

Short communication

High-Molecular-Weight Genomic DNA Isolation from *Doratomyces microsporus* and Synthesis of a Genomic DNA Library

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Abstract

A protocol for isolation of genomic DNA from a filamentous fungus was optimised and a genomic library was constructed. Various methods for preparing genomic DNA from different fungi are known: simplified methods to allow processing of large sample numbers and methods to increase the quality of DNA, which is especially important for synthesis of a genomic DNA library. An isolation of high-molecular-weight DNA from a fungus is a challenging process since cell wall composition and cellular components differ with the fungal species. A procedure for isolation of high-molecular-weight DNA from the keratinolytic filamentous fungus *Doratomyces microsporus*, strain MZKI B399, is described and compared to the method used for filamentous bacteria and to the method including commercially available DNA extraction buffer. The isolation protocol that gave the desired quality of DNA was optimised in the following steps: disruption of cells by grinding in liquid nitrogen, followed by removal of polysaccharides and proteins by a phenol extraction buffer and finally precipitation of DNA with isopropanol. The obtained DNA was used to produce the bacteriophage genomic DNA library of the fungus.

Keywords: Keratinolytic fungus, *Doratomyces microsporus*, genomic DNA library, DNA isolation

1. Introduction

Molecular biological studies of organisms with an as yet unsequenced genome require construction of a genomic DNA library. Isolation of high-molecular-weight genomic DNA from filamentous fungi is problematic due to a rigid cell wall, high polysaccharide content¹ and presence of nucleic acid hydrolysing enzymes. Numerous procedures for fungal DNA isolation have been developed that more or less successfully overcome the described difficulties. Due to specific characteristics of each particular group of fungi, a specific method often has to be designed for the chosen group.^{2–7}

For disruption of fungal cell wall, an enzymatic^{2–5} or a mechanical force, such as sequential rapid freezing and thawing (for yeasts),^{8,9} grinding lyophilized mycelium,⁶ and disruption of cell walls in micro-dismembrators⁷ could be used. The cell wall-degrading enzymes are used to form spheroplasts that can easily be disrupted with extraction buffers. However, a more efficient but time-consuming method for cell wall disruption is grinding the frozen mycelium.

Cetyltrimethylammonium bromide (CTAB) is usually used for removal of polysaccharides.^{10,11} Proteinase K^{6,12,13} or phenol along with detergents,⁷ or benzyl chloride¹⁴ are used for removal of proteins. However, high-molecular-weight DNA is essential for construction of a genomic DNA library therefore a high number of steps in the procedure should be avoided due to higher risk of shearing the DNA molecules.

A deuteromycetous fungus *Doratomyces microsporus* produces a serine proteinase with a high keratinolytic activity^{15–17} known to hydrolyse keratinous and also some non-keratinous proteins. Keratinases are important enzymes with potential application in pharmaceutical, cosmetic and leather industries, since their specific substrate is keratin, a fibrillar protein present in skin and its appendages.

Research is underway to further increase the enzymatic yield in a bioprocess by genetic modification of the fungal strain. In order to clone the keratinase from a not fully investigated filamentous fungus *D. microsporus* we intended to construct a genomic DNA library. The first, and the most important, step in the genomic DNA library

1 synthesis involves isolation of high-molecular-weight
2 DNA. Until now, no protocol for *D. microsporus* has been
3 described. Our aim was to develop a suitable protocol to
4 obtain high-molecular-weight genomic DNA from the
5 fungus, which could later be used to construct a genomic
6 DNA library.

2. Experimental

2.1. Enzyme and General Procedures

12 The restriction enzyme *Sau3AI*, T4 DNA ligase,
13 *Bam*HI-predigested LambdaGEM-11 *Bam*HI arms were
14 from Promega (Madison, WI, USA), a Gigapack III Gold
15 Packaging Extract was from Stratagene (La Jolla, CA,
16 USA) and DNase-free RNase, phenol equilibrated with
17 TRIS buffer pH 8.5 were from Sigma (St. Louise, MO,
18 USA). Basic procedures for DNA manipulations and hori-
19 zontal agarose gel electrophoresis are described by Sam-
20 brook et al.¹⁸ Other chemicals were DNase and RNase
21 free.

2.2. Microorganism and Cultivation

25 The keratinolytic filamentous fungus *Doratomyces*
26 *microsporus*, strain MZKI B399¹⁵ was obtained from the
27 Culture Collection of The National Institute of Chemistry,
28 Ljubljana, Slovenia. Spores were prepared by growing the
29 fungus on agar slants at 30 °C for seven days. A sterilized
30 complete medium, according to Benčina et al.,¹⁹ in ali-
31 quots of 100 mL in 0.5 L Erlenmayer flasks was inocu-
32 lated to give 10⁹ spores L⁻¹ of the medium and incubated for
33 20–22 h at 30 °C with shaking at 125 rpm (IS-200K,
34 Kambič, Slovenia).^{19, 20}

35 The fungal mycelium was harvested by filtration,
36 rinsed with physiological solution (10 mM sodium phosp-
37 hate buffer, 0.8% (w/v) NaCl, pH 6) and either frozen
38 with liquid nitrogen or resuspended in KMC buffer pH 5.8
39 (1 M KCl, 50 mM CaCl₂, 20 mM MES).

2.3. Disruption of Fungal Cell Wall

42 Different principles were used to disrupt the cell
43 wall of the filamentous fungus.

44 (i) *Grinding mycelium in liquid nitrogen*. The har-
45 vested mycelium was washed with a physiological solu-
46 tion, dried between two sheets of filter paper and frozen
47 under liquid nitrogen. The frozen mycelium was crushed
48 in a mortar with pestle under liquid nitrogen to form a fine
49 powder. Powdered mycelium was collected, weighed and
50 the DNA extracted.

51 (ii) *Rapid freezing and thawing of mycelium*. The
52 harvested mycelium was washed with a physiological so-
53 lution. Then it was consequently rapidly frozen in liquid
54 nitrogen followed by thawing in a water bath at 90 °C up to
55 10 times. During immersion of samples in water bath, they

1 were gently mixed and as soon as the frozen content melted
2 the samples were removed from the water bath and im-
3 mersed in liquid nitrogen. Finally, the DNA was extracted.

4 (iii) *Enzymatic degradation of fungal cell wall*. Fun-
5 gal protoplasts were prepared by resuspending the fresh
6 mycelial pellets (2 g) in 20 mL of KMC buffer with 100
7 mg of lysing enzymes Glucanex (NovoNordisk, Bagsv-
8 ærd, Denmark) by mixing and then incubating for 2 h at
9 30 °C. During the two-hour process occasional mixing of
10 the mycelium was performed to resuspend it in KMC buf-
11 fer. Upon completion of hydrolysis, the obtained proto-
12 plasts were separated from the remaining mycelia by fil-
13 tration through a nylon mesh (square side size between
14 0.1 to 0.2 mm). Protoplasts were collected from the filtra-
15 te by centrifugation at 3500 × g for 10 minutes (Andreas
16 Hettich, Tuttlingen, Germany, rotor 1617) and dissolved
17 in 0.2 mL of KMC buffer.

2.4. Isolation of DNA

21 Three different procedures were tested as follows.

22 (i) *Isolation of genomic DNA with the commercial*
23 *reagent DNAzol*. 0.5 g of the pulverised mycelium obtai-
24 ned after grinding in liquid nitrogen, or 0.2 mL of the pro-
25 toplasts obtained after enzymatic treatment were carefully
26 mixed with 5 mL of DNAzol (Life Technologies, Gait-
27 hersburg, MD, USA). After centrifugation at 10,000 rpm
28 for 10 minutes an upper water phase containing the DNA
29 was collected and DNA was precipitated with isopropanol
30 and dissolved in either TE buffer (10 mM Tris, 1 mM
31 EDTA, pH 7.6) or 8 mM NaOH.

32 (ii) *Isolation of genomic DNA with phenol extrac-*
33 *tion buffer*.⁷ 1 g of the pulverised mycelium or 0.2 mL of
34 the protoplasts was mixed with 2 mL of a phenol extrac-
35 tion buffer. The phenol extraction buffer contained 1.5 vo-
36 lume phenol equilibrated with Tris pH 8.0, 1 volume tri-
37 isopropyl naphthalene sulphonic acid (Acros Organics,
38 Belgium) (0.02 g mL⁻¹), 1 volume p-amino-salicylic acid
39 (0.12 g mL⁻¹) and 0.5 volume 5 × RNB buffer (1 M Tris;
40 1.3 M NaCl, 0.25 M EGTA pH 8.5). The buffer was hea-
41 ted to 55 °C. The mycelium and the phenol-extraction
42 buffer were mixed by gently inverting the tube for 1 min,
43 0.5 mL chloroform was added, and then mixed again for
44 another 1 min. The aqueous phase was separated from the
45 organic phase by centrifugation at 10,000 rpm (Centrifuge
46 5415R, Eppendorf, Germany) at room temperature for 10
47 minutes. The upper phase with DNA was additionally
48 treated with 1 volume of chloroform to remove remnants
49 of phenol. Chloroform was removed by centrifugation
50 (Figure 1). The DNA was precipitated with isopropanol.

51 (iii) *Salting-out method*.¹² 2 g of the pulverized
52 mycelium were resuspended in 5 ml SDS buffer (final
53 concentration 1% (w/v) SDS), the proteinase K (0.25 g
54 L⁻¹ of buffer containing 75 mM NaCl, 25 mM EDTA pH
55 8, 20 mM Tris pH 7.5) was added and the mixture was in-
56 cubated for 2 h at 55 °C. After incubation, NaCl to a final

1 concentration of 1.25 M and 1 volume of chloroform we-
2 re added. The precipitated proteins, polysaccharides and
3 cell wall remnants were removed by centrifugation for 15
4 minutes at 6,000 rpm (Andreas Hettich, Tuttlingen, Ger-
5 many, rotor 1615). The clear upper phase containing the
6 DNA was collected and the DNA was precipitated as des-
7 cribed below.

2. 5. DNA Precipitation

11 The DNA was precipitated from the aqueous solu-
12 tion with 0.7 volume of isopropanol at room temperature
13 and collected either onto a sealed Pasteur pipette or, if the
14 DNA pellet was not visible, with centrifugation at 4° C
15 and at 12,000 g (Centrifuge 5415R, Eppendorf, Germany)
16 for 15 minutes. The DNA pellet was then washed with
17 75% (v/v) ethanol and collected as described above. To re-
18 move RNA, the DNA was treated with DNase-free RNA-
19 ase (10 mg L⁻¹). The air dried DNA was resuspended in
20 TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) containing
21 DNase-free RNase. If necessary, an additional step of
22 phenol/chloroform extraction was performed (Figure 1),
23 followed by DNA precipitation with 0.1 volume sodium
24 acetate buffer (3 M sodium acetate, pH 5.2) and 2 volu-
25 mes of 96% (v/v) ice-cold ethanol.

2. 6. Analysis of DNA by Pulsed-Field Electrophoresis

30 The size and quality of the DNA were determined
31 using pulsed field electrophoresis with 1.2% (w/v) agar-
32 ose gel in TBE buffer (45 mM Tris, 45 mM boric acid, 1 m-
33 M EDTA). The electrophoresis was performed at 120 V to
34 180 V, overnight, with a pulse of 5 s. The DNA was visua-
35 lised by staining the gel in ethidium bromide (0.2 mg L⁻¹)
36 for 30 minutes.

2. 7. Genomic DNA Library Construction

40 A genomic DNA library in the form of bacteriophages
41 was constructed according to the manufacturers' proto-
42 col (Promega, Madison, WI, USA). The restriction
43 enzyme *Sau3AI* was used for partial digestion of high-
44 molecular-weight DNA. The concentration of the restric-
45 tion enzyme *Sau3AI* appropriate for generating 9 to 23 ki-
46 lo base pair (kbp) DNA fragments was determined as des-
47 cribed in the manufacturers' protocol (Promega, Madison,
48 WI, USA). Briefly, a defined amount (1 µg) of the high-
49 molecular-weight DNA was mixed with serial dilutions of
50 *Sau3AI*, from 1 to 0.0033 U per µg DNA and incubated at
51 37 °C for 30 min. The reaction was stopped with the addi-
52 tion of EDTA at pH 8.0 (10 mM final concentration). Pro-
53 ducts of partially digested DNA were precipitated and
54 analysed by pulse-field electrophoresis.

55 For the large scale partial digestion of high-molecu-
56 lar-weight DNA, for 100 µg of DNA, a 0.0125 U *Sau3AI*

1 restriction enzyme per µg DNA was used at 37 °C for 30
2 minutes in a final volume of 5 mL. The concentration of
3 the restriction enzyme (0.0125 U per µg DNA) was half
4 the amount determined in the optimization experiment
5 (0.025 U per µg DNA). The reaction was stopped with 0.1
6 mL 0.5 M EDTA pH 8.0. The DNA from the reaction mix-
7 ture was extracted with phenol/chloroform and precipita-
8 ted. The DNA fragments in the size range between 9 and
9 23 kbp were isolated by sucrose gradient centrifugation.²¹

10 The DNA fragments of the appropriate size were li-
11 gated with dephosphorylated *Bam*HI-predigested EMBL4
12 lambda arms (Promega). The molar ratio of the lambda
13 arms to the genomic insert varied from 1 : 1 to 1 : 3. The
14 ligation mixture was packaged into phages *in vitro* accord-
15 ing to the manufacturers' protocol (Gigapack III Gold
16 Packaging Extract, Stratagene, La Jolla, CA).

17 After the library was packed it was amplified by se-
18 condary transfection of bacteria *E. coli* LE392 and stored.
19 The freshly packaged and titrated library was adsorbed to
20 bacteria *E. coli* LE392. The mixture was plated at a high
21 density and allowed to grow until the plaques were still
22 subconfluent. The phages were eluted from the plate by
23 incubation overnight with a phage buffer (0.01% gelatin,
24 50 mM Tris pH 7.5, 100 mM NaCl, 8 mM MgSO₄) and
25 stored at -70 °C.

2. 8. Separation of DNA Fragments by Sucrose Density Gradient

30 The high-molecular-weight DNA, partially digested
31 with *Sau3AI*, was separated according to size using sucro-
32 se gradient centrifugation.¹⁸ The DNA solution [75 µg of
33 DNA in 500 µL STE buffer (1 M NaCl, 20 mM Tris, 5 m-
34 M EDTA pH 8.0)] was heated to 65 °C for 5 min to disso-
35 ciate the aggregated DNA fragments. It was then loaded
36 onto a high-salt sucrose gradient [10–40% (w/v) sucrose
37 gradient, 1 M NaCl, 20 mM Tris, 5 mM EDTA pH 8.0]
38 and centrifuged at 113,000 × g for 22 hours at 20 °C
39 (Beckman L7-55, rotor SW-28). Fractions (1.5 mL) were
40 collected and the DNA size was analysed using pulsed-
41 field electrophoresis. Fractions containing the DNA frag-
42 ments with a size between 9 and 23 kbp were pooled,
43 dialysed against TE buffer and precipitated with ethanol.

3. Results and Discussion

45 For the construction of a representative genomic
46 DNA library the quality of the starting material, the isolat-
47 ed genomic DNA, is of crucial importance. To obtain the
48 high-molecular-weight genomic DNA with a size above
49 100 kbp, shear forces during the isolation procedure
50 should be minimised. Due to the specificity of fungal
51 mycelial cells, a procedure for DNA isolation should be
52 optimised to achieve the required length of DNA for li-
53 brary synthesis. The most commonly used method⁷ which
54
55
56

includes grinding, phenol and chloroform extraction, and DNA precipitation is tedious and made up of several phases. On the other hand, this procedure was confirmed to give the best result⁷ for the isolation of genomic DNA from *Aspergillus sp.*

3. 1. Cell Wall Disruption

The fungal cell wall represents a rigid, mechanical barrier on the surface of the protoplast and in such a way it determines the shape of the cell. It is composed mainly of structural polysaccharides. Two basic principles to remove the fungal cell wall are: (i) enzymatic degradation²² and (ii) mechanical approach.^{7,8,23} Therefore, in the first stage we tested the efficiency of three different approaches: freezing the young mycelium in liquid nitrogen and then grinding;⁷ sequential rapid freezing and thawing of the mycelium;⁸ generating protoplast with lytic enzymes.²²

The efficient removal of cell wall is a prerequisite for good DNA yield and its length. It was expected that the last two methods mentioned above, namely, rapid freezing and thawing and generating protoplasts by enzymatic hydrolysis of the cell wall, would be less damaging for the DNA and would give better results since they include less friction and shear stress on the DNA molecule. Surprisingly, the longest DNA with 100–150 kbp and the best yield giving 125 µg DNA per g of mycelium was obtained by grinding in liquid nitrogen (Table 1). Therefore, this method was chosen for the first stage of genomic DNA isolation from *D. microsporus*.

A low DNA yield and poor DNA quality obtained with the other two methods could be assigned to long processing times from collecting mycelium and to extraction. The method with sequential rapid freezing and thawing yielded 67 µg DNA per g mycelium when mycelium was treated with 10 sequential rapid freeze/thaw cycles. When less than 10 cycles were performed DNA yield was lower. An increase in the number of cycles did not improve DNA yield and DNA quality. Nucleases were not inhibited during the first stage, which might be an explanation for low DNA yield and insufficient length. With the method used for cell wall disruption with lytic enzymes, we obtained a very low DNA yield. This could be explained with the low efficiency of protoplast formation. Not all of the proto-

Table 1. Amount of DNA and its length isolated with phenol-extraction buffer after three different methods of cell wall removal.

Cell wall disruption method	Amount of DNA [µg per g mycelium]	Length [kbp]
Grinding in liquid nitrogen	125	100–150
Sequential freezing and thawing	67	~ 50
Enzymatic hydrolysis	7	< 50

plasts stay intact which leads to leaking out cellular components into the lysis buffer.

Unfortunately, cell wall disruption by grinding in liquid nitrogen is a labour-demanding method and is not a first choice method if speed and high throughput are required.

3. 2. Extraction of High-Molecular-Weight DNA

In the second phase the removal of contaminants such as soluble proteins and polysaccharides is required. Three different procedures were tested: (i) a method adapted from protocols for filamentous fungal species

CELL WALL DISRUPTION

1. Grinding of washed and frozen mycelium in liquid nitrogen to generate fine powder.

DNA ISOLATION

2. Protein and polysaccharide precipitation with phenol extraction buffer (2 mL per 1 g mycelium); gentle mixing 5 minutes; incubation 10 minutes, 55 °C.
3. Addition of chloroform (0.5 mL); gentle mixing 1 minute; centrifugation (10,000 rpm, room temperature; 10 minutes).
4. Collection of upper water phase containing DNA; addition of chloroform (1 volume); gentle mixing 1 minute; centrifugation (10,000 rpm, 4 °C; 5 minutes).
5. Collection of upper water-DNA phase; DNA precipitation with isopropanol (0.7 volume); gentle mixing; centrifugation (10,000 rpm, 4 °C; 10 minutes).
6. Dissolving the DNA pellet in TE buffer with RNase.

RE-PURIFICATION

7. Addition of phenol : chloroform 1 : 1 (1 volume); gentle mixing 1 minute; centrifugation (10,000 rpm, 4 °C, 10 minutes).
8. Collection of upper water-DNA phase; addition of chloroform (1 volume); gentle mixing 1 minute; centrifugation (10,000 rpm, 4 °C, 5 minutes); repeated twice.
9. DNA precipitation with sodium acetate (0.1 volume) and ethanol (2 volumes); centrifugation (10,000 rpm, 4 °C; 10 minutes).
10. Washing the DNA pellet with 70% ethanol (1 volume); centrifugation (10,000 rpm, 4 °C, 5 minutes).
11. Dissolving the DNA pellet in TE buffer.

Figure 1. Flowchart of high-molecular-weight DNA isolation method for the fungus *D. microsporus*.

Table 2. Amount of DNA and its length isolated when using different extraction methods after cell wall removal by grinding in liquid nitrogen.

Extraction agent	Additional purification with phenol: chloroform (1:1)	DNA concentration [µg per g mycelium]	Length [kbp]
DNazol	–	30	50–100
	+	2	< 50
DNA extraction buffer	–	125	100–150
	+	50	100–150

Aspergillus sp., using a phenol-extraction buffer⁷ (ii) a rapid method for DNA isolation with a commercial product DNazol and (iii) a salting-out method that included proteinase K to remove proteins giving very good results with filamentous bacteria *Streptomyces* sp.^{6,12,13,23} Additionally, we have tested extraction by phenol alone,¹⁸ however, only a small amount of DNA with molecular length less than 10 kbp was obtained. According to the results (Table 2), DNA extraction with the phenol-extraction buffer yielded the best DNA quality with a suitable length of 100–150 kbp to proceed in the genomic DNA library synthesis. Isolation of DNA with reagent DNazol is rapid and simple. However, precipitated DNA poorly dissolved in TE buffer. On the other hand, when dissolved in sodium hydroxide as recommended by the manufacturer, the DNA was damaged as shown. In addition, the length of DNA was around 50 kbp, which is not good enough for synthesis of a bacteriophage DNA library. The yield of DNA obtained with the salting-out method was 20 µg of DNA per g of mycelium, with a length around 50 kbp.

Constituents of a phenol-extraction buffer are more lipophilic than sodium dodecyl sulphate. The phenol-extraction buffer contains EGTA and p-amino-salicylic acid to chelate metal ions and indirectly inhibit DNases,¹¹ and a detergent to improve precipitation of polysaccharides and proteins. The components of phenol-extraction buffer are fully dissolved at pH above 8.0 and a temperature higher than 50 °C which ensures correct buffer pH and temperature.

Based on the overall results a final protocol was designed, which is depicted in Figure 1.

Finally, the isolated DNA was detected by pulse-field electrophoresis as shown in Figure 2A.

3. 3. Construction of Genomic DNA Library

The quality of the obtained genomic DNA and restriction enzymes used for synthesis of a DNA library are crucial for a successful result. The DNA must be of high molecular weight. A useful insert fragment must have both ends generated by restriction enzymes to be able to ligate into the chosen vector. In order to generate a majority of the random fragments with two good ends, the starting DNA material must be on average at least three times

longer (> 70 kbp) than the insert of about 9–23 kbp to be used in the cloning procedure.

The isolated high-molecular-weight genomic DNA of *D. microsporus* was partially digested with *Sau3AI* restriction enzyme (Fig. 2B). With the use of the optimal dilution (1 : 1200) of the restriction enzyme the fractions with DNA fragment size between 9 and 23 kbp (Fig. 3A) were isolated by sucrose gradient centrifugation (Fig. 3B). A size separation of DNA fragments in sucrose gradient is time consuming but has high size resolution between 5 to 60 kbp DNA. Isolation of suitable fragments of the fungal genomic DNA is shown in Fig. 3C.

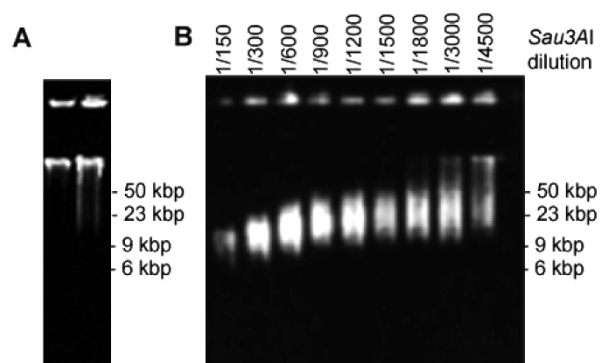


Figure 2. [A] Pulse-field electrophoresis of the isolated high-molecular-weight genomic DNA which was used as starting material for optimization of partial digestion. Procedure depicted in Figure 1 was used for DNA isolation. [B] Partial digestion of high-molecular-weight DNA of *D. microsporus*. Pulse-field electrophoresis of partially digested starting material after using different dilutions of the restriction enzyme *Sau3AI* (from 1/150 to 1/4500).

Ligation of the purified fragments with a size in the range between 9 and 23 kbp into *Bam*HI-predigested lambda arms (9 kbp and 20 kbp) is shown in Fig. 4. The appearance of a ligation product with a size of 50 kbp fragment, which is required for bacteriophage packaging, is clearly visible.

The obtained ligation mixture was *in vitro* packaged into bacteriophage particles and transfected into bacteria *Escherichia coli* LE392. After lysis of bacterial cells, bacteriophages were collected. A bacteriophage titer of the primary genomic DNA library was 10⁵ plaque-forming units (PFU) that presented a 1.6 Gbp DNA.

4. Conclusions

For isolation of high-molecular-weight DNA from the filamentous fungus *Doratomyces microsporus*, the method using phenol extraction buffer gave the largest quantity and the best quality of DNA regarding its length.

For the cell wall removal, grinding of mycelium in liquid nitrogen was preferable to disruption of the cell wall with lytic enzymes and to the method using sequential rapid freezing and thawing.

In the extraction step, the phenol-extraction buffer proved to be the reagent of choice over DNAzol and the salting-out method.

To obtain pure DNA, an extra precipitation of DNA had to be performed.

Using the complete protocol, including cell wall disruption, DNA isolation and re-purification, sufficiently long DNA fragments from 100 to 150 kbp could be obtained, which were suitable for genome library construction.

Using the optimised protocol a genomic DNA library of the filamentous fungus *D. microsporus* could be prepared with approx. 100 times genome coverage.

5. Acknowledgements

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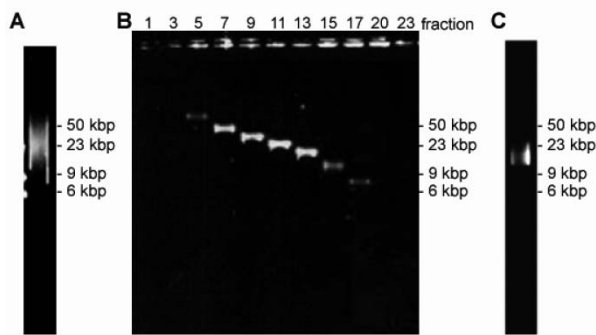


Figure 3. [A] Analysis of preparative partially digested DNA (an aliquot) resolved by pulse-field electrophoresis. Preparative partial digestion of the high-molecular-weight DNA was performed with the restriction enzyme *Sau3AI* (dilution 1/1200 which corresponds to 0,0125 U *Sau3AI* per mg of DNA). A band of partially digested DNA with sizes from 9 to 50 kbp was determined with the electrophoresis. [B] Fractions of the partially digested DNA after centrifugation in sucrose gradient. [C] Collected fractions of partially digested DNA suitable for construction of bacteriophage genomic DNA library. Fractions with sizes between 9 and 23 kbp (fractions from 11 to 15 see Fig. 3B) were collected and an aliquot was analyzed by pulse-field electrophoresis.

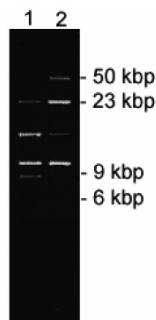


Figure 4. Ligation of partially digested genomic DNA into bacteriophage arms LambdaGEM-11 *Bam*HI arms. Legend: control without ligase [1] and ligation mixture [2].

To amplify the primary genomic DNA library from *D. microsporus* an additional step of *E. coli* transfection was introduced and a secondary bacteriophage stock was collected. A bacteriophage titer of secondary genomic DNA library from *D. microsporus* was 3×10^8 PFU, which accounted for 4500 Gbp DNA. Since the size of the genome for *D. microsporus* is not known an exact coverage of genome with the genomic library could not be determined. If we assume that the size of the *D. microsporus* genome is in the range of genome sizes of *Aspergillus nidulans* (30 Mbp), *Magnaporthe grisea* (39 Mbp) or *Neurospora crassa* (39 Mbp) the coverage is approximately 100 times. Approximately 10,000 independent clones would need to be screened to isolate a particular sequence with a probability of 99%²¹ from this bacteriophage DNA library.

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Povzetek

Razvili in optimirali smo protokol za izolacijo DNA filamentozne glive in pripravili gensko knjižnico. Poznane so številne metode priprave genomske DNA: metode, ki omogočajo obdelavo velikega števila vzorcev in metode, ki omogočajo izolacijo visoko kvalitetne DNA, kar je še posebej pomembno za sintezo genomske DNA knjižnice. Izolacija DNA visoke molekulske mase iz glive predstavlja precejšen izziv, ker se sestava celične stene in celična vsebina razlikujejo med posameznimi vrstami gliv. Opisan je optimiziran postopek izolacije dolge visoko kvalitetne DNA keratinolitične filamentozne glive *Doratomyces microsporus*, sev MZKI B399, ki smo ga primerjali z drugimi metodami. Kot najprimernejša metoda za izolacijo DNA se je izkazalo trenje micelija v tekočem dušiku v kombinaciji z odstranitvijo proteinov in polisaharidov iz raztopine DNA z ekstrakcijskim pufrom in obarjanjem DNA z izopropanolom. Tako pripravljeno DNA smo uporabili za pripravo genomske DNA knjižnice glive.