approximately the same in each case.

Restriction map for the rDNA repeat unit of 74-OR23-1A

Given the new information about the size of the rDNA repeat unit in 74-OR23-1A, restriction mapping experiments were done with the existing clones containing various pieces of the repeat unit. The current restriction map is presented in Fig. 5.

Conclusion

The results show that the rDNA repeat unit in 31 strains of *Neurospora*, representing five species, is very similar in size. In all but one strain, three *EcoRI* sites located in, or close to the presumptive primary transcript coding region are highly conserved in their positions. The exceptional strain has an extra *EcoRI* site in the non-transcribed spacer region. Evidence was also obtained for polymorphism among strains in the number and/or relative positioning of *HindIII* sites in the NTS region. This polymorphism is strain-specific and not species-specific.

The essential uniformity of the rDNA repeat unit among *Neurospora* species, four of which are heterothallic and one of which (*N. tetrasperma*) is secondarily homothallic, indicates that the rDNA unit has been highly conserved during evolution, including the size of the non-transcribed spacer. This is in accord with the hypothesis that selection has a significant role in maintaining the parallel evolution of genetically separate but homologous repetitive gene clusters. There is, however, substantial variation in

the number and position of *HindIII* sites in the non-transcribed spacer DNA. It is somewhat surprising that no polymorphisms – indeed no sites – have been found in this region for other enzymes with six basepair recognition specificity. This may be purely coincidence, or it could possibly reflect the existence of similar but not identical domains within the region, each containing either a *HindIII* site or a sequence which can become a *HindIII* site by change of a single base pair. Only much more extensive restriction mapping and/or sequencing will distinguish between these possibilities.

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