Partial contig map of the smallest chromosome in *Coprinus cinereus*

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We have constructed a chromosome-specific cosmid library from electrophoretically separated chromosomes of the basidiomycete *Coprinus cinereus* and performed contig mapping and analysis of chromosome length polymorphisms (CLPs) for the smallest chromosome of the 5302 strain. A contig map of about 300 kb indicated that the novel size chromosomes in the F_1 progeny were apparently recombinants containing physical markers derived from both ends and central regions in this map. This may be the first case in which the formation of CLPs in the F_1 generation has been explained using the contig map. The results obtained were consistent with the hypothesis that novel CLPs were produced by meiotic recombination between the parental homologous chromosomes of unequal sizes.

Detailed genetic and physical maps of genomes provide extremely useful starting points for the cloning of genes that are important to human health, agriculture, and industry (Hulbert et al., 1988; Leskiw et al., 1988; Viskochil et al., 1990; Wallace et al., 1990). In many instances, the only practical approach to the physical isolation of a gene involves "walking" from an identified, previously cloned chromosomal site across the locus of interest (O'Connell et al., 1989; Rommens et al., 1989). A genetic map consists of a set of genes whose linear order has been established by recombinational linkage analysis, whereas a physical map consists of an ordered set of specific sequences, for example, restriction sites, that often have been identified in cloned, overlapping segments of DNA. Such overlapping segments can correspond to large contiguous regions of chromosomes that are known as contigs. Contig maps have been completed for *Escherichia coli* (Migula) Castellani et Chalmers (Kohara et al., 1987; Smith et al., 1987) and they are nearly complete for the nematode *Caenorhabditis elegans* Daugherty (Coulson et al., 1986). Efforts are being made to complete similar maps for other organisms that include the yeast *Saccharomyces cerevisiae* Meyen ex Hansen and man. Progress in genome-related projects is helping us to unravel the details of the arrangements of the chromosomes of lower and higher eukaryotes (NIH/CEPH Collaborative Mapping Group, 1992; Oliver et al., 1992). The existence of contig maps facilitates gene cloning and allows novel investigations of relationships between genome structure and function.

The reconstruction in vitro of a genome as contigs is facilitated if libraries of recombinant DNA are divided into chromosome-specific subcollections. Detection of overlaps between clones within these restricted subcollections can generate contigs more readily than when unfractionated collections are used, by reducing the number of clones that are subjected to analysis. One approach to the formation of such libraries is to initiate the construction of a library from a chromosome that has been partially purified by flow cytometry (Bartholdi et al., 1987; McCormick et al., 1989) or by pulsed-field gel electrophoresis (PFGE). In many cases, however, it would be useful to be able to sort pre-existing random libraries that have been extensively characterized and have well-known properties into chromosome-specific subcollections. Such is the case, in particular, for two intensively investigated filamentous fungi, *Aspergillus nidulans* (Eidam) Winter (Brody et al., 1991) and *Neurospora crassa* Shear et Dodge (Mautino et al., 1993; Davis et al., 1994). Many well-characterized genes have been cloned from these organisms and are located in existing cosmid collections. Similar collections are being made for industrially important species, such as *Cephalosporium acremonium* Corda, the producer of the antibiotic cephalosporin (Samson et al., 1985; Dotzlaf and Yeh, 1987), and plant pathogens such as *Magnaporthe grisea* (Hebert) Barr, the causal agent of one of the most important diseases of plants, rice blast (Parsons et al., 1987).

Fungi have small genomes and their chromosomes can be separated by PFGE (Timberlake, 1978; Krumlauf and Marzluf, 1979; Orbach et al., 1988; Brody and Car-

bon, 1989). Most fungi contain low levels of repetitive DNA, almost all of which consists of rDNA that is distributed as a long tandemly repeated array of elements. The remainder of the reiterated DNA consists mainly of short, low-copy-number, interspersed repeats. From these observations, we reasoned that it should be possible to order existing genomic DNA libraries according to individual chromosomes using PFGE-isolated chromosomes.

We chose *Coprinus cinereus* (Schaeffer: Fries) S. F. Gray to test our hypothesis for four reasons. First, one incomplete cosmid library in the pLlc5200 (Zolan et al., 1992) vector has been constructed. pLlc5200 contains the *trp1* gene of *C. cinereus*, a cos site, and an ampicillin-resistance gene. Second, the genome of *C. cinereus* has been characterized by classical genetic analysis, and about 100 loci have been assigned to its thirteen linkage groups (North, 1987). Third, the genome has been investigated at the molecular level. Finally, PFGE has allowed the fractionation of chromosome-sized molecules of DNA from *C. cinereus* (Zolan et al., 1994; Arima and Morinaga, 1995). We used contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu et al., 1986) to resolve the chromosomes of *C. cinereus* and made a cosmid library for one of the individual chromosomes.

In this report, we describe our method for making a cosmid library and the contigs of regions of a specific chromosome of *C. cinereus*. In addition, we describe a simple model of meiotic recombination, based on our data, that resembles a model that was constructed previously (Arima et al., 1996) from a different perspective and confirms the validity of the earlier model.

Materials and Methods

Strains and culture conditions The *C. cinereus* wild-type strains 5302 and Dd 13 were used for the isolation of F_1 progeny (PR-1–PR-16) as described previously (Arima and Morinaga, 1995). Strains were maintained also as described previously (Arima and Morinaga, 1995).

Manipulation of DNA Standard protocols were used for digestion by restriction enzymes, ligation, agarose gel electrophoresis and Southern blotting (Maniatis et al., 1982). Gels were blotted onto Hybond-N$^+$ membranes (Amersham). Hybridization was performed with an ECL system (Amersham) according to the manufacturer's recommendations. Plasmid DNA was isolated by alkaline lysis, as described by Birnboim and Doly (1979). Transformation of bacteria was performed by standard procedures (Maniatis et al., 1982).

Pulsed-field gel electrophoresis Protoplasts of *C. cinereus* were prepared as described previously (Arima and Morinaga, 1995). Chromosome-sized DNAs were prepared from protoplasts and resolved by contour-clamped homogeneous electric field (CHEF) gel electrophoresis, as described previously (Arima and Morinaga, 1995). For intact chromosomes, electrophoretic conditions were as described elsewhere (Arima and Morinaga, 1995). For isolation of the smallest chromo-

some of strain 5302, CHEF gel electrophoresis was carried out in 1% chromosomal grade agarose (BioRad) in 0.5× TBE buffer (Maniatis et al., 1982) with a 3-min pulse time at 150 V for 16 h.

Construction of a chromosome-specific cosmid library We modified the previous method described by Zolan et al. (1992). Insert DNA was prepared from the smallest chromosome of strain 5302. The procedure followed those described elsewhere (Zolan et al., 1992; Arima et al., 1996) with the exception that the restriction enzyme used to digest the chromosome was *Bam*HI. The partially digested DNA was dephosphorylated with bacterial alkaline phosphatase (Boehringer Mannheim, as described by the manufacturer) to prevent ligation of fragments with each other, and then 1 μg of SuperCos 1 (Stratagene) cut with *Xba* I and dephosphorylated with bacterial alkaline phosphatase was digested with *Bam* HI to ligate with the partially-digested DNA fragments. The phage particles packaging the ligation mixture with Gigapack Gold III (Stratagene) were used to transfect *E. coli* strain VCS 257. We followed the manufacturer's instructions for packaging, preparation of bacteria, and transfection. Individual colonies selected on LB (Maniatis et al., 1982) agar plates containing ampicillin (100 μg/ml) were picked up and transferred into the wells of 96-well microtiter dishes containing the same LB medium including 20% glycerol. Colonies grown at 37°C overnight were stored at −80°C.

Results

Electrophoretic karyotype CHEF gel electrophoresis allows good separation of the intact chromosomes of *C. cinereus*, which range in size from about 1.3 to 5 Mb, as described previously (Arima et al., 1996).

Construction and characterization of the chromosome-specific cosmid library To construct the chromosome-specific library from the smallest chromosome of *C. cinereus* strain 5302, we cut the band of the smallest chromosome from the CHEF gel. Partial digestion with *Bam*HI was performed while the DNA was still in the agarose, and then the digested DNA was electroeluted. The efficiency was very low compared to that of the general procedure. All colonies were picked up, grown overnight in LB containing ampicillin and stored at −80°C. We maintained at least seven chromosome equivalents of the smallest chromosome in strain 5302 in this library. We also constructed an incomplete library of the smallest chromosome of strain 5302 with the pLlc5200 vector (data not shown). Characterization of a total of 350 clones from the library indicated that the inserts ranged in length from approximately 25 to 45 kb. After digestion of all clones with *Bam*HI and *Not* I and subsequent electrophoresis, the clones were divided into 20 groups.

Contigs of the smallest chromosome in strain 5302 First, we performed intragroup-hybridization to fractionate each group and constructed some subgroups within individual groups from our results. Second, the plasmids in each clone in a subgroup were digested with

*Bam*HI, and the digests were subjected to electrophoresis to compare the fragments generated by *Bam*HI. Third, we constructed the restriction map for *Bam*HI for the subgroup. Finally, we generated contigs based on the *Bam*HI restriction map and confirmed the results by hybridization. The probes used for hybridization were clones that overlapped the subgroups. As a result, we constructed a contig map of about 300 kb (Figs. 1, 2) and other small contig maps (70 kb–100 kb; data not shown) for the smallest chromosome of strain 5302. These contig maps covered about 50% of the smallest chromosome (about 1.3 Mb) in strain 5302.

Meiotic recombination in a 300-kb region We have previously presented a simple model of meiotic recombination (Arima et al., 1996), in which interfertile strains with homologous chromosomes of different sizes might contribute a chromosome of a new size to the F_1 progeny. However, this model was constructed on the basis of results obtained with only one probe, pRC-1, which was the smallest chromosome-specific clone of strain 5302. We attempted here to validate this simple model of meiotic recombination from a new perspective. We generated contigs of the smallest chromosome in strain 5302 and made a contig map of a 300-kb region of
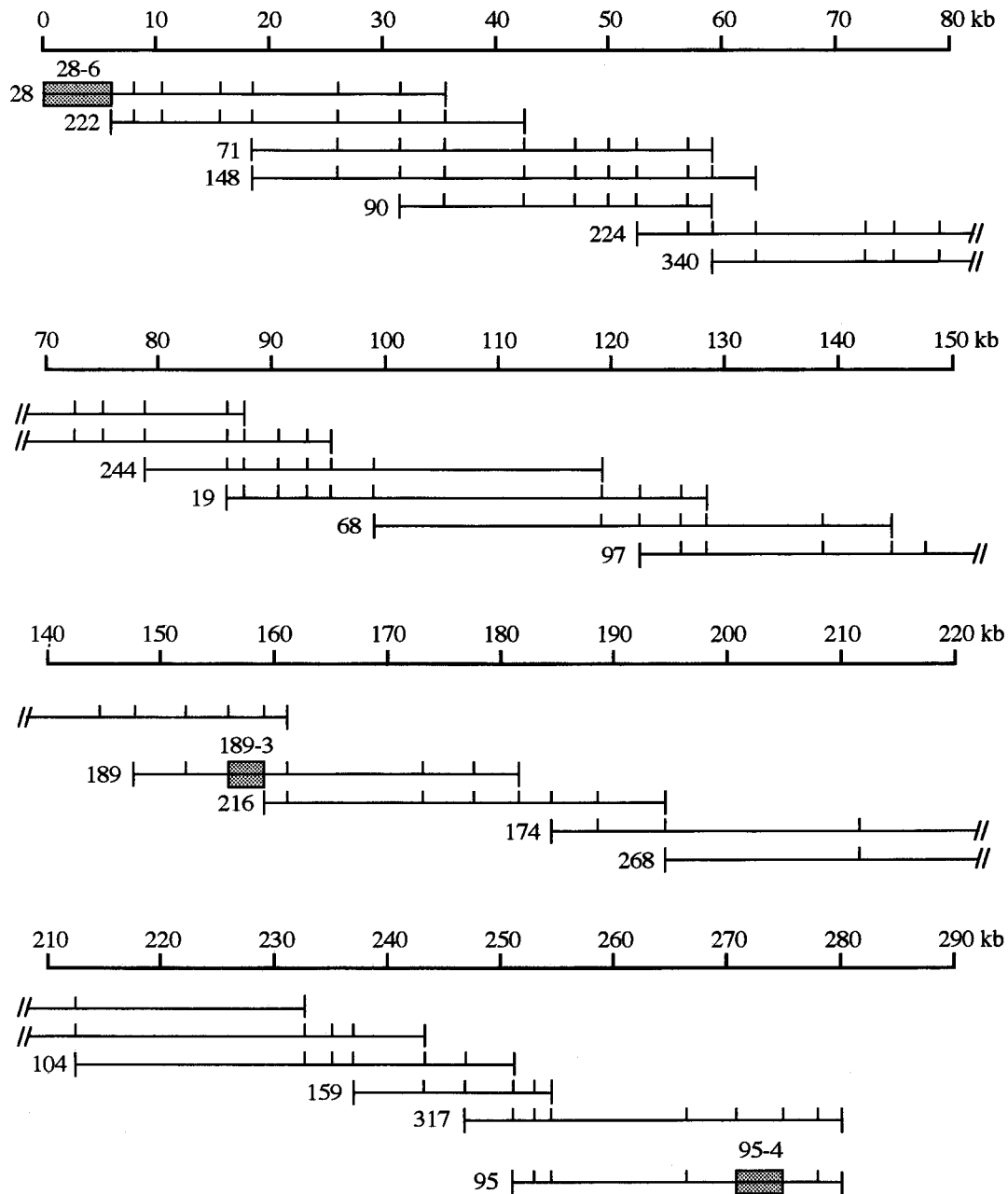

Fig. 1. Contig map of the cosmids used in a 300-kb region of the smallest chromosome in the 5302 strain. Cosmid inserts are represented by horizonal lines. Vertical lines above the horizontal indicate *Bam*HI restriction sites. The location of physical markers is represented by the speckled boxes.
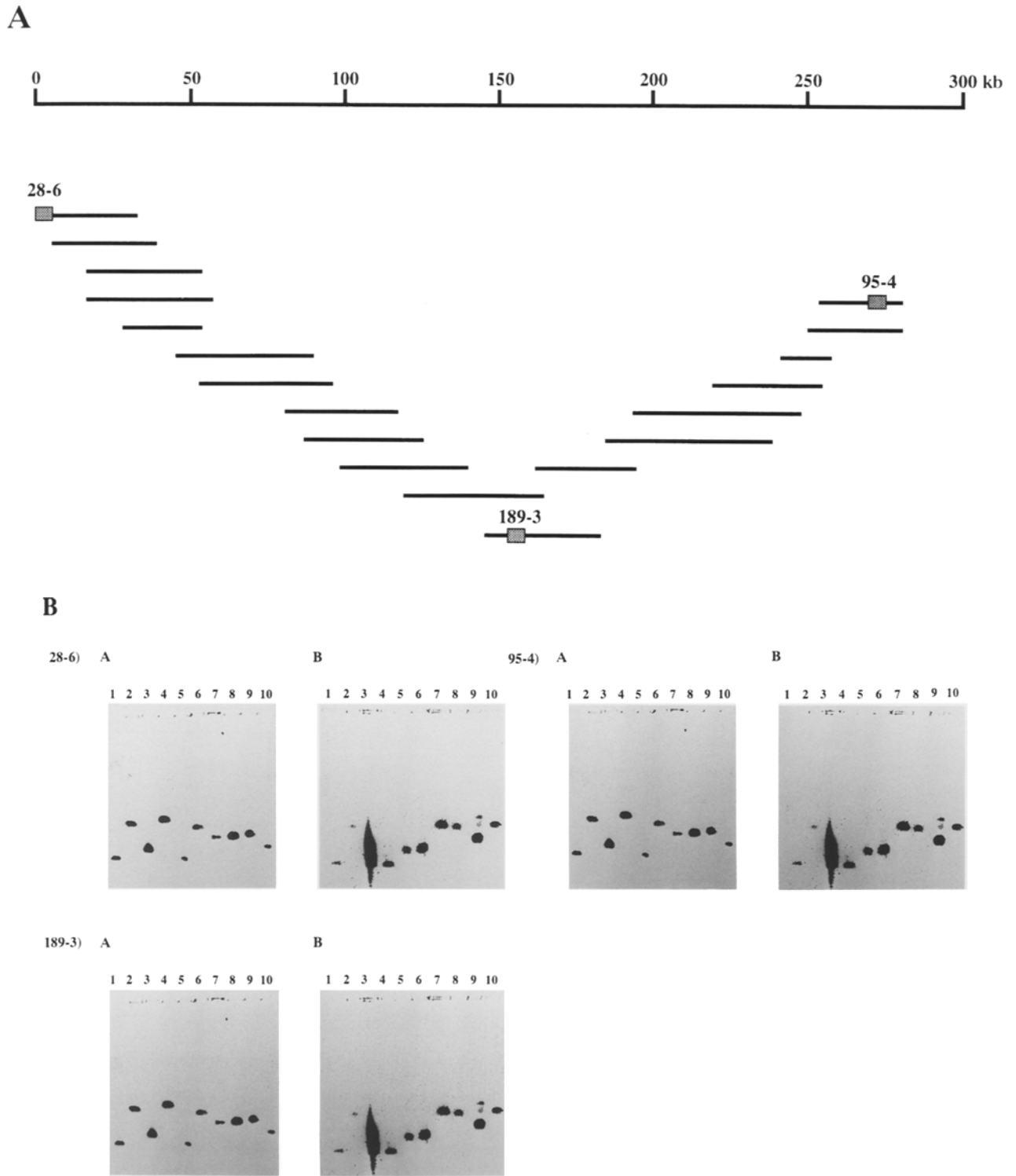
Fig. 2.   Physical analysis of chromosome length polymorphisms in the F_1 progeny using the smallest chromosome of the 5302 strain.
A. Physical map of a 300-kb region in the smallest chromosome.   Cosmid inserts are represented by horizontal bars and the location of the physical markers is indicated by speckled boxes.   B. Hybridization of CHEF-separated chromosomes of parental and F_1 progeny strains probed with physical markers, 28–6, 189–3, and 95–4, respectively.   A. Lane 1, 5302; lane 2, Dd 13; lane 3, PR-

this chromosome (Figs. 1, 2). We then selected chromosome-specific fragments that hybridized only to the smallest chromosome of strain 5302 from both ends and the central regions of the contig map (Fig. 2) and obtained three such fragments, designated 28-6, 189-3 and 95-4 (Figs. 1, 2). Using probes derived from the three chromosome-specific fragments, we obtained hybridization patterns (Fig. 2) that coincided with the results previously obtained with the pRC-1 probe (Arima et al., 1996).

Discussion

We have constructed a chromosome-specific cosmid library from CHEF-separated chromosomes of the basidiomycete *C. cinereus*. In this study, we were careful to avoid the co-ligation of insert DNA because our goal was to obtain a contig map of the smallest chromosome in strain 5302. We modified the previous method described by Zolan et al. (1992), who did not perform size-selection of insert DNA before ligation, resulting in the likelihood that inserts in individual clones were chimeras of two or more chromosomal fragments that had been ligated together. We used insert DNA that had been treated with bacterial alkaline phosphatase for ligation. This treatment ensured that only one insert fragment was cloned per vector. However, the efficiency was very low. Indeed, the low efficiency might be positive proof of the absence of co-ligation of insert DNA in our library. We estimated that we recovered about 2×10^5 clones/μg of insert DNA by the method described Zolan et al. (1992). This library greatly facilitated the isolation of genes by complementation of mutant phenotypes (Zolan et al., 1992). This library was, however, inadequate for use to generate the contigs and the map-based cloning that was performed in *N. crassa* (Mautino et al., 1993; Davis et al., 1994). Our library consisted of 350 clones with seven times the DNA in the smallest chromosome of strain 5302. This library did not cover all regions of the smallest chromosome, because some clones specific to the smallest chromosome that we had constructed previously with the pUC 18 vector (Arima et al., 1996) did not hybridize to this library. With respect to the telomeric sequence, we have evidence that the ends of chromosomes of *C. cinereus* are composed of TTAGGG repeats, as also observed in *Cladosporium fulvum* Cooke (Coleman et al., 1993) and *N. crassa* (Schechtman, 1990), and such repeats also did not hybridize to our library (data not shown). There may be regions of the chromosome that are easy to clone and others that are difficult. It might be possible to clone the latter regions by long-PCR using end-specific primers, based on clones adjacent to these regions.

We obtained contigs of the smallest chromosome of strain 5302 using our library. Although contigs have been obtained in general by use of terminal probes that have been synthesized under control of T3 and T7 promoters in SuperCos 1, we did not adopt this method because of the possibility that these probes might hybridize non-specifically. We employed the entire region of

one clone as a probe for the contigs of the smallest chromosome in strain 5302. As the result, we constructed one long-range and several short-range contig maps (Figs. 1, 2). These contig maps were equivalent in total to about 50% of the smallest chromosome (about 1.3 Mb) in strain 5302. From an estimated minimum genetic length of 1,300 cM (Holm et al., 1981) and a genome size of approximately 35,000 kb (Dutta, 1974), the average physical distance per genetic map unit in *C. cinereus* was 27 kb. The long-range contig map of about 300 kb, which corresponds to about 11 map units, might be divided into two parts, because the clones belonging to the former part were twice as numerous as those in the latter part. A similar phenomenon was observed for one of the short-range contig maps. In this case, the clones belonging to the contig map were more numerous than those that were included in the other contig maps. Francis and Michelmore (1993) showed that the small chromosomes of *Bremia lactucae* Regel that did not follow the rules of Mendelian inheritance were apparently composed primarily of repetitive DNA. They pointed out that, in the absence of information about the presence of centromeres and telomeres on the small chromosomes of *B. lactucae*, their status as true chromosomes or as linear, nuclear plasmids was unclear. Thus, a part of the smallest chromosome of *C. cinereus* might be composed of repetitive arrays. We have confirmed, however, that the smallest chromosome of strain 5302 is a true chromosome. The oligonucleotide probe (TTAGGG)$_3$, which is considered to represent the telomeric repeat sequence of *C. cinereus*, hybridized to all CHEF-separated chromosomes including the smallest chromosome of strain 5302 (data not shown). The smallest chromosome of strain 5302 had a homologous chromosome in the compatible strain Dd 13 (Arima et al., 1996).

The chromosomal rearrangements that can lead to the generation of chromosome length polymorphisms (CLPs) are of several types (Zolan, 1995). The genomes of fungi contain sequences that can lead to CLPs. Recombination among subtelomeric regions can lead to variability of chromosome ends. The subtelomeric repeats in *S. cerevisiae* have been shown to be composed of a mosaic of repeat units (Louis et al., 1994), and recombination among subtelomeric repeats has been documented both for this organism and for *M. grisea* (Farman and Leong, 1995). Transposable elements can act passively in the generation of CLPs, serving as mobile sites of homology that can act as substrates for ectopic recombination (Wilke et al., 1992). However, the deliberate doubling of the number of *Ty1* elements (from about 25 to 50) within a strain of *S. cerevisiae* did not lead to significantly decreased viability of spores in one meiotic generation, an indication that the rate of ectopic recombination was not increased (Boeke et al., 1991). Therefore, neither of these elements is an efficient substrate for ectopic recombination or, alternatively, ectopic recombination among them is actively suppressed. In addition, reciprocal recombination between homologous chromosomes with size polymorphisms can generate

chromosomes of novel sizes. In *C. cinereus*, crosses between strains with CLPs lead to new sizes of chromosomes in the F_1 progeny (Arima et al., 1996), indicating that recombination probably occurs between homologs (Zolan et al., 1994; Arima et al., 1996). Therefore, the new sizes of chromosomes observed in F_1 progeny are dependent for their formation on meiosis in a strain with CLPs. A logical conclusion from these studies is that these chromosomes with new sizes, which are often intermediate in size between the homologs of both parents, result from meiotic recombination between homologs (Zolan et al., 1994; Arima et al., 1996). However, it has not yet been demonstrated that chromosomes of new sizes are, in fact, recombinants that contain physical markers from the homologs in the two parental strains. In addition, although genetic markers are known for 10 of the 13 chromosomes (Casselton, 1995), the absence of a more complete genetic map hinders the interpretation of the genetic meaning of CLPs. We have used a physical approach to the analysis of CLPs in the F_1 progeny, using the smallest chromosome of strain 5302 to construct a simple model of meiotic recombination. We constructed a 300-kb contig map of this chromosome by this physical approach, choosing physical markers from both ends and the middle regions on this map (Figs. 1, 2). Although these markers hybridized to one chromosome in both parental strains, the size of the hybridized chromosomes differed considerably (Fig. 2). In the F_1 progeny, most of the hybridized chromosomes were situated in terms of size between those of the parents (Fig. 2). These results are consistent with the previous results obtained with the probe pRC-1, which does not correspond to the 300-kb contig region, and they support the simple model of meiotic recombination that we proposed previously (Arima et al., 1996). We have shown that most of the chromosomes, excluding the rDNA encoding-chromosome, follow our simple model of meiotic recombination (Tagashira et al., in preparation). Such results also confirm our model. Therefore, changes in chromosome length might be a common and prominent feature of the genome of *C. cinereus*, and the variety of karyotypes could represent the plasticity of the genome of this sexual fungus. Thus, we reiterate our proposal that novel CLPs are produced by reciprocal recombination between parental homologous chromosomes of unequal size during meiosis.

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