Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa*

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Genet. Mol. Res. 5 (4): 851-855 (2006)
Received September 26, 2006
Accepted November 6, 2006
Published December 14, 2006

ABSTRACT. DNA isolation from some fungal organisms is difficult because they have cell walls or capsules that are relatively unsusceptible to lysis. Beginning with a yeast *Saccharomyces cerevisiae* genomic DNA isolation method, we developed a 30-min DNA isolation protocol for filamentous fungi by combining cell wall digestion with cell disruption by glass beads. High-quality DNA was isolated with good yield from the hyphae of *Crinipellis perniciosa*, which causes witches' broom disease in cacao, from three other filamentous fungi, *Lentinus edodes*, *Agaricus blazei*, *Trichoderma stromaticum*, and from the yeast *S. cerevisiae*. Genomic DNA was suitable for PCR of specific actin primers of *C. perniciosa*, allowing it to be differentiated from fungal contaminants, including its natural competitor, *T. stromaticum*.

Key words: Genomic DNA extraction, *Crinipellis perniciosa*, PCR, Filamentous fungi

INTRODUCTION

The basidiomycete fungus *Crinipellis perniciosa* (Stahel) Singer is the cause of witches' broom disease of cacao (*Theobroma cacao* L.), which has drastically decreased cacao production in most of the western hemisphere (Griffith, 2004). *C. perniciosa* is endemic to the Amazon basin region of South America; this phytopathogenic fungus has spread to cacao plantations throughout the Americas and the Caribbean islands (Pereira et al., 1996; Purdy and Schmidt, 1996). The genome of *C. perniciosa* has been partially sequenced, allowing molecular analysis of genes of interest. Although great effort has been made during the last 10 years to understand the biological and molecular basis of witches' broom infection (Andebrhan et al., 1999; Scarpari et al., 2005; Rincones et al., 2006; Meinhardt et al., 2006), we lack standardized and specific protocols for the routine molecular biology research of this organism, which are commonly available in yeast research. Current methods of DNA extraction from *C. perniciosa* and other fungal pathogens are either time-consuming and require toxic chemicals or are based on expensive technologies (Muller et al., 1998; Faggi et al., 2005; Borman et al., 2006; Cheng and Jiang, 2006). They include use of SDS/CTAB/proteinase K (Wilson, 1990), SDS lysis (Syn and Swarup, 2000), lysozyme /SDS (Flamm et al., 1984), high-speed cell disruption (Muller et al., 1998), and bead-vortexing/SDS lysis (Sambrook and Russel, 2001). Additionally, some give poor yields of DNA, as cell walls or capsules are difficult to lyse (Muller et al., 1998).

The major challenge for isolation of DNA of good quality and quantity from fungi lies in breaking the rigid cell walls, as they are often resistant to traditional DNA extraction procedures (Fredricks et al., 2005). Fungal nucleases and high polysaccharide contents add to the difficulties in isolating DNA from filamentous fungi (Zhang et al., 1996; Muller et al., 1998). All methods have in common the use of detergents such as SDS for cell wall lysis, and this often inhibits further purification manipulations. As an alternative to lysis by SDS, toxic chemicals, e.g., phenol, have been used (Cheng and Jiang, 2006). According to Fredricks et al. (2005) no single extraction method amongst those currently available is optimal for all analyzed fungi.

We developed an alternative and rapid DNA isolation method adapted from a yeast protocol (Sambrook and Russel, 2001) that was successfully applied to *C. perniciosa*, three other filamentous fungi, and baker yeast. We also examined whether specific primers that were designed for the actin gene of *C. perniciosa* can be used to differentiate this fungus from other filamentous fungi, baker yeast, and from its natural competitor, *Trichoderma stromaticum.*

MATERIAL AND METHODS

Crinipellis perniciosa growth conditions and media were as described by Filho et al. (2006). *Lentinus edodes* and *Agaricus blazei* were grown at 28°C in medium containing 0.1% KH_2PO_4, 0.05% $MgSO_4$ $7H_2O$, 0.5% peptone, 1% glucose, 0.01% chloramphenicol, and 1.5% agar, pH 5.6. *T. stromaticum* was grown at 25°C in 3.9% potato-dextrose-agar media (Difco).

Using a sterile toothpick, hyphae of *C. perniciosa* and of the other fungi (0.1-1.0 mg) were scraped from a 7-15-day-old agar plate, transferred to a microcentrifuge tube and suspended in 200 µL buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA). When DNA was extracted from *C. perniciosa* that had been grown in liquid culture, with shaking (Filho et al., 2006), the balls that had formed were washed three times with cold-sterile distilled water and the DNA extraction buffer had 10-fold EDTA, 200 µL phenol-

chloroform-alcohol isoamylic (25:24:1) mixture and 0.3 g sterile glass beads (Sigma, G1277). The suspension was vortexed at top setting for 5 min. To each tube, 200 µL Tris-EDTA, pH 8.0, was added mixed, and the suspension was centrifuged for 5 min at 13,500 rpm. The supernatant was transferred to a new microcentrifuge tube, and the nucleic acids were precipitated by adding 1 mL absolute ethanol. Suspensions were mixed and centrifuged for 2 min (13,500 rpm). The pellet was resuspended in 400 µL Tris-EDTA, pH 8, 3 µL RNAse (10 mg/mL) and incubated for 5 min at 37°C. Then, 10 µL ammonium acetate (4 M) and 1 mL absolute ethanol were added and gently mixed. This mixture was centrifuged for 3 min at 13,500 rpm and the supernatant discarded. The DNA pellet was dried in airflow for 15 min and finally resuspended in 40 µL distilled sterile water. The genomic DNA was verified by 1% agarose gel electrophoresis.

PCR of actin

The extracted DNA was used for PCR, which was performed in 25-µL reaction volumes containing: 20 ng genomic DNA, 100 µM dNTPs, 1 mM $MgCl_2$, 2.5 µL 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.2 µM of each *primer* pair and 1 U *Taq* DNA polymerase (Invitrogen®); distilled water was added to complete the final volume of the reaction. Cycling conditions were: initial denaturation step at 95°C for 3 min, followed by 30 cycles, each consisting of 95°C for 50 s, annealing temperature 58°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

C. perniciosa-specific actin *primers* (forward: CCACAATggAggACgAAgTCg; reverse: CCCgACATAggAgTCCTTCTg) were added to the DNA extracts of the five different fungi and single reactions were performed in an Eppendorf MasterCycler® Thermocycler. The quality of the PCR reactions was monitored in 1% Tris-acetate-EDTA-agarose gel, and bands were visualized by staining with ethidium bromide. Images were made and stored with the Kodak-EDAS® system.

RESULTS AND DISCUSSION

Since the currently available DNA extraction protocols are rather costly and time-consuming (Wilson, 1990; Syn and Swarup, 2000; Sambrook and Russel, 2001), we adapted a rapid DNA isolation method from yeast (Burke et al., 2000), combining chemical reagent digestion with mechanical (glass beads) shearing for lysing the hyphae of *C. perniciosa* and three other hyphal fungi, followed by DNA isolation. The whole procedure required approximately 30-40 min and was not specific for *C. perniciosa*, as it also allowed rapid isolation of genomic DNA from *A. blazei*, *T. stromaticum* and *L. edodes*. In all cases, we obtained good yields of high-quality genomic DNA (Figure 1). As expected, the protocol also worked with the yeast *Saccharomyces cerevisiae* (Figure 1, lane 11).

This DNA extraction method has several advantages: a) the number of DNA extraction steps is minimal, b) it is low-cost, as only small amounts of chemicals and little equipment are employed, and c) it is efficient because as little as 0.05 g of *C. perniciosa* mycelium gives good DNA yields (Figure 1, lane 1). Using the same quantities of the reagents, up to 1.0 g *C. perniciosa* hyphae can be processed for extraction of genomic DNA (Figure 1, lanes 1 to 6); however, the quantity of extracted genomic DNA is not proportional to the input of hyphal mass. DNA extraction from *C. perniciosa* grown in liquid media necessitated slightly altered proce-

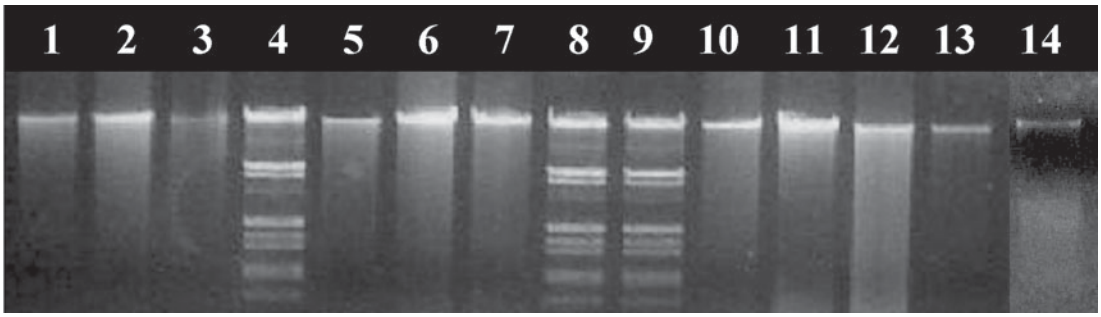

Figure 1. Agarose gel electrophoresis of extracted genomic DNA (1.0%): *Lane 1*, 0.05 g *Crinipellis perniciosa*; *lane 2*, 0.1 g *C. perniciosa*; *lane 3*, 0.2 g *C. perniciosa*; *lane 4*, λ/*Eco*RI/*Hin*dIII; *lane 5*, 0.1 g *C. perniciosa*; *lane 6*, 0.5 g *C. perniciosa*; *lane 7*, 1.0 g *C. perniciosa*; *lanes 8* and *9*, λ/*Eco*RI/*Hin*dIII; lane 10, 0.2 g liquid grown *C. perniciosa*; *lane 11*, 1 x 10^8 *Saccharomyces cerevisiae* cells; *lane 12*, 0.1 g *Agaricus blazei*; lane 13, 0.1 g *Lentinus edodes*, and *lane 14*, 0.1 g *Trichoderma stromaticum*.

dures (see Material and Methods), but yielded about the same amounts of DNA (Figure 1, lane 10). Nevertheless, the quantity as well as the quality of the extracted genomic DNA was high enough to perform hundreds of PCR-based reactions (Figure 2) and also to be used for other DNA manipulation techniques (Northern blot analysis, DNA library construction, etc.; data not shown). Further simplification of the protocol, i.e., omission of the phenol-chloroform step, reduced the yield of genomic DNA to zero (data not shown).

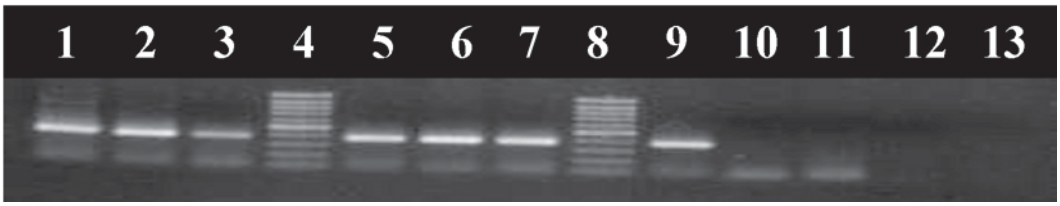

Figure 2. PCR products using *Crinipellis perniciosa*-specific actin primer. *Lane 1*, 0.05 g *C. perniciosa*; *lane 2*, 0.1 g *C. perniciosa*; *lane 3*, 0.2 g *C. perniciosa*; *lane 4*, λ 100 bp; *lane 5*, 0.1 g *C. perniciosa*; *lane 6*, 0.5 g *C. perniciosa*; *lane 7*, 1.0 g *C. perniciosa*; *lane 8*, λ 100 bp; *lane 9*, 0.1 g *C. perniciosa*; *lane 10*, 1 x 10^8 *Saccharomyces cerevisiae* cells; *lane 11*, 0.1 g *Agaricus blazei*; *lane 12*, 0.1 g *Lentinus edodes*, and *lane 13*, 0.1 g *Trichoderma stromaticum*.

One of the main problems with *in vitro* cultivation of *C. perniciosa*, especially when starting growth from basidiospores, is contamination with other fungi (some of which are very similar morphologically) or bacteria. We found that *C. perniciosa* can easily be differentiated from other possible fungal contaminants by specific PCR amplification of the conserved region of the fungal actin gene (Figure 2, lanes 1 to 7). Amplification with actin primers occurred only with DNA of *C. perniciosa* and not with the DNA from *A. blazei*, *L. edodes*, *T. stromaticum*, or *S. cerevisiae*, extracted with the same protocol (Figure 2, lanes 9 to 13), thus confirming species-specificity of the actin gene.

In summary, we developed a fast and reliable genomic DNA extraction protocol for four filamentous fungi, which facilitates work with *C. perniciosa*. The *C. perniciosa*-specific actin primers permit reliable discrimination between *C. perniciosa* and other filamentous fungi.

ACKNOWLEDGMENTS

We thank Dr. Eduardo Gross and Dr. Marcio G.C. Costa for kindly providing some fungal strains. Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Mars Symbiosciences (MARS/USA). S.C.S. Melo held a CAPES fellowship and is a doctoral student in the Genetics Post-Graduation Program of UESC. C. Pungartnik held a fellowship by PRODOC/FAPESB/CNPq and M. Brendel is a visiting scientist supported by Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB).

REFERENCES

Andebrhan T, Figueira A, Yamada MM, Cascardo J, et al. (1999). Molecular fingerprinting suggests two primary outbreaks of witches' broom disease *Crinipellis perniciosa* of *Theobroma cacao* in Bahia, Brazil. *Eur. J. Plant Pathol.* 105: 167-175.

Borman AM, Linton CJ, Miles SJ, Campbell CK, et al. (2006). Ultra-rapid preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter paper technology - a reusable DNA archiving system. *Med. Mycol.* 44: 389-398.

Burke D, Dean D and Tim S (2000). Methods in yeast genetics 2000: A Cold Spring Harbor laboratory course manual. Cold Spring Harbor Press, New York.

Cheng HR and Jiang N (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol. Lett.* 28: 55-59.

Faggi E, Pini G and Campisi E (2005). Use of magnetic beads to extract fungal DNA. *Mycoses* 48: 3-7.

Filho DF, Pungartnik C, Cascardo JC and Brendel M (2006). Broken hyphae of the basidiomycete *Crinipellis perniciosa* allow quantitative assay of toxicity. *Curr. Microbiol.* 52: 407-412.

Flamm RK, Hinrichs DJ and Thomashow MF (1984). Introduction of pAM beta 1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. *Infect. Immun.* 44: 157-161.

Fredricks DN, Smith C and Meier A (2005). Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. *J. Clin. Microbiol.* 43: 5122-5128.

Griffith GW (2004). Witches' broom and frosty pods. *Biologist* 51: 71-75.

Meinhardt LW, Bellato CM, Rincones J, Azevedo RA, et al. (2006). *In vitro* production of biotrophic-like cultures of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. *Curr. Microbiol.* 52: 191-196.

Muller FM, Werner KE, Kasai M, Francesconi A, et al. (1998). Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *J. Clin. Microbiol.* 36: 1625-1629.

Pereira JL, de Almeida LC and Santos SM (1996). Witches' broom disease of cocoa in Bahia: attempts at eradication and containment. *Crop Prot.* 15: 743-752.

Purdy LH and Schmidt RA (1996). Status of cacao witches' broom: biology, epidemiology and management. *Annu. Rev. Phytopathol.* 34: 573-594.

Rincones J, Mazotti GD, Griffith GW, Pomela A, et al. (2006). Genetic variability and chromosome-length polymorphisms of the witches' broom pathogen *Crinipellis perniciosa* from various plant hosts in South America. *Mycol. Res.* 110: 821-832.

Sambrook J and Russel DW (2001). Rapid isolation of yeast DNA. In: Molecular cloning, a laboratory manual (Sambrook J and Russel DW, eds.). Cold Spring Harbor Laboratory, New York, 631-632.

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AW, et al. (2005). Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. *J. Exp. Bot.* 56: 865-877.

Syn CK and Swarup S (2000). A scalable protocol for the isolation of large-sized genomic DNA within an hour from several bacteria. *Anal. Biochem.* 278: 86-90.

Wilson K (1990). Preparation of genomic DNA from bacteria. In: Current protocols in molecular biology (Ausubel FM and Brent R, eds.). Greene Publ. Assoc. and Wiley Interscience, New York, 241-245.

Zhang D, Yang Y, Castlebury LA and Cerniglia CE (1996). A method for the large scale isolation of high transformation efficiency fungal genomic DNA. *FEMS Microbiol. Lett.* 145: 261-265.