Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*

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Summary. We find three interesting characteristics of the genes encoding the ribosomal RNA (rRNA) in the basidiomycete *Coprinus cinereus*. First, there are only 60 to 90 copies of the genes, fewer than in other fungi. Second, the genes are organized in an unusual arrangement. The 5S rRNA genes are located in the repeat unit which encodes the other rRNAs and all four rRNAs are transcribed in the same direction. Third, meiotic recombination is inhibited within the ribosomal DNA.

Key words: Ribosomal RNA genes – Recombination – *Coprinus*

Introduction

The organization of the genes encoding ribosomal RNA (rRNA) has been studied in many organisms. In eukaryotes, the genes encoding the 17–18S, 5.8S and 25–28S rRNAs are transcribed as one precursor which is then processed to produce the three rRNAs. There are multiple copies of these genes arranged as head to tail repeats of coding and spacer regions. This is referred to as the ribosomal DNA (rDNA). In higher eukaryotes, the 5S rRNA genes are not part of this repeat unit. Instead they are found in one or more clusters elsewhere in the genome. In lower eukaryotes the location of the 5S rRNA genes is variable. In *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Saccharomyces rosei*, and *Torulopsis utilis*, all ascomycetes, in *Achlya ambisexualis*, an oomycete, and in *Mucor racemosus*, a zygomycete, the DNA repeat containing the three larger rRNA genes also contains the 5S rRNA gene (Bell et al. 1977; Verbeet et

al. 1983; Tabata 1980; Rozek and Timberlake 1979; Cihlar and Sypherd 1980). In the ascomycetes *Neurospora crassa*, *Schizosaccharomyces pombe*, and *Aspergillus nidulans* the 5S rRNA genes are not located in the repeat encoding the other rRNA genes. Instead they are dispersed as single genes throughout the genome (Free et al. 1979; Selker et al. 1981; Tabata 1981; Mao et al. 1982; Bartnik et al. 1981; Borsuk et al. 1982; Lockington et al. 1982). It was of interest to determine the organization of the rRNA genes in the basidiomycete *Coprinus cinereus* since the location of the 5S rRNA genes in members of this higher fungal group has not been reported.

Coprinus cinereus is also an excellent organism in which to study the inheritance of rDNA. Tetrads of basidiospores can be easily recovered from dried gill segments (Moore 1966) and over 70 loci have been assigned to 8 linkage groups in this basidiomycete (Lewis and North 1974). In a previous study, we reported the occurrence of restriction enzyme recognition site polymorphisms in the rDNA (Wu et al. 1983). Our own cytological observations and those of Holm et al. (1981) indicate that there is a single nucleolus organizer in each haploid chromosome set in *Coprinus*. In this study, we have used two previously characterized polymorphisms to determine if the ribosomal RNA genes segregate as a single Mendelian locus and to monitor meiotic crossing-over between these genes.

Materials and methods

Coprinus monokaryotic strains used are listed in Table 1. Growth of the cultures was in liquid YMG medium (Rao and Niederpruen 1969) as described in Wu et al. (1983). Alternatively, 30 ml of medium were innoculated and grown 7–10 days at 37 °C in 100 mm petri dishes. Tetrads were isolated as described by

Table 1. List of strains used

Strain	ATCC#[a]	Geographic source	Genotype	Source
Penn-a (117)	42726d	USA		Moore
12890/3 (125)	12890d	USA (?)		Moore
Okayama-7 (130)		Japan		Moore
C692 (104)		England	*ade*3, A3, B1	Casselton
PJP52		England	*trp*1, *met*9	This laboratory

[a] d indicates derivative

Moore (1966) except a micromanipulator was not used. The isolation of plasmid pCc1, which contains one complete repeat of the rDNA from *Coprinus cinereus*, has been described (Wu et al. 1983).

DNA extraction. DNA extraction from large amounts of tissue has been previously described (Wu et al. 1983). Alternatively, for DNA extraction from small amounts of tissue, the tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle containing liquid nitrogen. This powder was added to lysis buffer (10 mM Trizma-HCl, pH 8.0, 0.05 M EDTA, 0.5% Sarkosyl, 100 μg/ml proteinase K) and incubated in a rotary shaking water bath at 50 °C for 2 h. Cellular debris was removed by sedimentation at 6,000 rpm in a Sorvall SS-34 rotor for 10 min at 4 °C. Carbohydrates were removed by sedimentation at 35,000 rpm for 1 h in a Beckman A1-40 rotor at 20 °C. CsCl (Varlacoid Chemical Company) was added to the supernatant (starting $\rho = 1.58$) and ethidium bromide was added to a final concentration of 100 μg/ml. Centrifugation was in the A1-40 rotor at 35,000 rpm for 36–40 h at 20 °C. Ethidium bromide was removed from the DNA by extraction with isopropanol saturated with H_2O and NaCl. The DNA was dialyzed against TE buffer (10 mM Trizma-Hcl, pH 8.0, 1 mM EDTA) to remove the CsCl and was concentrated by ethanol precipitation.

RNA extraction. RNA extraction was performed as previously described (Wu et al. 1983). Alternatively, RNA was extracted by the method of Chirgwin et al. (1979) and stored as an ethanol precipitate at −20 °C. The 26S and 18S rRNAs were isolated from other RNAs and from each other by sucrose gradient centrifugation (Brown and Littna 1964). The 5.8S and 5S rRNAs were separated from other RNAs and from each other by electrophoresis on 8% polyacrylamide gel and eluted by the method of Maxam and Gilbert (1977).

Blotting and probing. Gel electrophoresis, Southern transfer, DNA and RNA labeling, and hybridization were as previously described (Wu et al. 1983) except AMV reverse transcriptase (Life Sciences, Inc.) was used to synthesize complementary DNA (cDNA) using random calf thymus deoxyoligonucleotide primers and 5S or 5.8S rRNA templates (Maniatis et al. 1982). To obtain strand separation, pCc1 DNA was digested with BamHI and BglII. To a 20 μl solution containing 1 μg of digested DNA was added 10 μl 0.25 M EDTA, pH 8.0, and enough 2 N NaOH to bring the solution to pH 13. This DNA was then electrophoresed on a 1% agarose gel containing 2.5 μg/ml ethidium bromide. The gel was soaked for 30 min in 20 x SSC and the DNA transferred to nitrocellulose as before.

Preparation of R-loops. The plasmid pCc1 was linearized with HindIII which cuts once in the vector and does not cut in the insert. This DNA was cross-linked with Trioxsalen (Kaback et al. 1979) and the DNA was incubated with rRNA at 56 °C for 3 h following the suggestions of Kaback et al. (1979) for incubation and spreading.

Results

RNA sizes

To determine the organization of the rDNA in *Coprinus cinereus*, it was necessary to establish the sizes of the two large rRNAs. This was done by electrophoresis of the 26S and 18S rRNAs on an agarose gel after denaturation with glyoxal (McMaster and Carmichael 1977). Size markers were denatured lambda DNA cut with HindIII and EcoRI. The 26S rRNA was shown to be approximately 3,000 nucleotides in length and the 18S rRNA was shown to be approximately 1,700 nucleotides in length (data not shown). The sizes were independently determined by electron microscopy. R-loops were formed using the cloned copy of the rRNA genes and the 18S and 26S rRNAs (Fig. 1A). Measurements of 17 molecules indicated that the 26S rRNA is 3,000 ± 270 nucleotides (2,400 nucleotides + 600 nucleotides) and the 18S rRNA is 1,600 ± 330 nucleotides (Fig. 1B). These sizes are comparable to those found in *Neurospora* (Free et al. 1979) and *Saccharomyces carlsbergensis* (Klootwijk and Planta 1973).

Number of copies of the rDNA repeat

The *Coprinus* genome is 37,500 kb (Dutta 1974). One repeat of the rDNA has previously been shown to be 9.3 kb (Wu et al. 1983). Therefore, in one μg of genomic DNA, there would be 248 pg of a single copy sequence 9.3 kb in length. One μg of genomic DNA from strain Penn-a was digested with BamHI and run on a gel. In the adjacent lanes were the equivalent of 50 and 100 copies (12.4 ng and 24.8 ng) of the rDNA insert from pCc1 which was also digested with BamHI. These DNAs were probed with labeled pCc1 DNA and the resulting auto-
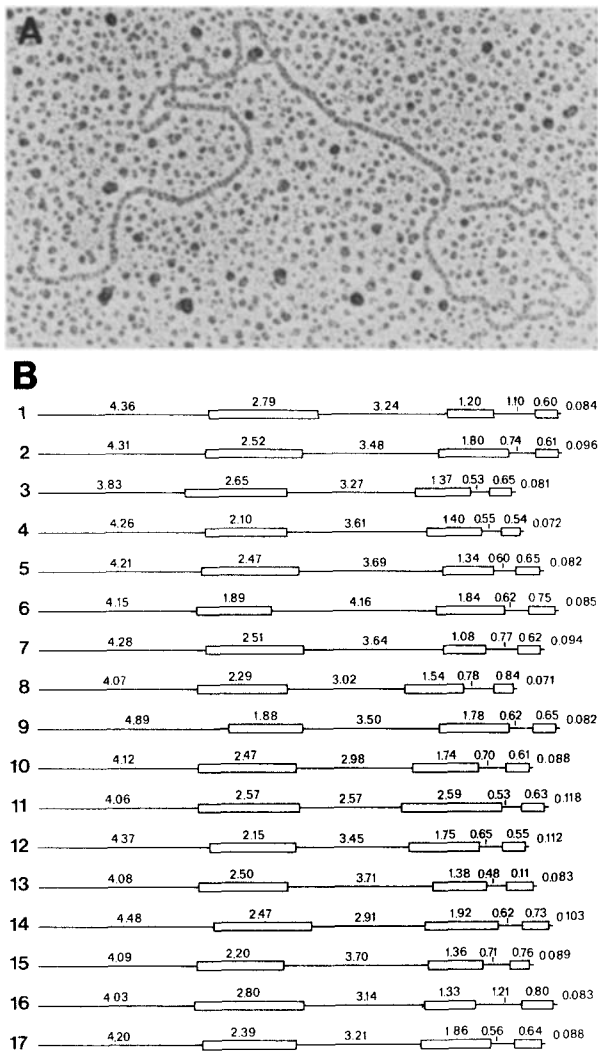
A

B

1	4.36	2.79	3.24	1.20	1.10 0.60	0.084
2	4.31	2.52	3.48	1.80	0.74 0.61	0.096
3	3.83	2.65	3.27	1.37	0.53 0.65	0.081
4	4.26	2.10	3.61	1.40	0.55 0.54	0.072
5	4.21	2.47	3.69	1.34	0.60 0.65	0.082
6	4.15	1.89	4.16	1.84	0.62 0.75	0.085
7	4.28	2.51	3.64	1.08	0.77 0.62	0.094
8	4.07	2.29	3.02	1.54	0.78 0.84	0.071
9	4.89	1.88	3.50	1.78	0.62 0.65	0.082
10	4.12	2.47	2.98	1.74	0.70 0.61	0.088
11	4.06	2.57	2.57	2.59	0.53 0.63	0.118
12	4.37	2.15	3.45	1.75	0.65 0.55	0.112
13	4.08	2.50	3.71	1.38	0.48 0.11	0.083
14	4.48	2.47	2.91	1.92	0.62 0.73	0.103
15	4.09	2.20	3.70	1.36	0.71 0.76	0.089
16	4.03	2.80	3.14	1.33	1.21 0.80	0.083
17	4.20	2.39	3.21	1.86	0.56 0.64	0.088

Fig. 1A, B. Size and position of 26S and 18S rRNA coding regions in pCc1. **A** Electron micrograph of R-loops formed from hybridizing 18S and 26S rRNAs with pCc1 linearized at the HindIII site. **B** Measurements of 17 such molecules, oriented as in Fig. 3. In these molecules, one sees vector, the 3' end of the 26S rRNA gene, which is interrupted by the cloning procedure, the nontranscribed spacer region, the 18S rRNA gene, the transcribed spacer region, and the 5' end of the 26S rRNA gene. Numbers indicate the size in kb

Fig. 2. Autoradiographic determination of the number of rDNA repeats in the *Coprinus* genome. Lane a contains 1 μg of genomic DNA digested with BamHI. The control lanes contain the following amounts of pCc1 insert plus accompanying vector: lane b 12.4 ng (50 copies), lane c 24.8 ng (100 copies). The filter was probed with pCc1

radiograms were examined by densitometry to determine the copy number. From replicative experiments, it was determined that there are 60–90 copies of the rDNA per haploid genome (Fig. 2) making it 1.5%–2.2% of the genome in this strain. The number of copies may vary slightly from strain to strain. In genomic digests, minor bands are seen in addition to the 9.3 kb band. In Fig. 2, one minor band is quite prominent. In other strains, these bands sometimes vary in size and are usually less prominent. It is unlikely that these minor bands result from artifacts of restriction enzyme digestion because
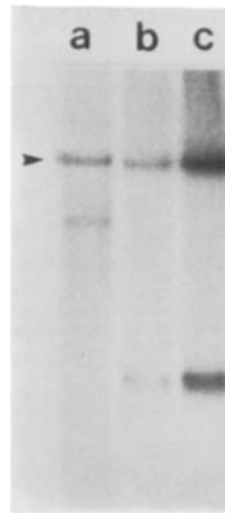
they are seen with all enzymes used and they differ in size between strains. Also, genomic DNA from strain Penn-a was digested with BamHI, BglII, and BglI (each of which cuts the rDNA once), the DNA run on gels, and probed with the rDNA. One minor band, smaller than the major band, was seen in all three cases (data not shown). Thus it appears likely that some or all of the minor bands result from one or a few copies of the rDNA which have gained or lost restriction sites or which contain insertions or deletions not found in most copies. Also, some minor bands could be the fragments which form the junction between the rDNA and adjacent genomic DNA.

Coding regions

Figure 3A shows the restriction map of the rDNA containing plasmid pCc1. The coding region for the 26S rRNA was determined by hybridization of ^{32}P labeled 26S rRNA to restriction digests of pCc1. The 26S rRNA hybridized to five EcoRI fragments, 4,400 bp, 1,700 bp, 900 bp, 700 bp, and 500 bp (faint) in size (Fig. 4, lanes a and b). The 4,400 bp and 500 bp fragments contain vector as well as rDNA sequences which explains why they hybridize less strongly than the others. The faint hybridization to the 2,400 bp fragment is probably due to slight contamination of the 26S rRNA probe with 18S rRNA. These fragments encompass 3,900 bp of rDNA sequence, easily encoding the approximately 3,000 nucleotide 26S rRNA. This analysis places the 26S rRNA coding sequence in the area indicated in Fig. 3B.

The 18S rRNA coding region was localized in the same way to a 2,400 bp EcoRI-XhoI restriction fragment (Fig. 4, lanes c and d). This is easily large enough to encode the 1,700 nucleotide 18S rRNA. Hybridization of the
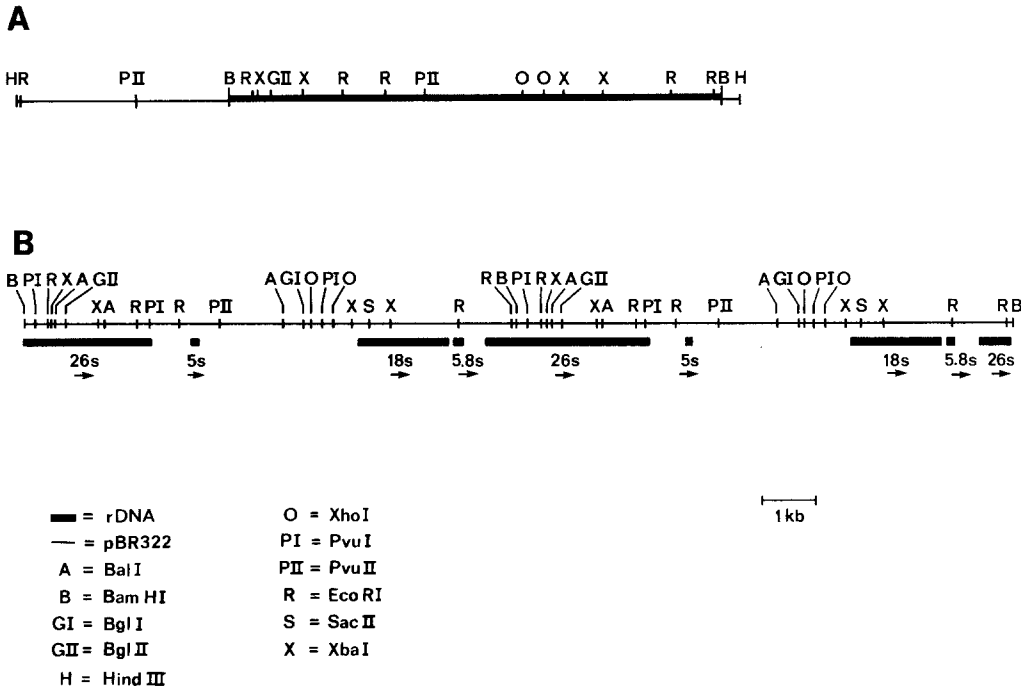
A

B

Fig. 3A, B. Map of restriction enzyme recognition sites in *Coprinus* rDNA. A A partial restriction map of the plasmid pCc1, which contains one complete repeat of the rDNA. The plasmid has been linearized by HindIII digestion and the position of restriction enzyme sites used to determine the location and direction of transcription of the rRNA genes are shown. B A more complete restriction map showing two repeat units of rDNA as found in the genome
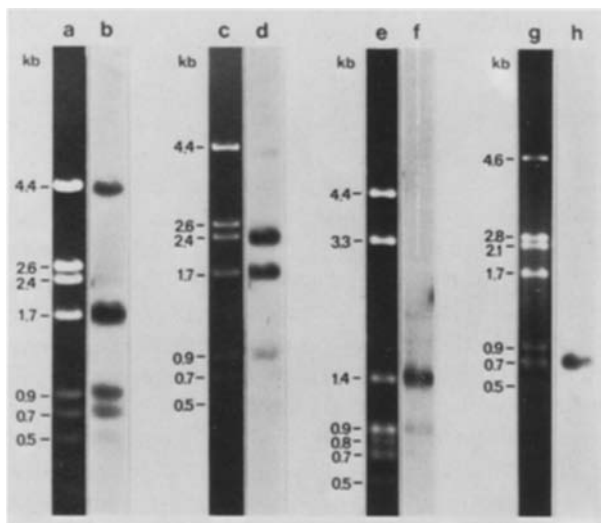

Fig. 4. Determination of coding regions in *Coprinus* rDNA. Lanes a, c, e, and g are ethidium stained gels of pCc1 digested with EcoRI and XhoI (lanes a and c), EcoRI and XbaI (lane e), or EcoRI and PvuII (lane g). Lanes b, d, f, and h are autoradiographs of the same digests after probing with labeled 26S rRNA (lane b), labeled 18S rRNA (lane d), labeled cDNA to 5.8S rRNA (lane f) or labeled cDNA to 5S rRNA (lane h)

18S rRNA to the 1,700 bp fragment is apparently due to contamination of the 18S rRNA with 26S rRNA sequences. The 1,700 bp fragment and the 2,400 bp fragment are not contiguous and the data from R-loop formation support placement of the 18S rRNA gene in the 2,400 bp fragment.

The 5.8S rRNA coding region was localized using a ^{32}P labeled cDNA made from the 5.8 rRNA. The cDNA hybridized to a 1,400 bp XbaI-EcoRI fragment (Fig. 4, lanes e and f). The slight hybridization to the adjacent 900 bp fragment indicates that the EcoRI site cuts within the gene near one end. This places the 5.8S rRNA gene between the 18S and 26S rRNA genes where it has been found in all eukaryotes examined to date.

Because the location of the 5S rRNA gene varies in fungi, it was of interest to determine where it is located in *Coprinus*. To localize the coding region for the 5S rRNA gene, genomic DNA and pCc1 DNA were digested and probed with a ^{32}P labeled cDNA made from the 5S rRNA. The cDNA hybridized to the clone and to the rDNA repeat in the genome. There was no obvious hybridization elsewhere in the genome to this DNA (data not shown). The gene was localized to a 700 bp PvuII-EcoRI restriction fragment (Fig. 4, lanes g and h).
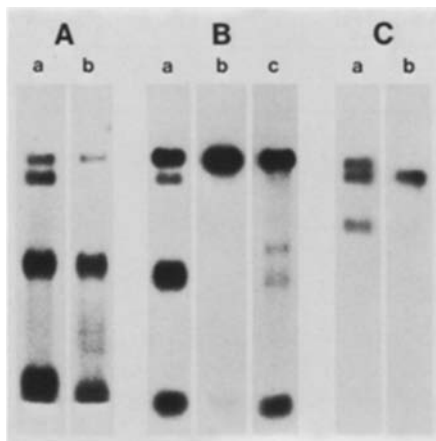
Fig. 5A–C. Autoradiographic determination of the direction of transcription of rRNAs. A Identification of separated strands of pCc1 digested with BamHI and BglII and denatured. In lane a both 5' BamHI ends and 5' BglII ends are labeled. The top two bands are the two strands of the 8.7 kb rDNA fragment, the wide middle band is the vector, and the wide lower band is the remaining 500 bp BamHI-BglII fragment. In lane b, only the 5' BamHI ends are labeled. B Hybridization with 18S and 26S rRNAs. Digestion and strand separation are as in part A. In lane a all strands are labeled to serve as markers. In lanes b and c, the DNA was not labeled. Lane b was probed with 18S rRNA and lane c was probed with 26S rRNA. Only the 8.7 kb fragment contains sequences homologous to the 18S rRNA but both the 8.7 kb fragment and the 500 bp fragment contain sequences homologous to the 26S rRNA. C Hybridization with cDNA using 5S rRNA as a template. Unlabeled pCc1 was digested with BamHI and BglII and denatured as in part A. Lane a was probed with pCc1 to mark the position of the strands. Lane b was probed with cDNA that was made using 5S rRNA as a template (note this probe has the opposite polarity as the 5S rRNA)

Direction of transcription

Having determined that the 5S rRNA gene is in the rDNA repeat, it was of interest to determine if the 5S rRNA is transcribed in the same direction as the other three rRNAs. To determine the direction of transcription, pCc1 was digested with BamHI, 5' end labeled with ^{32}P, and then cut with BglII which cuts the BamHI fragment asymmetrically (Fig. 3A). The DNA was then denatured and run on an agarose gel to separate the two strands of the large BamHI-BglII fragment as described in Materials and methods. This DNA was transferred to nitrocellulose and the strands were detected by autoradiography. The strand which migrates more slowly was the one which was labeled (Fig. 5A, lane b); therefore, its 5' end must be at the BamHI site and the 5' end of the strand which migrates more quickly must be at the BglII site. The nitrocellulose filter was probed with either 5' end labeled RNA (18S or 26S) or with labeled cDNA (5S). The 18S and 26S rRNAs hybridized to the strand whose 5' end is

at the BamHI site (Fig. 5B, lanes b and c), indicating that the direction of transcription is as shown in Fig. 3B. The cDNA probe from 5S rRNA hybridized to the strand whose 5' end is at the BglII site (Fig. 5C, lane b). The 5S rRNA is, therefore, transcribed in the same direction as the other rRNAs.

The sequence of the 5S rRNA (Liu and Nazar 1983) was used to confirm the location and direction of transcription of the 5S rRNA gene. The EcoRI–PvuII fragment believed to encode the 5S rRNA was gel purified and digested with restriction enzymes which, according to the sequence data, cut within the gene. These enzymes, HaeIII, HpaI, FnuDII, RsaI, and HhaI all cut the fragment in the predicted manner (data not shown) indicating that the 5S rRNA gene is present in this piece of DNA, that there is only one 5S rRNA gene in each repeat, and that it is transcribed in same direction as the other rRNAs.

Recombination and genetic linkage

Previously we discovered several strains of *Coprinus* with polymorphisms in the restriction enzyme digestion patterns of their rDNA (Wu et al. 1983). These restriction polymorphisms were used to look for linkage between the rDNA and several genetic markers and to determine whether recombination within the rDNA is inhibited. A total of 17 tetrads isolated from two crosses were examined. The first cross was between strains C692 and 12890/3. The markers followed in this cross were *ade3*, the two mating type loci, A and B, and the restriction pattern of the rDNA obtained with BamHI. The difference in the restriction patterns between the parental strains is due to a 200 bp insert which contains a BamHI site in the rDNA of strain 12890/3 between the 5S rRNA gene and the 18S rRNA gene (Wu et al. 1983). The restriction patterns of a typical tetrad (lanes a–d) and the parents (lanes e and f) are shown in Fig. 6A. The data from this cross are summarized in Table 2. The second cross was between strains PJP52 and Okayama-7. The markers followed in this cross are *trp1*, *met9* and the EcoRI restriction pattern of the rDNA. The difference in the restriction pattern between the parental strains is due to the loss of the EcoRI site between the 5S rRNA gene and the 26S rRNA gene in Okayama-7 (Wu et al. 1983). The restriction patterns of a typical tetrad (lanes a–d) and the parents (lanes e and f) of this cross are shown in Fig. 6B. The data from this cross are summarized in Table 3. The rDNA segregates as a Mendelian gene. That is, segregation is 2:2 in all 17 tetrads and all progeny contain one or the other but not both of the parental rDNA patterns. This indicates that all or virtually all copies of the rDNA repeat are located in one cluster or several tightly linked clusters on one chromosome.
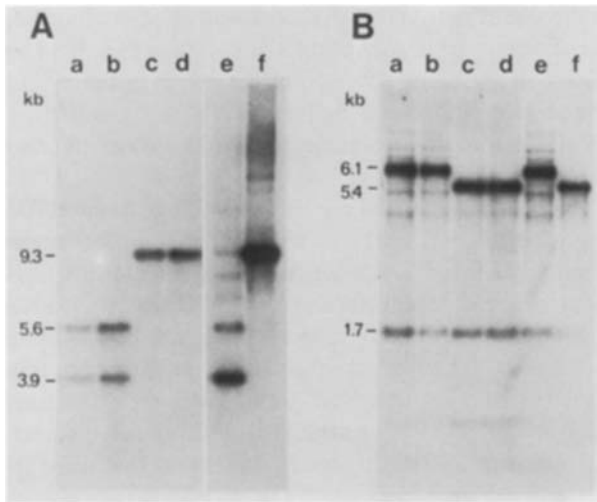
Fig. 6A, B. Autoradiographic determination of rDNA segregation. A Digestion of DNA from a tetrad from the cross C692 × 12890/3 and the parental DNAs with BamHI. Lanes a–d contain DNA from the four members of the tetrad. Lane e contains 12890/3 DNA. Lane f contains C692 DNA. B Digestion of DNA from a tetrad from the cross PJP52 × Okayama-7 and the parental DNAs with EcoRI. Lanes a–d contain DNA from the four members of the tetrad. Lane e contains Okayama-7 DNA. Lane f contains PJP52 DNA. All DNAs were probed with pCc1

Table 3. Tetrad analysis of progeny from PJP52 × Okayama-7

Markers	PD	NPD	T
*trp*1-rDNA	4	7	1
*met*9-rDNA	7	3	2
*trp*1-*met*9	4	7	1

ity that no recombination would occur in the rDNA in any of the 17 tetrads is $(0.696)^{17}$ or 0.002. We therefore conclude that recombination is inhibited within the rDNA.

Discussion

In this paper we have described three characteristics of the rDNA of *Coprinus cinereus* which are of interest. First, there are only 60–90 copies of the rDNA in *Coprinus*. This number is lower than that reported for other fungi. There are 100–150 copies in yeast (Schweizer et al. 1969; Rubin and Sulston 1973) 190 copies in *Neurospora* (Free et al. 1979) and 120 in *Schizophyllum* (Dons and Wessels 1980). It is perhaps due to this low number of rDNA repeats that we see minor bands in digestions of genomic DNA since polymorphisms involving a small number of copies would represent a greater percent of the rDNA in this organism. These minor bands may be due to the ends of the rDNA repeat or may be one or a few copies of the rDNA which contain insertions, deletions, or base changes which result in different restriction patterns. Single copy units with restriction site polymorphisms are seen in *Caenorhabditis elegans* which has only 55 copies of the rDNA (Files and Hirsh 1981). The low copy number of the rDNA in *Coprinus* will aid us in determining which, if any, of these minor bands represent internal or terminal junctions between the rDNA and other chromosomal sequences.

Second, we have shown that *Coprinus* rDNA has an unusual organization. The 5S rRNA genes are in the repeat as is the case for *Saccharomyces* and several other fungi. In *Saccharomyces cerevisiae* and *Torulopsis utilis*, the 5S rRNA is transcribed in the direction opposite to that of the other rRNAs (Valenzuela et al. 1977; Tabata 1980). This is not the case in *Coprinus*, where the 5S rRNA is transcribed in the same direction as the other rRNAs. It will be of interest to determine if this unusual arrangement occurs in other basidiomycetes and in members of other fungal groups. The organization of the 5S rRNA genes would appear to be an important factor to consider in addition to the 5S rRNA sequence in determining accurate phylogenetic relationships among the fungi (Walker and Doolittle 1982, 1983; Huysmans et al. 1983).

Table 2. Tetrad analysis of progeny from C692 × 12890/3

Markers	PD	NPD	T
A-rDNA	3	2	0
B-rDNA	3	1	1
*ade*3-rDNA	1	1	3
A-B	3	1	1

PD = parental ditype
NPD = non-parental ditype
T = tetratype

The rDNA is unlinked to any of the markers used. The markers A, B, *trp*1, and *met*9 are all within 10 map units of their centromeres (Lewis and North 1974). The low frequency of tetratype tetrads indicates that the rDNA is also centromere linked.

In the above recombination analysis, none of the progeny contained both parental rDNA patterns. This indicates that there was no recombination within the rDNA in any of these tetrads. It has been estimated that there are a minimum of 24 chiasmata formed in each meiotic cell, indicating 24 recombination events per meiosis (Holm et al. 1981). Because the rDNA is at least 1.5% of the genome, the probability that, in any given tetrad, no recombination events would occur in the rDNA if recombination is random is $(0.985)^{24}$ or 0.696. The probabil-

Third, we have established that recombination within the rDNA is inhibited. This is consistent with what has been found in yeast (Petes and Botstein 1977), and may be due to a general suppression of crossing-over between genes in tandem arrays (Fogel et al. 1983). If recombination were allowed to occur between homologous chromosomes aligned out of register, the drastic changes in the number of genes in the tandem array could be fatal to the organism. The relative stability of tandem arrays in eukaryotes may depend on mechanisms which suppress genetic exchange in these regions.

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