

# Application of fibre-FISH (fluorescence *in situ* hybridization) to filamentous fungi: visualization of the rRNA gene cluster of the ascomycete *Cochliobolus heterostrophus*

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**Fibre-FISH (fluorescence *in situ* hybridization) has not been used in filamentous fungi before to the authors' knowledge. In this study, this technique was applied to a filamentous ascomycete, *Cochliobolus heterostrophus*, to visualize the organization of the rRNA gene clusters (rDNA). Using protoplasts embedded in agarose, DNA fibres were released from interphase nuclei and extended on a glass slide. Four kinds of probes (0.5–9.0 kb in size) that correspond to specific regions in the repeat unit of rDNA were hybridized singly or in combination to the DNA fibres, and the hybridization was detected with fluorescein- and/or rhodamine-conjugated antibodies after one round of signal amplification. The alternating arrangement of 18S and 28S rRNA genes as well as the tandem repetitive nature of the repeat units were clearly visualized by this single- or two-colour fibre-FISH. With a probe targeting the 5.8S or 18S rRNA gene, a region spanning over 800 kb could be visualized in a single fibre, allowing estimation of both the copy number of the repeat unit in rDNA and the stretching degree of the DNA fibre. It was shown that *C. heterostrophus* has more than 90 copies of the repeat unit in its rDNA and the stretching degree was similar to the value based on the Watson–Crick model. Visualization of individual genes on an extended DNA fibre was accomplished in filamentous fungi by this study.**

Keywords: ascomycete, chromatin, nucleolus, rDNA, ribosome

## INTRODUCTION

Highly sensitive fluorescence *in situ* hybridization (FISH) has recently been developed (Heng *et al.*, 1992; Wiegant *et al.*, 1992; Parra & Windle, 1993; Heiskanen *et al.*, 1994). This technique is called fibre-FISH because extended DNA fibres released from lysed nuclei are used as specimens for hybridization instead of nuclei or chromosomes as for conventional FISH. Fibre-FISH can distinguish two probes separated by 1 kb on a DNA fibre (Heng & Tsui, 1998), and because of this resolution, has been used in mammals and plants for physical mapping (Parra & Windle, 1993; Heiskanen *et al.*, 1995; Raap *et al.*, 1996; Jackson *et al.*, 1998), detection of genomic rearrangement (Parra & Windle, 1993; Heiskanen *et al.*, 1995), direct visualization of DNA replication (Rosenberg *et al.*, 1995), positional

cloning (Courseaux *et al.*, 1996; Leppanen *et al.*, 1996), analysis of gene organization (Fransz *et al.*, 1996; Ersfeld *et al.*, 1998; Liu *et al.*, 1998), and so on.

In spite of the proven usefulness of fibre-FISH in higher eukaryotes, no report using this technique has been published so far for fungi. Considering the extreme smallness of their chromosomes and nuclei compared with those of higher organisms, it is not surprising that application of conventional FISH to the metaphase chromosomes or interphase nuclei has been very limited in this group of organisms (Taga & Murata, 1994; Taga *et al.*, 1999). Regarding fibre-FISH, however, there seems to be no innate hindrance to its use in fungi. Once DNA fibres are properly prepared, FISH techniques should be applicable to fungal DNA fibres without difficulty, as in other organisms.

In this study, we aimed to establish the fibre-FISH technique for filamentous fungi using an ascomy-

**Abbreviation:** FISH, fluorescence *in situ* hybridization.

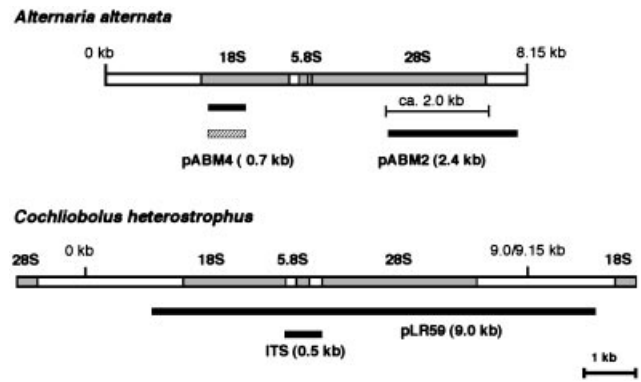
cetous corn pathogen, *Cochliobolus heterostrophus* (Drechsler) Drechsler [anamorph, *Bipolaris maydis* (Nishikado and Miyake) Shoemaker]. This fungus serves as a model fungal pathogen in plant pathology with which extensive molecular genetic studies have been conducted. As the target DNA sequences, we chose the rRNA gene cluster, or rDNA, because relatively easy detection of this region, owing to its repetitive nature and occupation of a large part of a nucleolar chromosome (M. Taga, D. Tsuchiya & M. Murata, unpublished results), was thought to make it suitable for screening and establishing experimental conditions for fibre-FISH techniques in this fungus.

In this study, procedures for preparing DNA fibres for FISH were established in *C. heterostrophus*. With these fibres, the *in situ* organization of rDNA in terms of the arrangement of rRNA genes in the cluster was visually revealed by single- or two-colour fibre-FISH. Data for the number of repeat units in rDNA and the stretching degree of the DNA fibre were also obtained. To our knowledge, this is the first report of visualization of individual genes on an extended DNA fibre in fungi.

## METHODS

**Preparation of protoplast-embedded agarose block.** Protoplasts from hyphal cells of *C. heterostrophus* strain B30.A3.R.45 (a gift from C. R. Bronson, Department of Plant Pathology, Iowa State University, Ames, IA, USA) were embedded in low melting agarose (hereinafter called protoplast-agarose block), and used as the source of nuclei from which DNA fibres were to be released. The methods for protoplasting and embedding of cells were the same as those used for PFGE. Briefly, conidia harvested from 1-week-old cultures were incubated in liquid complete medium (Leach *et al.*, 1982) at a concentration of  $1 \times 10^6$  ml<sup>-1</sup>, and allowed to germinate on a reciprocal shaker at 110 strokes min<sup>-1</sup> for 12–15 h at 27 °C. The germlings were vacuum-filtered on filter paper (Whatman no. 4), washed with 0.7 M NaCl, then suspended in 6–10 ml filter-sterilized enzyme solution [Novozym 234 (Novo Biolabs) 2 mg; Kitalase (Wako Pure Chemical) 3 mg;  $\beta$ -glucuronidase (type HA-4, Sigma) 3 mg ml<sup>-1</sup> of 0.7 M NaCl]. After 2–4 h incubation at 30 °C on a reciprocal shaker (65–75 strokes min<sup>-1</sup>), protoplasts were filtered through four layers of Kimwipe (Jujo Kimberly), collected by centrifugation at 550 g for 10 min, and the pellet was washed twice with 0.7 M NaCl by centrifugation. The final protoplast pellet was suspended in SE (1 M sorbitol, 50 mM EDTA, pH 8.0), embedded in 0.5% (w/v) low melting agarose (Bio-Rad) in SE at a final concentration of approximately  $1 \times 10^8$  protoplasts ml<sup>-1</sup>, and then incubated in NDS (0.5 M EDTA; 10 mM Tris/HCl, pH 8.0; 1%, w/v, sodium lauroyl sarcosinate) for 14 h at 37 °C to lyse cells. After being rinsed three times in 50 mM EDTA (pH 8.0) for 30 min each at 37 °C, samples were stored in this solution at 4 °C until they were used.

**Preparation of DNA fibres.** Glass slides were coated with poly-L-lysine (Sigma) by soaking for 5 min and dried at room temperature overnight. A tiny protoplast-agarose block (approx. 20  $\mu$ l) was placed on a slide and mounted with 40  $\mu$ l sterile water. The slide was placed on a heat block at 85 °C for 20–30 s to melt the agarose. The liquified agarose drop was mechanically extended with a coverslip as described by Heiskanen *et al.* (1994) and air-dried. The slides were treated



**Fig. 1.** Probes used for FISH and their target sites in rDNA. pABM2 and pABM4 are from *Alternaria alternata*. Probes represented as black and hatched bars were labelled with biotin and digoxigenin, respectively. Hybridization of these probes with target sites was visualized with fluorescein-conjugated (for biotinylated probes) or rhodamine-conjugated (for digoxigenin-labelled probes) secondary antibodies. Physical maps of the repeat unit of rDNA of *C. heterostrophus* and *A. alternata* are based on Garber *et al.* (1988) and Tsuge *et al.* (1989), respectively.

with 100  $\mu$ g RNase A ml<sup>-1</sup> in  $2 \times$  SSC ( $1 \times$  SSC: 0.15 M NaCl, 0.015 M sodium citrate) for 45 min at 37 °C, dehydrated through an ethanol series (70–80–99%), and air-dried.

**DNA probes.** Four probes, each corresponding to a specific part of the repeat unit of rDNA, were used (Fig. 1). pABM2 and pABM4 are heterologous probes from the filamentous imperfect fungus *Alternaria alternata*, each of which contains *Xba*I fragments of the rDNA repeat unit of this fungus in Bluescribe M13 (Tsuge *et al.*, 1989). pLR59 contains a 9 kb *Pvu*II fragment corresponding to the whole repeat unit of *C. heterostrophus* in pBR322 (Garber *et al.*, 1988). These three plasmid DNAs were isolated as described by Maniatis *et al.* (1982). ITS, which covers the whole region of the internal transcribed spacers and the 5.8S rRNA gene, was amplified by PCR with universal primers ITS1 and ITS4 (White *et al.*, 1990) using total genomic DNA of B30.A3.R.45 as template. The PCR reaction mixture contained approximately 100 ng template DNA, 200  $\mu$ M each of dNTPs, 1.5 mM MgCl<sub>2</sub>, and 1.25 U *Taq* polymerase (Toyobo) in a 50  $\mu$ l reaction volume. The thermal conditions were 10 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, then final extension for 10 min at 72 °C. After completion of the reaction, an aliquot of the mixture was directly used as a template for PCR labelling.

**Probe labelling.** For pABM2 and pLR59, the whole plasmid (vector plus insert) was labelled with biotin-14-dATP by nick translation using the BioNick Labelling System (Gibco-BRL). For pABM4, the insert was excised from the plasmid, and labelled with digoxigenin-11-dUTP by nick translation using the Nick Translation Kit (Boehringer Mannheim). ITS was biotin-labelled by PCR with the primers ITS1 and ITS4 under the following conditions. The reaction mixture included 1  $\mu$ l of the amplified product of the first PCR, 160  $\mu$ M biotin-16-dUTP (Boehringer Mannheim), 80  $\mu$ M dTTP, 200  $\mu$ M each of the other dNTPs, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 1.25 U *Taq* polymerase (Toyobo) in a 50  $\mu$ l reaction volume. The thermal conditions were 5 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 62 °C, 3 min at 72 °C, and a final extension of 10 min at 72 °C. The

amplified DNA was subsequently purified by ethanol precipitation to remove free oligonucleotides and unincorporated dNTPs.

**In situ hybridization.** Hybridization mixture (50%, v/v, formamide; 10%, w/v, dextran sulfate; 100 ng sonicated salmon sperm DNA  $\mu\text{l}^{-1}$ ; and the proper amount of labelled probe DNA/ $2 \times$  SSC) was applied to the specimen, covered with a coverslip (18  $\times$  32 mm), and sealed with rubber cement. The slide was heated for denaturation on a hot plate at 80 °C for 5 min, and incubated for hybridization at 37 °C for 16–20 h. After hybridization, the coverslip was removed by floating it off in  $2 \times$  SSC. Subsequently, the slide was washed once for 15 min in 50% formamide dissolved in  $4 \times$  SSC at 37 °C, twice for 8 min in  $2 \times$  SSC, once in  $4 \times$  SSC for 5 min, and then blocked with 1% Block Ace (Dainippon Pharmaceutical)/ $4 \times$  SSC for 10 min at room temperature. Hybridization of biotin-labelled probes was detected with goat anti-biotin antibody (Vector Laboratories) followed by staining with fluorescein-conjugated rabbit anti-goat antibody (Boehringer Mannheim). Hybridization of digoxigenin-labelled probes was detected with mouse anti-digoxigenin (Boehringer Mannheim) followed by amplification with rhodamine-conjugated rabbit anti-mouse antibody (Chemicon International). All of the antibodies were diluted in  $4 \times$  SSC containing 1% Block Ace. Incubations for detection and staining were 1 h each at 37 °C, followed by washing for 5 min in  $4 \times$  SSC, for 5 min in  $4 \times$  SSC containing 0.1% Triton X-100, and for 5 min in  $4 \times$  SSC. Finally, the specimens were mounted with an antifading solution, Vectashield (Vector Laboratories).

**Fluorescence microscopy.** Observations were made with an epifluorescence microscope (Olympus BHS-RFC) equipped with an IB excitation filter cube (Olympus BH2-DMIB) for fluorescein, and triple band pass filter (Chroma) for two-colour FISH. Photographs were taken on 800 ASA/ISO colour print film (Fujicolor Super HG800). The colour images on the negatives were digitized with a film scanner (Coolscan II; Nikon), and processed by personal computer software Photoshop version 5.0 (Adobe).

## RESULTS AND DISCUSSION

### Visualization of the rDNA region on DNA fibres

Extended DNA fibres were easily prepared from protoplast–agarose blocks. The feasibility of FISH to the DNA fibres was first confirmed by using a plasmid probe, pLR59, whose insert encompasses an entire rDNA repeat unit (Fig. 2a). As expected, a long linear track consisting of consecutive signals was observed by this FISH, indicating that our procedures worked satisfactorily to visualize rDNA on the DNA fibre. At least 20–30 DNA fibres with such signals for rDNA were usually observed per slide. The protoplast–agarose blocks used in this study had been kept in a refrigerator for almost 2 years. Together with our experience of other fungi in which DNAs in protoplast–agarose blocks were mostly intact after at least 7–8 years' storage (D. Tsuchiya & M. Taga, unpublished result), considerably long-term storage of the block may be possible for fibre-FISH. Although proteinase K treatment was reported to be important for preparing DNA fibres of good quality (Heiskanen *et al.*, 1995), our result suggests that such treatment is not essential, at least in filamentous fungi.

### Analyses of the copy number of a repeat unit and the stretching degree of DNA fibres

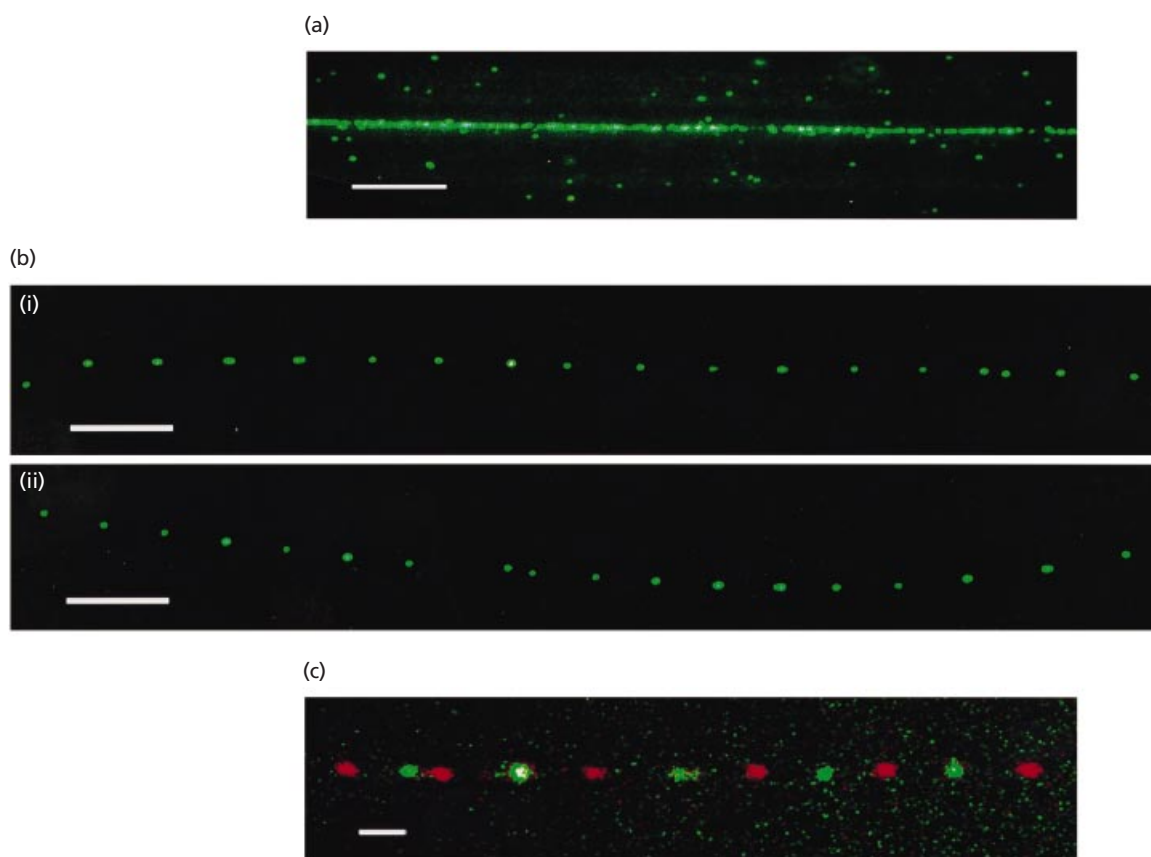
Two probes, pABM4 and ITS, were used for hybridization to detect the 18S and 5.8S rRNA genes. With either probe, a long string of signals with similar signal intervals was observed [Fig. 2b, parts (i) and (ii)]. These results apparently showed the regularly repeated arrangement of the two genes in the rDNA. The observed number of signals on each DNA fibre, which should represent the copy number of a repeat unit, varied depending on the fibre. For the total of 110 DNA fibres counted for both probes, the number ranged from 37 to 92, with a mean of 62. More than two-thirds of the total counts fell between 50 and 80. Considering that the smaller numbers in these counts were probably artefacts caused by several factors such as shearing of DNA fibres, fading out of signals, and poor hybridization, it seems to be reasonable to state that *C. heterostrophus* has at least 92 copies in a cluster. This estimation is compatible with the previous estimate of 130 copies by conventional Southern hybridization analysis for this fungus (Garber *et al.*, 1988), the reliability of which, however, has never been examined. Since FISH is superior to Southern hybridization analysis in measuring copy number, a firm conclusion as to the number of repeats could be derived from future FISH analysis on the whole rDNA region.

As to the interval length between signals, there was no significant difference between the two FISH experiments; i.e. the mean lengths in the FISH with pABM4 and that with ITS were  $3.47 \pm 0.11 \mu\text{m}$  [observed number ( $n$ ) = 256] and  $3.35 \pm 0.09 \mu\text{m}$  ( $n$  = 221), respectively. Adopting 9.0 or 9.15 kb as the size of the repeat unit of this fungus (Garber *et al.*, 1988), the stretching degree of DNA fibres was calculated to be 2.64 (=  $9.0/3.41$ ) kb  $\mu\text{m}^{-1}$  (weighted mean length for both experiments) or 2.68 (=  $9.15/3.41$ ) kb  $\mu\text{m}^{-1}$ . These are in reasonable agreement with the extension of B-DNA of the Watson–Crick model (2.9 kb  $\mu\text{m}^{-1}$ ) as well as with the values obtained in other organisms, i.e. 2.77 kb  $\mu\text{m}^{-1}$  for mammalian rDNA (Shiels *et al.*, 1997) and 3.27 kb  $\mu\text{m}^{-1}$  for plant rDNA (Fransz *et al.*, 1996).

In addition, these experiments showed that preparation of DNA fibres exceeding 800 kb (9 kb  $\times$  90 copies = 810 kb) is possible in fungi. The upper limit of fibre length in fibre-FISH had been claimed to be approximately 500 kb in mammals (Heng & Tsui, 1998; Heiskanen *et al.*, 1996), whereas de Jong *et al.* (1999) indicated that long fibres up to 1 Mb are obtainable in plants. Preparation of fibres 1 Mb long may be possible irrespective of the kind of organism.

### Analysis of arrangement of 18S and 28S rRNA genes in rDNA

Two-colour FISH was carried out to analyse the arrangement of 18S and 28S rRNA genes in the cluster. The biotinylated insert that was excised from pABM2 and the digoxigenin-labelled pABM4 were simultaneously hybridized to the same specimen, and the



**Fig. 2.** (a) Fibre-FISH showing an rDNA region on a DNA fibre of *C. heterostrophus*. The biotinylated probe pLR59 that includes the entire rDNA repeat unit of the fungus was hybridized. Detection and fluorescence staining were done with fluorescein-conjugated antibody after one round of signal amplification. A long linear green track represents an rDNA region. Observation was made under IB excitation. Bar, 5  $\mu\text{m}$ . (b) Fibre-FISH targeting a specific site in the rDNA repeat unit of *C. heterostrophus*. (i) Internal transcribed spacer region including the 5.8S rRNA gene detected with the 0.5 kb probe ITS; (ii) 18S rRNA gene detected with the 0.7 kb probe pABM4. Both probes were biotinylated and the hybridization was visualized as in part (a). Bar, 5  $\mu\text{m}$ . (c) Two-colour fibre-FISH showing the alternating arrangement and tandem repeat of 18S and 28S rRNA genes in an rDNA cluster of *C. heterostrophus*. The two probes pABM2 and pABM4 targeting the 28S and 18S rRNA gene, respectively, were used. Red signals represent 18S rRNA genes and green signals represent 28S rRNA genes. Observations were made with a triple band pass filter. Bar, 1  $\mu\text{m}$ .

hybridization was detected with fluorescein- and rhodamine-conjugated secondary antibodies. As shown in Fig. 2(c), alternating green and red signals corresponding to the sites of 28S and 18S rRNA genes were observed. Using a rather simpler detection system in which biotinylated and digoxigenin-labelled probes were detected with avidin-FITC and rhodamine-conjugated anti-digoxigenin antibodies, respectively, signals were hardly visible. The result of this FISH presents proof for the head-to-tail tandem repetition of the units in rDNA. The space between neighbouring green or red signals was roughly constant, the mean of which was  $3.89 \pm 0.22 \mu\text{m}$  ( $n = 22$ ). With this value, the stretching degree of the DNA fibre was calculated as above to be 2.31 or 2.35  $\text{kb} \mu\text{m}^{-1}$ .

### Concluding remarks

In this study, the organization of rDNA in terms of the arrangement of rRNA genes was visualized for the first

time in fungi. Except for 5S rDNA, as far as we know, there have been only two papers that have used fibre-FISH for visualizing the array of rDNA repeats. In a plant, Fransz *et al.* (1996) revealed the tandem repeated array of 18S and 28S rRNA genes in *Arabidopsis thaliana* by two-colour FISH. In a mammal, the repeated arrangement of 18S rRNA genes was shown by single-colour FISH with a probe containing a partial region of the gene (Shiels *et al.*, 1997). In contrast with the present study, counting of the copy number of repeat units was not performed in either paper. As to the FISH procedures used for the prepared DNA fibre, two rounds of signal amplification were done by Fransz *et al.* (1996), but a single round of amplification gave satisfactory results in the work of Shiels *et al.* (1997) and our work.

This study was done using an ordinary epifluorescence microscope equipped with a camera for 35 mm film. As claimed for other organisms (Wiegant *et al.*, 1992; Zhong *et al.*, 1998), instruments such as a cooled CCD

camera are not essential for performing fibre-FISH in fungi as well. Therefore, studies similar to ours can be conducted in modestly equipped laboratories.

With the same procedures as described here, we have succeeded in visualizing 28S rRNA genes in another filamentous ascomycete, *Nectria haematococca* (anamorph, *Fusarium solani*), as well as detecting a single copy gene, *DEC1*, which encodes decarboxylase in *C. heterostrophus* (D. Tsuchiya & M. Taga, unpublished results). This indicates the versatility of our procedures in filamentous fungi. Thus the merits of fibre-FISH, such as higher mapping resolution and detection sensitivity, will be enjoyed in various aspects of genetic study for many fungi. The introduction of this method will especially accelerate high-resolution physical mapping of genomic clones and contribute to the elucidation of genome organization of fungi.

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