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An efficient method for DNA extraction from Cladosporioid fungi

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ABSTRACT. We developed an efficient method for DNA extraction from Cladosporioid fungi, which are important fungal plant pathogens. The cell wall of Cladosporioid fungi is often melanized, which makes it difficult to extract DNA from their cells. In order to overcome this we grew these fungi for three days on agar plates and extracted DNA from mycelium mats after manual or electric homogenization. High-quality DNA was isolated, with an A_{260}/A_{280} ratio ranging between 1.6 and 2.0. Isolated genomic DNA was efficiently digested with restriction enzymes and produced distinct banding patterns on agarose gels for the different *Cladosporium* species. Clear DNA fragments from the isolated DNA were amplified by PCR using small and large subunit rDNA primers, demonstrating that this method provides DNA of sufficiently high quality for molecular analyses.

Key words: DNA extraction; *Cladosporium* spp; PCR; rDNA-ITS1

INTRODUCTION

Cladosporioid hyphomycetes are common, widespread dematiaceous fungi that can be isolated from the environment almost anywhere in the world (Yano et al., 2003; Schubert, 2005). The most common species of the genus *Cladosporium* include *Cladosporium herbarum*, *C. sphaerospermum*, *C. cladosporioides*, and *C. elatum* (Qiu-Xia et al., 2008). The genus contains many saprophytic and some pathogenic species and several species are used in industry as efficient biodegraders of aromatic compounds. In spite of the importance of *Cladosporium* in medicine, agriculture and industry, very few molecular and phylogenic data are available from this cosmopolitan group of fungi (Ghahfarokhi et al., 2004). Good-quality DNA is required to perform conventional polymerase chain reaction (PCR) for phylogenic analyses with specific primers (Pitkäranta et al., 2008). The cell walls of *Cladosporium* species are often melanized, which makes difficult to disrupt them for extraction of DNA. Cell disruption for DNA extraction from this type of fungi can be achieved after freeze-thawing or treatment with liquid nitrogen or dry ice (Loeffler et al., 2001; Griffin et al., 2002). Incubation of mycelium with cocktails of cell-wall degrading enzymes prior to DNA extraction is also frequently used (Williamson et al., 2000). Melanized cell walls contain complex polysaccharides and various secondary metabolites, including complex phenolic compounds, which hamper successful isolation of DNA (Rogers, 1994). These cell wall properties require rigorous techniques to isolate DNA from these fungi (Yeo and Wong, 2002) compared to mammalian cells (Wong et al., 2007). Due to the complexity of these fungal cell walls, conventional methods employed for extracting DNA are often not suitable (Karakousis et al., 2006). Liquid nitrogen is often not available at remote locations and a method for DNA extraction that does not require liquid nitrogen would be useful (Sharma et al., 2003). The need for a rapid and efficient method is urgent, especially when hundreds of samples need to be analyzed. This is especially true for the analysis of *Cladosporium* species that occur worldwide and are of medical and economic importance. For this purpose, we describe here a rapid and efficient method for extraction of DNA that can be used for molecular analyses including PCR.

MATERIAL AND METHODS

Cladosporium isolates

Nine different species of *Cladosporium* were used in this study. Five species were obtained from the Saudi MycoBank (SMB) of King Saud University (Riyadh) including *Cladosporium herbarum* (Persoon) Link, *C. macrocarpum* Preuss., *C. chlamydosporis* Matsushima, *C. fulvum* Cooke, and *Cladosporium* sp and four were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) including *C. cladosporioides* (CBS 131.29), *C. herbarum* (CBS 673.69), *C. sphaerospermum* (CBS 114326), and *C. tennuissimum* (CBS 117134).

Fungal growth on duplex media

We used Petri dishes instead of liquid shake cultures to grow the different species for DNA isolation. Disposable polystyrene Petri dishes (4 cm) were filled with 1800 µL solid medium (potato dextrose agar), on which a layer of liquid medium (1400 µL peptone yeast

glucose) was added. The fungal species isolates were cultured by inoculating a small mycelial disk from stock onto the prepared Petri dishes that were subsequently incubated for 2-3 days at 28°C. Mycelium was lifted from the medium using sterilized inoculating loops and transferred into sterile 1.5-mL microfuge tubes. For some fungal species, the mycelium mats were pelleted by centrifugation for 15 min at 4000 *g* in a deepwell swing-bucket rotor (microcenrifuge 5804 R; Eppendorf).

The mycelium pellet was washed with 600 µL TE buffer and centrifuged again for 5 min at 4000 *g*. Finally, the TE buffer was decanted.

Fungal mat homogenization and DNA isolation

A hand-operated or electric grinder (Retsch, Germany) was used to homogenize fresh mycelial mats in a 1.5-mL microfuge tube. To prevent cross-contamination, the pestle tip was cleaned between isolations (the pestle tip was immersed in 70% EtOH and the grinder was turned on for 3 s and subsequently the pestle tip was rinsed in sterile distilled water and dried with sterilized filter paper. The step-by-step DNA isolation procedure is shown in Table 1.

Table 1. DNA extraction protocol.

Before you begin
1. Provide >99% isopropanol (2-propanol) and 80% ethanol (both not included in the kit).
2. Pre-heat incubator to 65°C.
3. Set an ice bucket with ice on your benchtop work area.

Cell lysis and RNAse treatment
1. Weigh out 100 mg homogenized fresh mycelium into a 1.5-mL tube.
2. Add 600 µL DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.6% SDS).
3. Mix the fresh mats with extraction buffer as much as possible.
4. Add 6 µL RNAse A (15 mg/mL final concentration); vortex briefly, incubate at 65°C for 10 min.

Protein precipitation
1. Add 140 µL Protein Precipitation Reagent (3 M sodium acetate, pH 5.3), mix thoroughly by inverting the tube.
2. Incubate at -20°C for 5 min. Centrifuge the tube at 13,000 *g* for 5 min.

DNA precipitation
1. Carefully transfer 600 µL of the supernatant directly into a new microtube, and add 600 µL 100% isopropanol (2-propanol). Mix the sample by inverting gently 30 times or by vortexing.
2. The microtube is stored at room temperature for 5 min.
3. Centrifuge at 12,000 *g* for 2 min at 4°C; the DNA will be visible as a pellet that ranges in color from off-white to light green.
4. Pour off the supernatant, while being careful to leave the pellet behind.

DNA wash
1. Wash the DNA precipitate twice with 650 µL 75% ethanol. At each wash, suspend the DNA in ethanol by inverting the tubes 3-6 times. Store the tubes vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting or decanting.

Elution of highly pure DNA
1. Add 90 µL DNA elution buffer (prewarmed at 65°C). Store DNA at 4°C until usage. For long-term storage, store at -20° or -80°C.

DNA quantification

DNA was quantified either by a) measuring the absorbance at 260 nm (A_{260}) using the NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies) or by b) analysis on 1% agarose gels and comparison with a known concentration of 100-bp DNA marker (Jena Bioscience, Germany).

DNA digestion

Genomic DNA (5 µg) was incubated with 10 units each of *Eco*RI, *Eco*RV, *Cfo*I, and *Xba*I (Boehringer Mannheim, Mannheim, Germany) in the recommended buffer at 37°C for 3 h. DNA digestion was assayed by visual inspection after agarose gel electrophoresis.

PCR assays

Ribosomal DNA amplification

The fungal primers of the internal transcribed spacers 3 and 4 (ITS3 and ITS4; White et al., 1990) amplify a region of ribosomal DNA (rDNA) that is common to all fungi. Each 25-µL reaction mixture contained buffer [(20 mM Tris-HCl, pH 8.4, 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates, 25 pmol of both the primers, 0.5 U Taq DNA polymerase (JenaBioscience) and 5 µL DNA sample. Cycling conditions were 95°C for 10 min, then 30 cycles at 94°C for 30 s, 50°C for 2 min and 72°C for 2 min.

Universally primed PCR

Amplification reactions were performed in 0.2-mL microcentrifuge tubes in a 25-µL reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.8 mM NaCl, 3.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 mM dNTPs, 20 pmol for primer L21 or AS4, 1.0 U Taq DNA polymerase (JenaBioscience) and 10 to 15 ng genomic DNA. PCR amplification was performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, CA, USA) programmed for 30 cycles of denaturation at 94°C for 30 s (first denaturation step at 94°C for 3 min), annealing at 56°C for 70 s and polymerization at 72°C for 60 s, with a final extension step of 72°C for 5 min. The reaction tubes were held at 4°C following the final amplification cycle. Two microliters universally primed PCR (UP-PCR) products (1/10 of the total reaction volume) was electrophoresed on 2% agarose gel at 150 V with TAE buffer for 50 min.

Microsatellite-primed PCR

Microsatellite-primed PCR (MSP-PCR) profiles were generated following the protocol of Abd-Elsalam et al. (2007), using T3B primer (20 pmol per 25-µL reaction volume) and extending the elongation step (72°C) to 2 min. Ten microliters reaction mixture was applied on 1.5% (w/v) agarose gels in 0.5X TBE (Tris-borate EDTA) buffer and the amplicons separated by electrophoresis at 80 V for 3.5 h. The gels were subsequently stained with ethidium bromide (EtBr) and photographed under ultraviolet light.

RESULTS AND DISCUSSION

In our laboratories, we perform phylogenic studies on a diverse variety of fungi. Efficient extraction of DNA is required for these studies, but routine DNA extractions often do not work for all fungi. Grinding of frozen or lyophilized mycelium or bead beating work usually for most fungi and require universal DNA extraction methods (Jin et al., 2004). However, for high throughput analysis these methods are labor-intensive and are not suited for analysis of multiple isolates. For DNA extraction typically fungi are grown in liquid shake cultures in Erlenmeyer flasks, Roux bottles or even microtubes (Cenis, 1992). We report here a simple method for culturing Cladosporioid fungi for isolation of DNA that can be completed within three days from start to finish. The growth of mycelia on Petri dishes eliminates the need for still or shaking liquid cultures. Fungal mycelium

mats (100 mg) from 3-day-old colonies grown on duplex agar medium was homogenized by an electric grinder prior to DNA extraction. The resulting medium is not a true solid medium, but rather looks like a dense gel.

The mechanical grinding procedure for isolation of DNA from cells in DNA extracting buffer is very simple and cost effective and it does not need the use of liquid nitrogen, which is often problematic and hazardous, especially when large numbers of samples need to be examined. Motorized pulverization of mycelium generates cell lysates, and often works faster than using cell-wall degrading enzymes or high temperatures (Lugert et al., 2006).

We evaluated the quality of the extracted DNA in a six-step process. The pellets were all colorless and could be dissolved in TE or water. Figure 1 shows the results of the separation of the extracted DNA on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. It shows that our method yields high-quality DNA, which is transparent, non-viscous and lacks visible contamination of RNA. No smearing of DNA occurred on the gel, and the 100-bp DNA marker fragment was clearly visible, indicating that DNA degradation had not occurred.

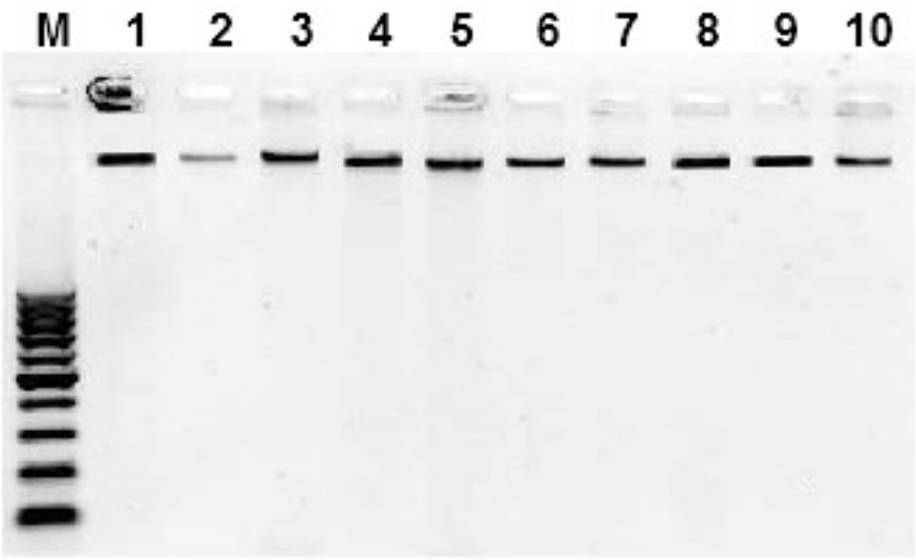

Figure 1. Agarose gel (1.5%) electrophoresis of undigested genomic DNA isolated from 10 different *Cladosporium* isolates. For each isolate 10 ng/µL genomic DNA was loaded (*lanes 1-10*); M = 100-bp DNA molecular weight ladder. *Lane 1 = C. cladosporioides*; *lane 2 = C. tennuissimum*; *lane 3 = C. sphaerospermum*; *lane 4 = C. herbarum*; *lane 5 = C. herbarum*; *lane 6 = C. macrocarpum*; *lane 7 = C. chlamydosporis*; *lane 8 = C. fulvum*; *lane 9 = Cladosporium* sp; *lane 10 = Cladosporium* sp.

DNA yields from fungal mats using the modified SDS method ranged from 120 to 160 µg/g fresh weight mycelium with an A_{260}/A_{280} ratio close to 2.00 and an A_{230}/A_{260} ratio around 1.6, indicating very little contamination of the DNA fraction by proteins, polysaccharides or aromatic compounds. Results obtained with the NanoDrop spectrophotometer for the DNA purified by the current method are shown in Table 2.

Table 2. DNA yield and quality from *Cladosporium* species as determined by NanoDrop spectrophotometer measurements.

Strain number	*Cladosporium* species	ng/2 µL	A_{260}/A_{280}	A_{230}/A_{260}
1	*C. cladosporioides*	21.63	1.93	1.81
2	*C. tennuissimum*	22.93	1.93	1.79
3	*C. sphaerospermum*	20.26	1.68	1.50
4	*C. herbarum*	20.65	1.65	1.47
5	*C. herbarum*	22.44	1.65	1.45
6	*C. macrocarpum*	22.19	1.66	1.36
7	*C. chlamydosporis*	22.86	1.60	1.57
8	*C. fulvum*	23.25	1.64	1.35
9	*Cladosporium* sp	22.52	1.89	1.65
10	*Cladosporium* sp	23.17	1.87	1.60

DNA was digested completely with 4 different restriction enzymes (*Eco*RI, *Eco*RV, *Cfo*I, and *Xba*I), further confirming the quality of the extracted DNA. Figure 2 gives an example of DNA digested with restriction endonuclease *Cfo*I. From these results we conclude that the purity and quality of the isolated genomic DNA was sufficient for efficient digestion by restriction enzymes.

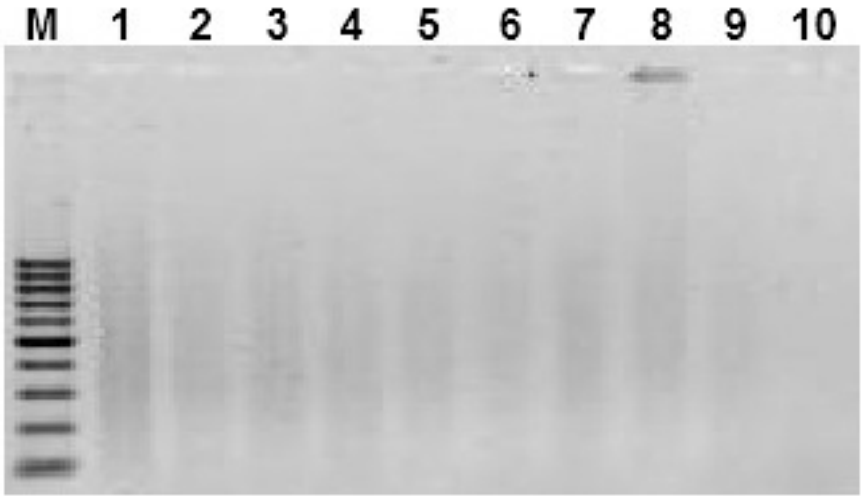

Figure 2. Agarose gel (1.5%) electrophoresis of genomic DNA digested with the restriction enzyme *Cfo*I. M = 100-bp DNA molecular weight ladder. For identification of lanes, see legend to Figure 1.

The suitability of extracted DNA for additional molecular analyses is shown by performing PCR. All primers tested were shown in Table 3.

Table 3. Primers used to amplify fungal DNA.

Primer code	Sequence	Amplified region	References
ITS3	5'-GCA TCG ATG AAG AAC GCA GC G-3'	ITS1 nDNA	White et al., 1990
ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'		
AS4	5'-TGT GGG CGC TCG ACA C-3'	Universally primed-PCR-derived sequence	Bulat et al., 1998
L21	5'-GGA TCC GAG GGT GGC GGT TCT-3'		
T3B	5'-AGG TCG CGG GTT CGA ATC C-3'	Microsatellite repeats	Meyer et al., 2001

ITS = internal transcribed spacer.

From the isolated DNA, the 18S rRNA genes of the ITS regions were amplified. When using the primer pair ITS3/ITS4, a single band of approximately 460 bp was obtained from all isolates studied (Figure 3). All genomic DNA samples obtained from the different isolates showed clear, sharp and reproducible PCR products when primer UP-PCR was used for amplification. UP-PCR products of various sizes, ranging from 200 to 1300 bp, were obtained (Figure 4).

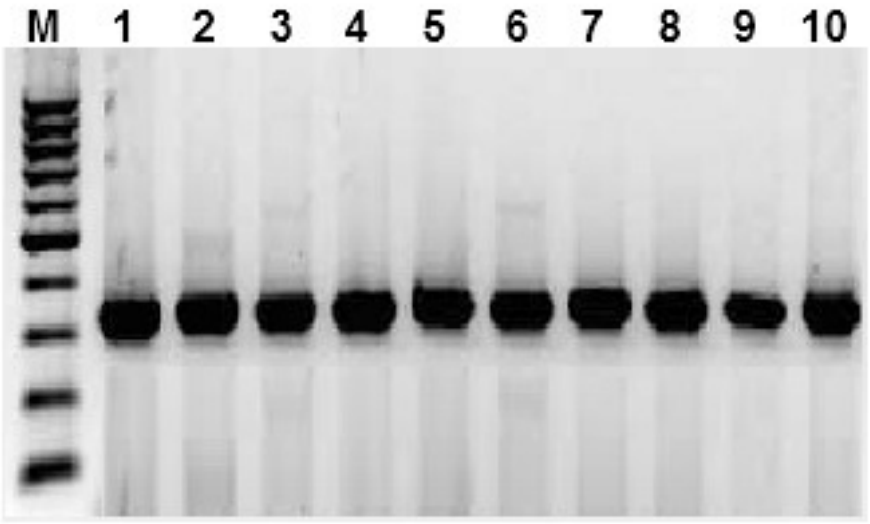

Figure 3. Agarose gel (1.5%) electrophoresis of the amplified internal transcribed spacer (ITS) products obtained with the ITS3/ITS4 primer pair. M = 100-bp DNA molecular weight ladder. For identification of lanes, see legend to Figure 1.

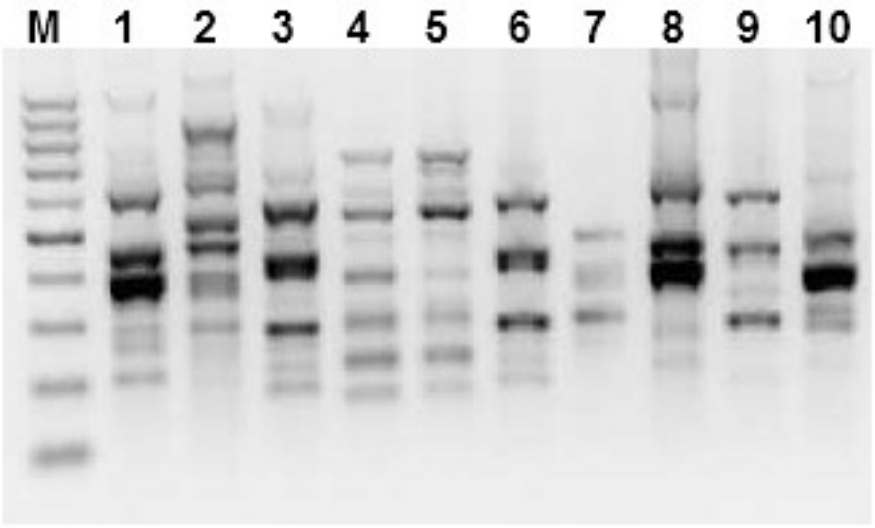

Figure 4. Agarose gel (1.5%) electrophoresis of universally primed-PCR products obtained by using the L 21 primer. M = 100-bp DNA molecular weight ladder. For identification of lanes, see legend to Figure 1.

DNA was also suitable for MSP-PCR, a technique analogous to MSP-PCR but using the T3B microsatellite core sequence as a unique PCR primer. The microsatellite DNA fragments within the expected range of length (100-1300 bp) were amplified in most cases from all *Cladosporium* isolates. About two thirds of microsatellites were not polymorphic among the 10 isolates, and could not be used for distinction of *Cladosporium* species (Figure 5).

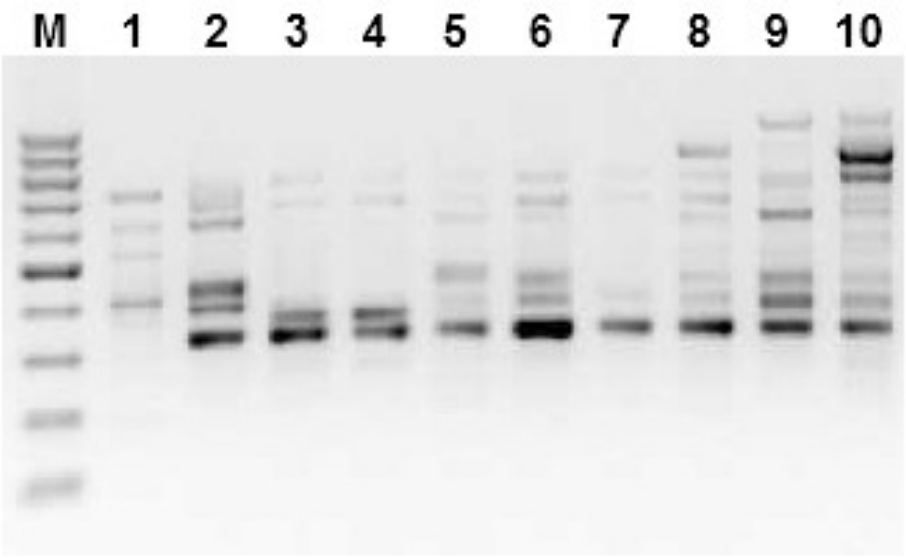

Figure 5. Agarose gel (1.5%) electrophoresis of the microsatellite-primed PCR products obtained by using the T3B primer. M = 100-bp DNA molecular weight ladder. For identification of lanes, see legend to Figure 1.

The DNA isolated from all *Cladosporium* isolates generated reproducible PCR amplification products that were well resolved on 1.5% agarose gels. This further confirmed that the isolated DNA was free of polysaccharide and polyphenols, which are known to inhibit Taq DNA polymerase and restriction endonucleases (Moyo et al., 2008). RNA can be removed by incubation with RNase A, either after the nucleic acids have been extracted or by inclusion in the extraction buffer (Abd-Elsalam et al., 2007). Pure DNA eluted from the agarose gels ensures reliable DNA amplification by PCR. Our method does not require liquid nitrogen or expensive commercial DNA extraction kits, which significantly decreases costs and time for DNA analysis. The growth of the fungus for only a few days before DNA extraction also facilitates homogenization and disruption of mycelium as it is not yet melanized. The time required for our DNA extraction method is less than 60 min, which is fast when compared with other genomic DNA extraction methods described. Our extraction method generated DNA that can be used in various molecular analyses. The method is especially useful in laboratories that lack the facilities to work with liquid nitrogen. We will use the method to further analyze global populations of *C. fulvum* and other *Cladosporium* species in the future.

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