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Cloning and Organization of the Ribosomal RNA Genes of the Mushroom *Tricholoma matsutake*

HWANG, SEON-KAP AND JONG-GUK KIM*

Department of Microbiology, Kyungpook Nat'l University, Taegu 702-701, Korea

A portion (7.4 kb) of ribosomal DNA tandem repeat unit from a genome of the mushroom *T. matsutake* has been cloned. A 1.75 kb *Eco*RI fragment was cloned first using *S. cerevisiae* 25S rRNA gene as a probe, and this was then used for further cloning. A chromosomal walking experiment was carried out and the upstream region of the 1.75 kb fragment was cloned using *Sma*I/*Bam*HI enzyme, the size was estimated to be 5.2 kb in length. Part of the downstream region of the 1.75 kb fragment was also cloned using *Xba*I/*Bam*HI enzymes. Restriction enzyme maps of three cloned DNA fragments were constructed. Northern hybridization, using total RNA of *T. matsutake*, and the restriction fragments of three cloned DNAs as probes, revealed that all four ribosomal RNA genes (large subunit[LSU], small subunit [SSU], 5.8S and 5S rRNA genes) are present in the cloned region. The gene organization of the rDNA are regarded as an intergenic spacer [IGS]2 (partial) - SSU rRNA - internal transcribed spacer [ITS]1 - 5.8S rRNA - ITS2 - LSU rRNA - IGS1 - 5S rRNA - IGS2 (partial).

The mushroom *Tricholoma matsutake* (S. Ito et Imai) Singer, Song-yi, is an ectomycorrhizal fungus which belongs to Tricholomataceae, Agaricales, Hymenomycetes, and Basidiomycotina. It is known to grow symbiotically and form ectomycorrhiza with *Pinus densiflora* (14, 15). The mycelia of the mushroom colonize around the roots of *P. densiflora* and supposedly receive the nutrients, such as many kinds of water-soluble polysaccharides (21) from the host. The mushroom has been used as a foodstuff for a long time in Eastern Asia and its economic importance is ever increasing because of growing demand as a high-price mushroom, low productivity in recent years, and its unsuitability for cultivation *in vitro*. A number of investigations to promote understanding the mushroom have been conducted over the past ten years or more (14, 15, 21) but, however, they are restricted to the cultivation of fruiting bodies *in vitro*, basic information to promote understanding of the syntrophism, environmental factors affecting its 'shiro', and so on.

Eukaryotic ribosomal RNA genes are tandemly repeated many times with copy numbers per haploid genome ranging from about 50 to 10,000 in different species. They are composed of highly conserved genes coding for the small subunit (SSU), 5.8S, large subunit (LSU), and 5S rRNAs, along with less conserved regions, internal transcribed spacers (ITSs) and intergenic spacers (IGSs) (1, 13). Since the highly conserved regions and less conserved regions are present in the repeat, their application to the use of probes may be useful in the identification of the *Tricholoma* species in soils. Recently, the ribosomal DNA (rDNA) repeat unit has been also used as a source for the restriction fragment length polymorphism (RFLP) which can offer taxonomic (2, 7, 9, 19), phylogenetic (4), and population information (16).

As a preliminary study for phylogeny, taxonomy, and to search for new and specific probes to *T. matsutake*, this report presents a cloning of ribosomal RNA genes which are tandemly repeated, a restriction enzyme map, and a genetic organization of ribosomal DNA of *T. matsutake*.

MATERIALS AND METHODS

Mushroom and Bacterial Strains

Fruiting bodies of *Tricholoma matsutake* were harvested mainly in Uljin and Gachang near Taegu. Immediately after harvesting, they were extensively washed with sterilized water to remove soil and other contaminants, and then frozen at -70°C. Two-day lyophilized fruiting bodies were used in this work.

The bacterial strains used for cloning and subcloning were *Escherichia coli* JM109 (*recA1 supE44*

*Corresponding Author
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endA1 hsdR17 gyrA96 relA1 thi△(lac-proAB)/F'[*traD 36 proAB*$^+$ *lacI*^{q} *lacZ△M15*]) (22) and XL-1-Blue (*supE hsdR lac*/F'[*proAB*$^+$ *lacI*^{q} *lacZ△M15*]) (5). Plasmid vectors employed were pUC18/19 (Ap^r *lacZ lacI*) (11).

Chemicals and Enzymes

Most restriction enzymes, RNase, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from Promega Co. or Boerhinger-Mannheim (BM, Germany). Enzymes were used according to the recommendations of the manufacturers. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactoside) were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). The α-[^{32}P]-dCTP and autoradiography film were obtained from Amersham (Buckinghamshire, UK). GENECLEAN kit (BIO 101 Inc., USA) was used for recovery of DNA fragments from agarose gel. The random primed DNA labelling kit was from Boerhinger-Mannheim. Guanidinium thiocyanate and detergents were from Sigma. A 50× Denhardt's solution composed of 1% Ficoll (Type 400), 1% polyvinylpyrrolidone and 1% bovine serum albumin (fraction V) in water was purchased from Sigma, and used for Southern and Northern hybridizations. All other chemicals and enzymes were reagent grade obtained from commercial sources.

Media and Cultural Conditions

Luria-Bertani (LB) medium (1.0% Bacto-tryptone, 0.5% Bacto-yeast extract, 1.0% NaCl) was used for cultivation of *E. coli* cells. Plasmid-harboring *E. coli* cells were cultured in LB medium supplemented with ampicillin (50 μg/ml). X-Gal agar plates (LB agar medium, 50 μg/ml ampicillin, 0.1 mM IPTG and 40 μg/ml X-Gal) were used for subcloning with pUC series of vectors in *E. coli* JM109 or XL-1-Blue. All *E. coli* cells were cultured aerobically at 37°C.

DNA and RNA Manipulation

Plasmids were isolated by the SDS/alkaline lysis procedure of Birnboim and Doly (3). Endonuclease digestion, ligation, and transformation into *E. coli* cells by the $CaCl_2$ method were done as described in Sambrook *et al.* (17).

T. matsutake genomic DNA was extracted according to the method described by Kim *et al.* (8) with some modifications. The 250 mg lyophilized fruiting bodies were ground to a fine powder for 10 min in liquid nitrogen in a precooled mortar and pestle at 4°C. Twenty mililiters of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4) was added and stirred for 10 min. SDS was added to a final concentration of 2% (w/v) and incubated at 65°C for 1 hour. Centrifugation was carried out at 10,000 g for 10 min and the supernatant was removed. NaCl was then added to a final concentration of 1.4 M followed by adding a-tenth volume of 10% CTAB buffer (10% CTAB, 100 mM EDTA, 500 mM Tris-HCl, pH 8.0). After being mixed thoroughly and incubated at 65°C for 10 min, chloroform: isoamyl alcohol (24: 1, v/v) extraction was repeated until the interface was clear. Nucleic acids were then concentrated by precipitation in two volumes of absolute ethanol and dissolved in 0.1× TE buffer (0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0). RNase A was treated and 1.5 volumes of 6 M guanidinium thiocyanate was added and mixed gently to remove proteins. Following incubation at 37°C for 30 min, ethanol precipitation was repeated. The purity of the final DNA solution was measured and yielded OD_{260}/OD_{280} values of 1.7 to 1.8.

Total RNA of *T. matsutake* was extracted by the guanidinium thiocyanate-acid-phenol extraction method described by Farrell (6) except that 100 mg of the lyophilzed fruiting bodies of *T. matsutake* was ground in a mortar and pestle prechilled at -70°C as in the DNA extraction procedure described above, and 4 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauroylsarcosine, 100 mM β-mercaptoethanol) was added.

Electrophoresis

Agarose gel electrophoresis of DNA was performed as described by Sambrook *et al.* (17). Agarose/formaldehyde gel (1.2%) eletrophoresis of total RNA was carried out to separate the SSU and LSU rRNAs, as described by Farrell (6). Polyacrylamide gel (8%) electrophoresis using 1×TBE buffer was carried out to analyze the small rRNAs, 5S and 5.8S rRNAs (6, 17).

Southern, Colony, and Northern Hybridization

Southern hybridization of genomic DNA and recombinant plasmid was done as follows (17, 18): DNA digested with different restriction enzymes was electrophoresed in 0.8 to 1.0% agarose gel (1×TAE buffer). DNA was denatured in 0.5 N NaOH/3.0 M NaCl, and then transferred to nylon membrane by capillary action with 0.4 N NaOH/1.0 M NaCl for 16 hours. After crosslinking under ultra-violet light (Stratagene), hybridization was carried out at 60°C in prehybridization and hybridization solution (6×SSC, 2× Denhardt's solution, 100 μg/ml denatured herring sperm DNA, and 0.5% SDS) containing isotope-labeled probe. The membrane was washed with 2× SSC/0.5% SDS for 10 min at room temperature, 2× SSC/0.1% SDS for 20 min at room temperature, and finally 0.1×SSC/0.5% SDS for 1 hour at 60°C followed by drying at room temperature. The clones were screened by colony hybridization and chromosomal walking experiments (17) for cloning ribosomal RNA genes. The hybridization conditions

were as described above.

Northern hybridization was performed according to the method described by Farrell (6). The total RNA was separated at constant voltage (50 volts) in agarose/formaldehyde gel, transferred to a nylon membrane with 10×SSC by capillary action, and then baked at 80°C for 2 hours. For polyacrylamide gel the total RNA was denatured in the presence of 50% formamide at 75°C for 15 min, separated in the gel at 35°C with constant current of 15 mA, treated in 7.5 mM NaOH for 20 min, and then transferred to a nylon membrane with 10×SSC by capillary action. Prehybridization and hybridization solutions used were 5×SSPE (3.6 M NaCl, 20 mM EDTA, 0.2 M sodium phosphate, pH 7.7), 50% formamide, 5× Denhardt's solution, 100 μg/ml denatured herring sperm DNA and 0.5% SDS. After prehybridization for 2 hours and hybridization for 20 hours at 42°C, the membrane was washed with two cycles of 2×SSC/0.1% SDS, once at room temperature for 10 min and once at 42°C for 20 min. In all cases autoradiography was conducted for appropriate periods.

RESULTS AND DISCUSSION

Cloning of Ribosomal RNA Genes

T. matsutake genomic DNA was digested with different restriction endonucleases and then transferred to a nylon membrane. A 2459-base fragment (*Hin*dIII/*Xba*I) of *S. cerevisiae* 25S rRNA gene (20) was used as a probe for Southern blotting. As shown in Fig. 1, various restriction patterns were obtained, and three bands of DNAs digested with *Hin*dIII, *Bam*HI, and *Sma*I are likely to be identical in size, and therefore it is assumed that the size of the tandem repeat unit is 11.5 kb. It is also assumed that there are no *Kpn*I, *Sac*I and *Pst*I sites, and that two or more *Eco*RI, *Xba*I, *Hin*cII, and *Sph*I enzyme sites are present in the repeat unit. For cloning the rRNA genes, the restriction enzymes, *Bam*HI, *Hin*dIII, and *Eco*RI, were used to digest the genomic DNA, and then, using pUC19 plasmid and JM109 cell, each small-scale genomic library was established. Colony hybridization was carried out with the same DNA probe as described in MATERIALS AND METHODS, and we first obtained three clones from the *Eco*RI digested genomic DNA (Fig. 2A). However, we could not obtain any clone from other libraries. They are all the same in size (1.75 kb) and in restriction endonuclease patterns (data not shown). To identify the authenticity of the cloned fragment by comparing it with *T. matsutake* genomic DNA digested with *Eco*RI, Southern hybridization was carried out using the same DNA probe. Fig. 3A shows that the cloned fragment originated from *T. matsutake* genomic DNA. The recombinant DNA was named pTMr101 and the restriction map of the plasmid was constructed (Fig. 4A). In order to clone the remainder of rDNA which was assumed to harbor all other rRNA genes, we used the DNA inserts of pTMr101 as probes depending on restriction data.

In order to clone the DNA fragment upstream of the *Sma*I site of pTMr101, a 0.45 kb DNA fragment from *Eco*RI/*Sma*I digestion was employed as a probe. Southern hybridization was carried out (Fig. 3B). Using pUC18 plasmid as a vector, we constructed small-scale genomic libraries from various sets of restriction enzyme digestions, and then colony hybridizations were carried out. From a library established with *Sma*I/

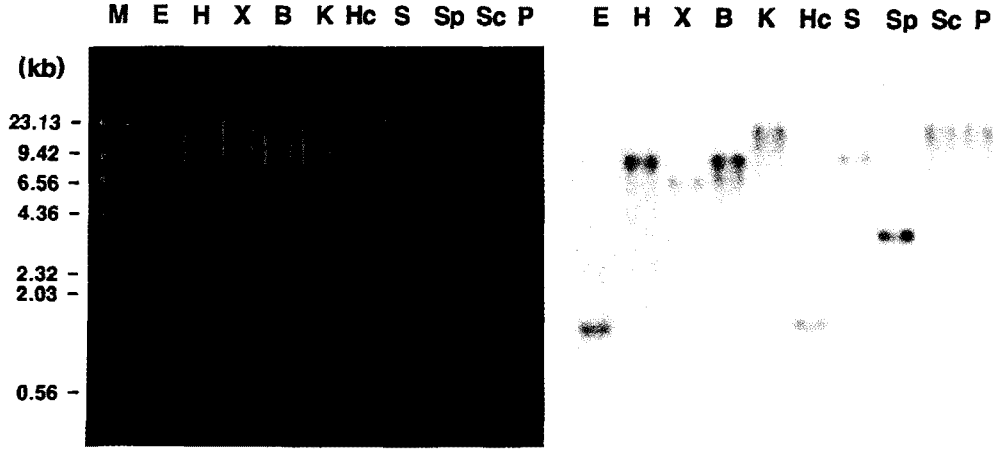

Fig. 1. Hybridization of *S. cerevisiae* 25S rRNA gene to the *T. matsutake* genomic DNA digested with different restriction endonucleases.

The left hand panel shows ethidium bromide stained genomic DNA and right panel the hybridization pattern. M, marker DNA (*Hin*dIII digested lambda DNA); E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; B, *Bam*HI; K, *Kpn*I; Hc, *Hin*cII; S, *Sma*I; Sp, *Sph*I; Sc, *Sac*I; P, *Pst*I.

*Bam*HI enzymes, we obtained a clone showing stronger signal than any other colonies (Fig. 2B). The recombinant plasmid, containing 5.2 kb insert, was named pTMr102, and its restriction enzyme map was also constructed (Fig. 4B).

For clonining the other fragment, part of the repeat unit which is downstream to the insert of pTMr101, a 0.2 kb DNA probe from *Xho*I/*Eco*RI digestion, was hybridized to the genomic DNA digested with various sets of restriction enzymes (Fig. 3C). From this result, and colony hybridization using the same probe, we obtained a clone (Fig. 2C), named pTMr80, and constructed its restriction enzyme map (Fig 4C) which was represented by closed bar. The pTMr80, however, did not harbor the intended size (about 7.1 kb) of DNA insert, but contain a 4.2 kb DNA insert. Furthermore, the plasmid contained parts of the the DNA inserts which have already been cloned in pTMr101 and pTMr102, and the restriction site which should be recognized by *Bam*HI enzyme was not restricted. Further trials of restriction failed to cut out the insert DNA from pTMr80 except on a 1.0 kb *Eco*RI fragment, named pTMr108. It is assumed that this unexpected result comes from the change of sequence in *Bam*HI site and the sequences surrounding it.

Gene Organization of the rRNAs

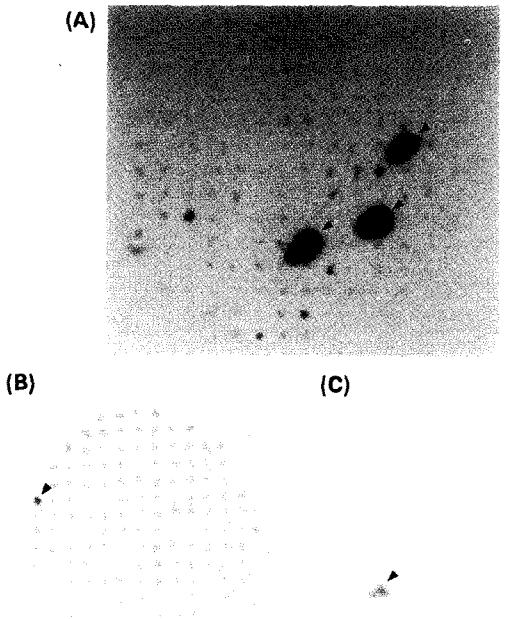

Fig. 2. Autoradiographs of colony hybridization experiments.

The genomic DNAs digested with *Eco*RI (A), *Sma*I/*Bam*HI (B), and *Xba*I/*Bam*HI (C) were inserted into pUC19, pUC18, and pUC19, respectively, and transformed into JM109 or XL-1-Blue cells. From hundreds of white colonies on X-Gal plates, three different clones, indicated by arrow heads, were obtained by colony hybridization.

From the restriction enzyme maps of the three cloned DNA fragments (Fig. 4A, 4B, and 4C), we examined the presence and order of all four rRNA genes, that is, LSU, SSU, 5.8S, and 5S rRNA genes, to elucidate the genetic organization of the rDNA cluster. To carry out Northern hybridization, three DNA probes, as in Fig. 4, a 2.9 kb fragment (*Bam*HI and *Hin*dIII digested, #1), a 2.3 kb fragment (*Hin*dIII and *Sma*I digested, #2) from pTMr102, and a 1.0 kb fragment (*Eco*RI digested, #3) from pTMr80 were prepared as DNA probes for locating the SSU and LSU rRNA genes. As shown in Fig. 5, the 2.9 kb fragment

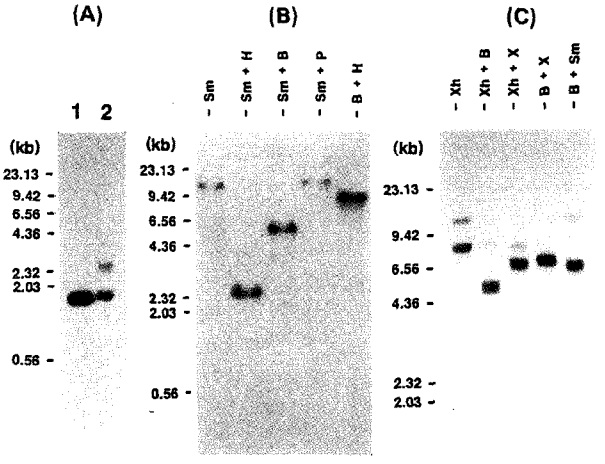

Fig. 3. Hybridization of pTMr101 to *T. matsutake* genomic DNA digested with various restriction endonucleases.

(A) Authenticity confirmation of pTMr101. Lane 1 and 2 represent pTMr101 and genomic DNA digested with *Eco*RI, respectively. (B) Southern hybridization pattern of *T. matsutake* genomic DNA using a 0.45 kb *Sma*I/*Eco*RI DNA fragment of pTMr101 as a probe. (C) Southern hybridization pattern of *T. matsutake* genomic DNA using a 0.2 kb *Xho*I/*Eco*RI DNA fragment of pTMr101 as a probe.

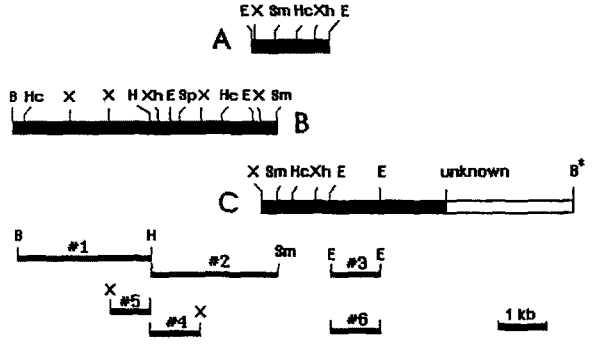

Fig. 4. Restriction map of the three cloned *T. matsutake* DNAs.

About 11.5 kb of *T. matsutake* rDNA repeat unit are shown with closed bars (the cloned DNA fragment in this work) and open bar (not cloned). The lower part of the figure also represents the DNA probes used for Northern hybridizations of Fig. 5 and Fig. 6. For abbreviations of restriction enzymes, refer to Fig. 1. The rightmost *Bam*HI, indicated by an asterisk, is the speculated restriction site from the result of Fig. 1.

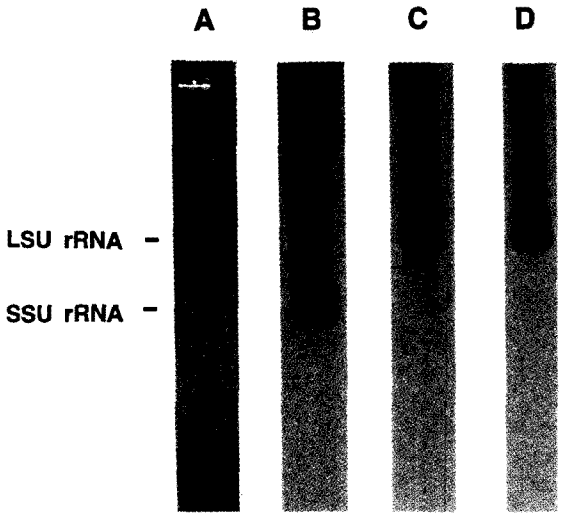

Fig. 5. Northern blot analysis of SSU and LSU rRNAs of *T. matsutake*.
Photograph of Agarose/formaldehyde gel electrophoresis of total RNA are shown (A). The probe #1, in Fig. 4, was hybridized to SSU rRNA (B), and the probes #2 and #3 were also hybridized to LSU rRNA (C and D, respectively).

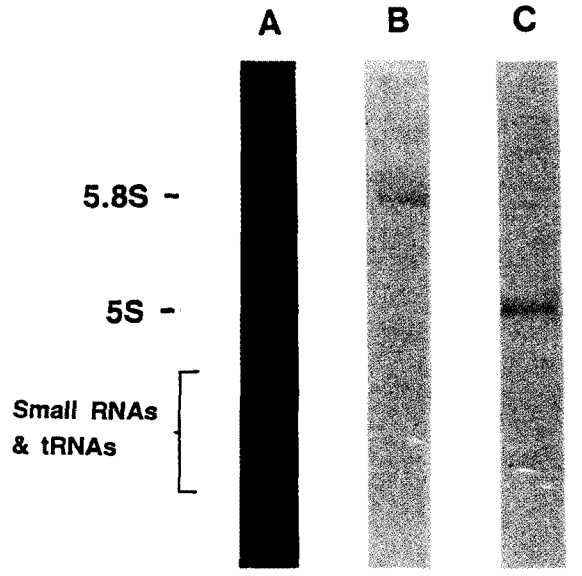

Fig. 6. Northern blot analysis of 5.8S and 5S rRNAs of *T. matsutake*.
Photograph of total RNA separated in 8% polyacrylamide gel (A), and hybridization patterns of 5.8S (B) and 5S rRNA (C) using the probes #4 and #6 (Fig. 4), respectively.

(#1) was hybridized to SSU rRNA (Fig. 5B) and, both the 2.3 kb (#2) and the 1.0 kb (#3) fragments to LSU rRNA (Fig. 5C and 5D, respectively). These results indicate that the order of rRNA genes are from SSU to LSU rRNA in our clones, which is the same result as in other studies elsewhere (12, 13). Especially, a faint band is detected in the upper site of the LSU rRNA bands, and it is assumed that the band is the precursor rRNA which is firstly transcribed in a precursor form containing SSU, 5.8S and LSU rRNAs, and then spliced into each mature form.

In order to prove the presence and location of the 5.8S and 5S rRNA genes in the rDNA repeat unit, three DNAs probes, a 0.75 kb fragment (*Hin*dIII and *Xba*I digested pTMr102, #4 in Fig. 4) for 5.8S rRNA, and a 1.2 kb fragment (*Bam*HI and *Xba*I digested pTMr102, #5) and a 1.0 kb fragment (*Eco*RI digested pTMr80, #6) for 5S rRNA, were prepared. From the results of Northern hybridization, it is considered that a 0.75 kb fragment was hybridized to 5.8S rRNA (Fig. 6B) and a 1.0 kb fragment to 5S rRNA (Fig. 6C), but the 1.2 kb fragment was not hybridized to 5.8S and 5S rRNA (data not shown). These results propose that 5.8S rRNA gene is located between the SSU and LSU rRNA genes, and that the 5S rRNA gene is right to the the LSU rRNA gene. The presence of the 5S rRNA gene on the same genomic repeat harboring SSU, 5.8S, and LSU rRNA genes, like *S. cerevisiae* (20) and *Tilletia* species (23), is not common among most eukaryotes studied so far that do not contain the 5S rRNA gene linked to those for other rRNA species (10). Additionally, we can speculate regarding the presence of ITS1, ITS2, IGS1, and partial IGS2 from these results. Therefore, we can also speculate that the organization of the rRNA genes is IGS2 (partial) - SSU rRNA- ITS1 - 5.8S rRNA - ITS2 - LSU rRNA - IGS 1 - 5S rRNA- IGS2 (partial). The assumed IGS2 region whose complete sequence has not yet been isolated in this work, is about a 4.7 kb in length, and located between 5S and SSU rRNA genes. The region is unusually large compared to those from other eukaryotes (20, 23). It is unclear why this region is so large, and whether the region contains the genes coding for proteins or other RNA molecules, and the structural elements essential to gene regulation. We are now determining the nucleotide sequence of ribosomal DNA fragments cloned in this work. When further studies are completely finished, using all four rRNA genes and noncoding sequences, a number of phylogenetic data concerning *T. matsutake* can be obtained, and RFLP analysis in the genus *Tricholoma* using rDNA as a probe will also be possible. The information about the structure of rDNA repeat may possibly be applied in the development of probes specific to *T. matsutake*.

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