



Electrophoretic molecular karyotype of the dermatophyte *Trichophyton rubrum*

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Abstract

The electrophoretic karyotype of the dermatophyte *Trichophyton rubrum* was established using contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Five chromosomal bands of approximately 3.0 to 5.8 megabase pairs (Mbp) each were observed and together indicated that 22.05 Mbp of the total genome are organized as chromosomal macromolecules. In addition to establishing the number and size of *T. rubrum* chromosomes, these results open perspectives for the construction of chromosome-specific libraries and for the physical mapping of genes of interest, thus permitting future gene linkage studies in this pathogen. A detailed understanding of the karyotype and genomic organization of *T. rubrum* should contribute to further genetic, taxonomic and epidemiological studies of this dermatophyte.

Key words: *Trichophyton rubrum*, fungal chromosomes, electrophoretic karyotype, dermatophyte, contour-clamped homogeneous electric field.

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Introduction

Trichophyton rubrum is a cosmopolitan filamentous fungus that can infect human keratinized tissue (skin, nails and, rarely, hair), and is one of the most frequently encountered dermatophytes (Costa *et al.*, 2002; Jennings *et al.*, 2002; Monod *et al.*, 2002). This pathogen, which normally causes well-characterized superficial infections, also produces skin infections in unusual parts of the body in immunodepressed patients (Squeo *et al.*, 1998; Sentamilselvi *et al.*, 1998; Smith *et al.*, 2001). Despite the incidence and medical importance of this dermatophyte, little is known about its genetic and biological characteristics. Classic genetic analysis is not possible with *T. rubrum* because no sexual phase is known. In addition, the number of chromosomes in this species has not been determined by light microscopy since mitotic material is generally unsuitable for the study of fungal chromosomes (Zolan, 1995).

Pulse-field gel electrophoresis (PFGE) is an effective method for separating chromosome-sized DNA, and is an important tool for basic genetic studies, especially in lower eukaryotes such as fungi and yeasts. Furthermore, summa-

tion of the sizes of the different molecules separated by this technique provides an estimate of the genome size. Pulse-field gel electrophoresis and a refinement known as contour-clamped homogeneous electric field (CHEF) gel electrophoresis, has been used to determine the molecular karyotype of many fungi, including *Aspergillus nidulans* (Brody and Carbon, 1989), *Aspergillus niger* (Verdoes *et al.*, 1994), *Neurospora crassa* (Orbach *et al.*, 1988), *Paracoccidioides brasiliense* (Montoya *et al.*, 1999), *Aspergillus fumigatus* (Tobin *et al.*, 1997), and pathogenic yeasts such as *Candida albicans* and *Candida glabrata* (Fodor *et al.*, 2002).

Since the karyotype of *T. rubrum* is unknown we have standardized the conditions for obtaining protoplasts in order to determine the electrophoretic karyotype of this species using CHEF gel electrophoresis and to estimate its genome size.

Material and Methods

Strain

A clinical isolate of *T. rubrum* (H6) obtained from a patient admitted to the University Hospital of Ribeirão Preto, SP, Brazil, and identified by standard methods (McGinnis, 1980; Fachin *et al.*, 1996) was used in this study.

Preparation of intact chromosomal DNA

After incubation of isolate H6 in Sabouraud glucose agar for 20 days at 28 °C, conidia and mycelium from one petri dish were carefully harvested and incubated with 100 mL of liquid Sabouraud glucose medium at 28 °C and 250 rpm for 18 h. The resulting mycelium was harvested by vacuum filtration through Whatman paper (Whatman International, Maidstone, UK) and washed with sterile water. Protoplasts were obtained by incubating the mycelium in 30 mL of lytic solution (600 mg of Glucanex (Novo Nordisk, Swiss), 50 mM KH₂PO₄, pH 7.5, and 0.7 M KCl) for 3–4 h at 28 °C. The suspension of protoplasts was filtered through glass wool and the protoplasts were collected by centrifugation (1575 g, 5 min). The pellet was resuspended and washed twice in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂). The final pellet was resuspended in protoplasting buffer (0.9 M sorbitol, 0.125 M EDTA, pH 7.5) to a protoplast concentration of 1.0×10^8 – 1.5×10^8 cells per mL. The suspension was heated to 42 °C and an equal volume of molten 1.4% (w/v) InCert agarose (Seaken Gold agarose, FMC Bioproducts) in protoplasting buffer pre-cooled to 42 °C was added. The agarose/protoplast mixture was then poured into a plug mold and solidified at room temperature for 30 min. Protoplast lysis was induced by adding proteinase K (2 mg/mL) in digestion buffer (0.5 M EDTA, pH 8.0, 10 mM Tris-HCl, 1% Na-*N*-laurylsarcosinate) at 50 °C for 72 h. Finally, the plug was washed three times in 50 mM EDTA, pH 8.0, at 50 °C and once at room temperature, and then stored at 4 °C in 50 mM EDTA.

CHEF gel electrophoresis conditions

Agarose plugs were sealed in the wells of 0.7% (w/v) agarose gels (Seaken Gold agarose, FMC Bioproducts). Plugs containing *Schizosaccharomyces pombe* and *Hansenula wingei* chromosomal preparations (BIO-RAD) were used as size standards. The chromosomes were separated by CHEF gel electrophoresis (CHEF-DR III BIO-RAD) under the following electrophoretic conditions: 72 h, 1.5 Vcm⁻¹, 60 min pulse, ±120° field angle; 144 h, 1.4 Vcm⁻¹, 45 min pulse, ±120° field angle; 76 h, 1.8 Vcm⁻¹, 25 min pulse, ±120° field angle; 0.5 X TBE (0.045 M Tris-borate, 0.001 M EDTA pH 8.0) at 12 °C (Tobin *et al.*, 1997). The gels were stained with ethidium bromide and photoimaged using Kodak Digital Science ID image analysis software. To achieve a better separation of small chromosomes, the agarose plugs were sealed in the wells of a 0.8% (w/v) agarose gel (Seakem Gold agarose, FMC Bioproducts) and a second CHEF gel was run under the following conditions: 48 h, 3.0 Vcm⁻¹, 500-second pulse, ±106° field angle; 0.5 X TBE at 12 °C.

Results

Intact chromosomes of strain H6 of *T. rubrum* were identified by CHEF gel electrophoresis (Figure 1). The ini-

tial electrophoretic conditions did not adequately separate the smallest chromosomes (Figure 1A), but a second CHEF gel with conditions optimized for this size range showed a more efficient separation of these chromosomes (Figure 1B). Under the conditions used here, at least five chromosome bands were detected. Assuming a linear separation, comparison with the *S. pombe* and *H. wingei* molecular weight standards showed that these bands were 3.0, 3.05, 4.6, 5.6 and 5.8 Mbp in size. The chromosomes shown in Figure 1 have been numbered according to size, starting with the largest. This numbering is arbitrary and does not take into consideration the presence of possible co-migrating chromosomes.

Discussion

CHEF gel electrophoresis showed that the molecular karyotype of the clinical isolate H6 of *T. rubrum* consists of five chromosomal bands which range in size from 3.0 Mbp to 5.8 Mbp, and correspond to a total genome size of 22.05 Mbp. Thus, at least 22.05 Mbp of the genome are organized as chromosomal macromolecules. Table 1 shows the chromosome and genome sizes of other fungi compared to *T. rubrum*.

Chromosome length polymorphisms are common in fungi (Skinner *et al.*, 1991) and variations in the molecular karyotype of *T. rubrum* may exist among different isolates. Such variability has been observed in yeast and fungal species such as *Candida albicans* (Pittet *et al.*, 1991) and *Septoria nodorum* (Cooley and Caten, 1991), and may reflect chromosomal rearrangements, such as deletions, duplications and translocations, as well as different degrees of pathogenicity in the same species. CHEF analysis is useful in fungal epidemiological studies, and has revealed inter- and intraspecies variations in *Candida* infections (Pittet *et al.*, 1991). The genetic variability of *T. rubrum* isolates has been demonstrated by sequence analysis of the 18S rDNA gene combined with morphological and enzymatic analysis (Nascimento and Martinez-Rossi, 2001).

The ability to separate intact chromosomes has led to the assignment of cloned genes to chromosomal locations without the need for crosses or progeny tests. Any cloned DNA segment may be assigned to a chromosome by Southern hybridization experiments (Skinner *et al.*, 1991; Scherer and Magee, 1990). This strategy has been used to locate the MDR-like genes *AfuMDR1* and *AfuMDR2* of *A. fumigatus* (Tobin *et al.*, 1997).

In addition to establishing the number and size of *T. rubrum* chromosomes, the present results open perspectives for constructing chromosome-specific libraries, for determining the presence of translocations, duplications and deletions, and for identifying the chromosomal location of genes of interest. This information should facilitate genetic linkage studies in this pathogen. The construction of a genetic map for *T. rubrum* will be useful for cloning genes of interest by “chromosome walking” to cognate

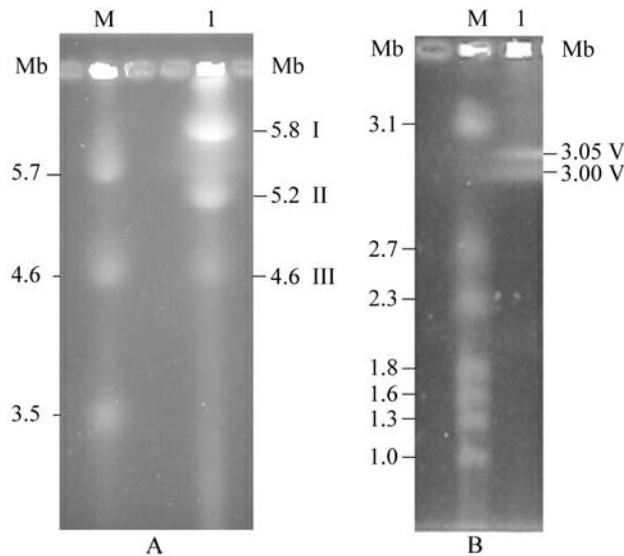


Figure 1 - CHEF electrophoretic karyotype of strain H6 of *T. rubrum* (1). (A) Separation of intact chromosomal DNAs (I to III) by CHEF in a 0.7% (w/v) agarose gel. M is the *S. pombe* standard. (B) Optimized CHEF separation of chromosomes IV and V in a 0.8% (w/v) agarose gel. M is the *H. wingei* standard. The approximate lengths of the chromosomal bands are indicated.

genes (Agnan *et al.*, 1997). The information obtained will contribute to our understanding of the genomic organization of this dermatophyte. Finally, determination of the *T. rubrum* genome size is important in order to determine the genome coverage of any genomic library of this fungus to be used in genome projects. The sequencing of the *T. rubrum* genome, together with that of 14 other fungi, has recently been proposed as part of the Fungal Genome Initiative (FGI) (<http://www-genome.wi.mit.edu/seq/fgi>). This project will allow the application of genomic technologies to the study of fungal pathogenicity, and is particularly important given the limited usefulness of classic genetic approaches for studying this species.

Table 1 - Electrophoretic karyotypes of various fungi.

Fungus	Number of chromosomes (haploid)	Chromosome size range (Mbp)	Genome size (Mbp)	Reference
<i>Aspergillus fumigatus</i>	5	1.7-4.8	15.8	Tobin <i>et al.</i> (1997)
<i>Aspergillus nidulans</i>	8	0.4-4	31	Brody and Carbon (1989)
<i>Aspergillus niger</i>	8	3.5-6.6	35.5-38.5	Verdoes <i>et al.</i> (1994)
<i>Aspergillus sydowii</i>	8	4-8	40	Schmitt <i>et al.</i> (2002)
<i>Cercospora kikuchii</i>	8	2.0-5.5	28.4	Higtower <i>et al.</i> (1995)
<i>Cochliobolus sativus</i>	15	1.25-3.8	33	Zhong <i>et al.</i> (2002)
<i>Neurospora crassa</i>	7	4-12.6	47	Orbach <i>et al.</i> (1988)
<i>Paracoccidioides brasiliensis</i>	5	3.2-10	29.7	Montoya <i>et al.</i> (1999)
<i>Penicillium purpurogenum</i>	5	2.3-7.1	21.2	Chavez <i>et al.</i> (2001)
<i>Trichophyton rubrum</i>	5	3.0-5.8	22.05	Present paper
<i>Ustilago hordei</i>	20	0.17-3.15	18.4-25.9	McCluskey and Mills (1990)

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