DNA extraction from frozen field-collected and dehydrated herbarium fungal basidiomata: performance of SDS and CTAB-based methods

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Resumo

Extração de DNA de basidiomas congelados e de Herbário, através dos métodos SDS e CTAB. A extração de DNA de basidomas coletados e congelados e de espécimes de herbário coletados em diferentes períodos e mantidos de diferentes formas foi avaliada de acordo a condição geral, cor, razão de absorvância espectral, concentração final e amplificação PCR, utilizando dois dos métodos mais comuns de extração SDS (Sodium Doecyl Sulphate) e CTAB (Cetyl Trimetyl Ammonium Bromide). A

maioria dos basidiomas congelados apresentou uma condição de DNA boa a muito boa e sem degradação. Todos os espécimes de herbário exibiram DNA em má condição com bandas de baixo peso molecular. Praticamente todas soluções de DNA das amostras foram coloridas e as taxas de absorbância foram consideravelmente variáveis, com valores menores nas zonas escuras. Todas as amostras foram amplificadas em PCR com sucesso. A extração de DNA de espécimes com alto conteúdo de polisacarídeos e de espécimes de herbário foram obtidas unicamente pelo método CTAB. Para uma proposta geral de extração de DNA de basidiomas coriáceos e lenhosos recém coletados e desidratados, com alto conteúdo de metabólitos secundários e/ou polissacarídeos, o método CTAB é a melhor escolha.

Unitermos: métodos PCR, basidioma, Basidiomycetes

Abstract

Extracted DNA from basidiomata of frozen field-collected and herbarium specimens collected in different periods and maintained in a variety of ways was evaluated according to general condition, color, spectral absorbance ratio, final concentration and PCR amplification, using two distinct and most common extraction methods: SDS (Sodium Doecyl Sulphate) and CTAB (Cetyl Trimetyl Ammonium Bromide). Most of the frozen field-collected basidiomata presented a very good to good DNA condition and no degradation. All the herbarium specimens exhibited poor DNA condition with low molecular weight bands. Practically all the DNA solutions of the samples were colored, and spectral absorbance ratios were considerably variable, with lower values observed in darker ones. All the samples were successfully PCR-amplified. DNA extraction of Herbarium specimens and also specimens with high polysaccharide content was only achievable using the CTAB method. For an all-purpose DNA extraction from field-collected and dehydrated coriaceous

and woody basidiomata bearing a high content of secondary metabolites and/or polysaccharides, the CTAB-based method is definitely best the choice of method.

Key words: PCR methods, basidiome, Basidiomycetes

Introduction

DNA from basidiomycetous fungi is usually extracted from living pure cultures, although sometimes isolation, development and conservation of cultures are not achieved due to several distinct factors such as contamination, difficulties in culture preservation or even a total impossibility of culturing certain species. Furthermore, cultivating even small amounts of fungi requires more time and resources than would a collection from nature (Blackwell and Chapman, 1993).

Frozen field collections of basidiomata or even dehydrated ones can be substituted by cultures, since DNA can be extracted directly without the necessity of cultivating the specimens. However, there are some problems associated with direct DNA extraction from field-collected and herbarium basidiomata: (a) high content of polysaccharides and/or secondary metabolites, (b) action of phenoloxidases and (c) DNA degradation (Moncalvo *et* al., 1995).

Several protocols have been proposed for the extraction of DNA from fungal tissues, such as SDS-based (Lee and Taylor, 1990), CTAB-based (Rogers and Bendich, 1985), glass-bead-beating (Smit *et* al., 1999) and various commercial DNA extraction kits (Loffler *et* al., 1997). Although the first two methods are time-consuming, the others require the purchase of specialized instrumentation (glass-bead-beating) or have a high cost per sample (commercial DNA extraction kits) and thus may be prohibitive for routine work in Mycology laboratories.

This work evaluates the performance of two traditional and easily made (in-house) DNA extraction methods (SDS-based and CTAB-based) for both field-collected and dehydrated basidiomata of Basidiomycetes taxa having a high content of secondary metabolites and/or polysaccharides, mainly exhibiting coriaceous and woody habit.

Material and Methods

DNA was extracted from the basidiomata of 21 specimens of Hymenochaetaceae, Polyporaceae and Auriculariaceae, comprising a total of 19 distinct Basidiomycetes species. Specimens were collected in different localities over different periods of time, and they were submitted to distinct conditions of preservation (Table 1).

Two extraction methods were utilized: (i) CTAB-based (100 mM Tris-HCl pH 8, 1.4 M NaCl, 2% CTAB, 20 mM EDTA, 1% PVP) (Rogers and Bendich, 1985) and (ii) SDS-based (50 mM Tris-HCl, pH 7.2, 3% SDS, 50 mM EDTA, 1% β-mercaptoethanol) (Lee and Taylor, 1990).

Bits of basidiome (60 mg) were ground with a pestle in a porcelain mortar containing liquid nitrogen. The resulting powder was transferred to a 1.5-mL pre-warmed (65°C) microcentrifuge tube with extraction buffer and incubated at 65°C for 1 hour. In the CTAB-based procedure, DNA was extracted once with chloroform-isoamyl alcohol (24:1), precipitated with isopropanol, washed with ethanol 70%, and resuspended in 30 µL sterilized deionized water containing RNase A (100 µg/mL). In the SDS-based procedure, chloroform-isoamyl alcohol was substituted for phenol-chloroform (1:1); the other steps were the same.

The extracted DNA solution was evaluated according to five parameters: (i) DNA condition, (ii) color, (iii) spectral absorbance ratio ($A_{260/280}$), (iv) final concentration (ng DNA/µL purified DNA), and (v) PCR amplification of nuclear 25S rDNA.

The quality of the DNA was assayed by means of 1% agarose gel electrophoresis, using a high weight DNA mass ladder, and by UV light spectrophotometry ($A_{260/280}$). The DNA condition was categorized as (1) high molecular weight DNA, no degradation, (2) somewhat degraded, but still showing a band with high molecular weight DNA, or (3) highly degraded and/or with a low molecular weight DNA band. The color of DNA solutions was classified, as (1) hyaline, subhyaline or whitish, (2) colored (yellowish) but still clear, (3) dark, opaque.

TABLE 1: Studied specimens.

Sample[1]	Voucher	Species	Material	Locality	Collection Date
1*	HUEFS 41459	*Aurificaria luteoumbrina* (Rom.) Reid Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil 12°34'S, 38°59'W	24/IX/1999
2*	HUEFS 41457	*Trichaptum byssogenum* (Jungh.) Ryv. Polyporaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	24/IX/1999
3	HUEFS 41461	*Datronia caperata* (Berk.) Ryv. Polyporaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	24/IX/1999
4	HUEFS 41456	*Phellinus gilvus* (Schw.) Pat. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	24/IX/1999
5*	HUEFS 41462	*Datronia caperata* (Berk.) Ryv. Polyporaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	24/IX/1999
6*	HUEFS 41465	*Phellinus gilvus* (Schw.) Pat. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	10/X/1999
7*	HUEFS 41468	*Phellinus rimosus* (Berk.) Pil. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	07/XI/1999
8*	HUEFS 41469	*Phellinus extensus* (Lév.) Pat. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	07/XI/1999
9*	HUEFS 41470	*Hymenochaete sallei* Berk. & Curt. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	07/XI/1999
10*	HUEFS 41471	*Phellinus melleoporus* (Murr.) Ryv. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	07/XI/1999
11*	HUEFS 41500	*Auricularia polytricha* (Mont.) Sacc. Auriculariaceae	Frozen	Salvador, BA, Brazil, 13°30'S, 38°24'W	25/VII/2000
12*	HUEFS 41499	*Phellinus membranaceus* Wright & Blumenf. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	15/VII/2000
13*	HUEFS 42783	*Phellinus palmicola* (Berk. & Curt.) Ryv. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	15/VII/2000

Sample[1]	Voucher	Species	Material	Locality	Collection Date
14*	HUEFS 42787	*Phylloporia pectinata* (Kl.) Ryv. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	16/VII/2000
15*	HUEFS 42789	*Phellinus grenadensis* (Murr.) Ryv. Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	16/VII/2000
16*	HUEFS 41790	*Phellinus apihaynus* (Speg.) Rajch. & Wright Hymenochaetaceae	Frozen	Cachoeira, BA, Brazil, 12°34'S, 38°59'W	17/IX/2000
17	FLOR 10709	*Inonotus patouillardii* (Rick) Imazeki Hymenochaetaceae	Herbarium	Florianópolis, SC, Brazil, 48°30'S, 38°24'W	27/I/1989
18	FLOR 10675	*Coltricia spathulata* (Hooker) Murrill Hymenochaetaceae	Herbarium	Florianópolis, SC, Brazil, 48°30'S, 38°24'W	20/I/1989
19	ICN 95786	*Stiptochaete damaecornis* (Link) Ryvarden Hymenochaetaceae	Herbarium	Municipality not indicated, RS, Brazil	30/VIII/1986
20	FLOR 11059	*Cyclomyces iodinus* (Montagne) Patouillard Hymenochaetaceae	Herbarium	Florianópolis, SC, Brazil, 48°30'S, 38°24'W	16/III/1995
21	FLOR 10648	*Hydnochaete peroxidata* (Berk. & Curt.) Dennis Hymenochaetaceae	Herbarium	Florianópolis, SC, Brazil, 48°30'S, 38°24'W	25/IV/1990

[1] D3 divergent domain of the nuclear gene that codes for 25S large subunit of ribosomal RNA of the samples marked with an asterisk (*) were sequenced and the data used in a phylogenetic study (Góes-Neto et al., 2002). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) database under accession numbers AF450252-AF450265, and the matrix and phylogenetic tree were deposited in TreeBASE (http://www.treebase.org/treebase/) under S841 and M1358.

Double-stranded symmetric PCR reactions were performed using a Perkin-Elmer Cetus DNA thermal cycler (GenAmp 2400). Reactions were carried out in 0.2-mL tubes in 100 µL reaction volume, containing the following components: 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 200 µM dNTP, 1 pmol/µL of each oligunucleotide primer, 0.02 U/µL *Taq* DNA polymerase, and 1 ng/µL genomic DNA template. Cycle parameters were according to Gardes and Bruns (1993): an initial denaturation at 94°C for 1 min 25 s, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 55°C for 55 s, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. Negative controls, without DNA template, were prepared in every series of amplification in order to exclude the possibility of contamination in reagents or reaction

buffers. The primers used for the amplification of the 5' end of the 25S nuclear ribosomal RNA gene consisted of the forward primer LR0R (5'-ACCCGCTGAACTTAAGC-3') and the reverse primer LR7 (5'-TACTACCACCAAGATCT-3') (Moncalvo et al., 2000). A total of 5 µL of each PCR reaction was electrophoresed on 1% agarose gels, and a DNA molecular weight marker was used as standard. The quality of amplified fragments was evaluated by 1% agarose gel electrophoresis and categorized as follows: (1) positive in dilutions up to 1:1000, (2) positive only in very high dilutions, 1:10.000 or more.

In order to analyze the multivariate binary and semiquantitative non-binary data set (Table 2), principal coordinates analysis (PCO) was performed in Multiv (Pillar, 1998), using the Gower index as a resemblance measure between sampling units.

Results

All results are summarized in table 2, which shows the success/failure in DNA extraction of the two tested methods for each sample as well as the associated values of evaluated variables of successful extractions. Figure 1 exhibits an order of samples using principal coordinates analysis.

All herbarium samples (17, 18, 19, 20 and 21) and one frozen field-collected sample (11) formed a group (Group A), which was clearly distinct from the group constituted by all other field-collected samples (Group B).

DNA extraction of Group A samples was only achievable using the CTAB method while both SDS-based and CTAB-based methods were effective in the DNA extraction of Group B samples. Although all Group A samples were successfully PCR-amplified, they were all characterized by poor DNA condition and, with the exception of the frozen field-collected sample, all DNA solutions of herbarium samples presented similar color and concentrations

and spectral absorbance ratios with small variability, indicating the remarkable homogeneity of Group A, in which all samples appear rather close to each other, reflecting low variances on both axes (Figure 1).

TABLE 2: Performance of the two DNA extraction methods (SDS and CTAB) tested.

Sample	Species	Method[1]	Color[2]	DNA condition[3]	$A_{260/280}$[4]	Cc(ng/µl)[5]	PCR[6]
1	*Aurificaria luteoumbrina*	SDS/CTAB	2/2	1/1	2/2	2/2	1/1
2	*Trichaptum byssogenum*	SDS/CTAB	3/3	1/1	3/3	1/1	2/2
3	*Datronia caperata*	SDS/CTAB	2/2	2/2	3/3	2/2	1/1
4	*Phellinus gilvus*	SDS/CTAB	2/2	1/1	2/2	2/2	1/1
5	*Datronia caperata*	SDS/CTAB	2/2	2/2	1/1	1/1	1/1
6	*Phellinus gilvus*	SDS/CTAB	2/2	2/2	1/1	2/2	1/1
7	*Phellinus rimosus*	SDS/CTAB	2/2	1/1	2/2	2/2	1/1
8	*Phellinus extensus*	SDS/CTAB	2/2	1/1	3/3	3/3	1/1
9	*Hymenochaete sallei*	SDS/CTAB	2/2	2/2	1/1	1/1	1/1
10	*Phellinus melleosporus*	SDS/CTAB	2/2	2/2	2/2	2/2	1/1
11	*Auricularia polytricha*	SDS/CTAB	-/1	-/3	-/1	-/3	-/1
12	*Phellinus membranaceus*	SDS/CTAB	2/2	1/1	3/3	2/2	1/1
13	*Phellinus palmicola*	SDS/CTAB	2/2	1/1	2/2	2/2	1/1
14	*Phylloporia pectinata*	SDS/CTAB	2/2	1/1	3/3	2/2	1/1
15	*Phellinus grenadensis*	SDS/CTAB	2/2	2/2	1/1	1/1	1/1
16	*Phellinus apihaynus*	SDS/CTAB	2/2	2/2	1/1	2/2	1/1
17	*Inonotus patouillardii*	SDS/CTAB	-/2	-/3	-/2	-/2	-/1
18	*Coltricia spathulata*	SDS/CTAB	-/2	-/3	-/1	-/2	-/1
19	*Stiptochaete damaecornis*	SDS/CTAB	-/2	-/3	-/1	-/2	-/1
20	*Cyclomyces iodinus*	SDS/CTAB	-/2	-/3	-/2	-/2	-/1
21	*Hydnochaete peroxidata*	SDS/CTAB	-/2	-/3	-/1	-/2	-/1

[1] Method for DNA extraction: a hyphen (-) in subsequent variables indicates failure in DNA extraction using the corresponding method.

[2] (1) Hyaline, subhyaline or whitish, (2) colored (yellowish), but still clear, (3) dark, opaque.

[3] (1) High molecular weight DNA; no degradation, very good DNA condition, (2) somewhat degraded, but still showing a band with high molecular weight DNA, good DNA condition, (3) highly degraded and/or with low molecular weight DNA band, poor DNA condition.

[4] (1) $A_{260/280}>1.8$, (2) $1.5< A_{260/280}<1.8$, (3) $1.2< A_{260/280}<1.5$.

[5] (1) Final concentration: Cc >1000ng/mL, (2) 100ng/mL<Cc<1000ng/mL, (3) Cc<100ng/mL.

[6] (1) Positive in dilutions up to 1:1000, (2) positive only in very high dilutions (= or >1:10.000).

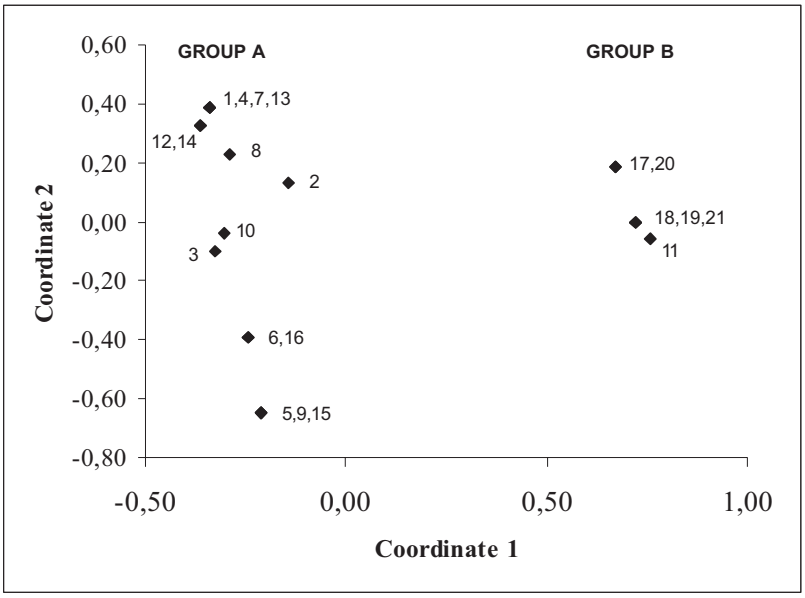

FIGURE 1: Scatter diagram of principal coordinates analysis of observed data.

In contrast to Group A, Group B samples exhibited, in both SDS and CTAB-based extraction methods, very good to good DNA condition, although they all presented colored DNA solutions, including a sample with a dark and opaque DNA solution. The final concentration and spectral absorbance ratio of Group B DNA solutions were considerably variable, reflecting the wide range on the vertical axis (Figure 1). Although all samples were PCR-amplified with success, one sample (2), which presented the darkest DNA solution, needed to be highly diluted in order to be PCR-amplified.

Discussion

The extraction of DNA from fungi has sometimes been notoriously difficult. Fungi often produce polysaccharides and a variety of secondary metabolites, including many phenolic

compounds, in copious amounts, that cause problems in DNA extraction (Rogers, 1994). Furthermore, long-lived basidiomata of many Basidiomycetes species are coriaceous or even woody hard, which poses additional difficulties in DNA extraction.

In the studied species, all these problems occur: Hymenochaetaceae are characterized by the extensive production of styrylpyrones and complex phenolic compounds in their basidiomata (Fiasson, 1982) as well as Polyporaceae (Parmasto and Parmasto, 1979), and Auriculariaceae have gelatinous basidiomata in which the content of polysaccharides can be as high as 95% (Miles and Chang, 1997). Moreover, most studied specimens have coriaceous or woody basidiomata.

The failure of the SDS-based method in Group A samples indicates that it must be avoided for extracting the DNA from polyssacharide-rich basidiomata as well as dehydrated ones, irrespective of their polysaccharide content. Using a SDS-based protocol to extract DNA from several herbarium Basidiomycetes specimens, Bruns et al. (1990) successfully extracted and PCR-amplified DNA from 31 distinct species. Nevertheless, all taxa used in their work were from the order Agaricales, embrassing species with fleshy and short-lived basidiomata, which are in sharp contrast with the long-lived, coriaceous and woody basidiomata of Hymenochaetales and Polyporales used in the current study.

Herbarium collections are now an immense source for obtaining molecular data, and molecular systematic studies are no longer strictly dependent on cultures or fresh/frozen basidiomata collections. DNA extracted from herbarium material is of low molecular weight, but is still capable of being PCR amplified, especially if the predicted PCR products are not long. PCR products as long as 1.5kb were obtained from all herbarium specimens.

Also observed by Rogers (1994), who studied DNA extraction methods for six Basidiomycetes species (all of them

Agaricales), the spectral absorbance ratio did not correlate with utility. PCR amplification was achieved for extracted DNA solutions with a ratio ($A_{260/280}$) as low as 1.2.

For a better removal of contaminants, centrifugation times and speeds may be increased just after the addition of organic solvents, but not in the precipitation step. The cleaning step, using 70% ethanol after DNA precipitation, can be repeated up to five times, as also indicated by Moncalvo et al. (1995) who studied polysaccharide and pigment-rich *Ganoderma* (Basidiomycetes) species. As also pointed out by Rogers (1994), the antioxidant agents PVP (polyvinylpyrollidone) and â-mercaptoethanol can be increased by up to 5% in extraction buffer to prevent the action of phenoloxidases, mainly in the case of a high content of phenolic compounds.

Although the studied specimens were collected in different periods (1986-1999) and maintained in distinct ways, DNA extraction and amplification was successfully performed in all cases, using either the CTAB-based method alone or both CTAB- and SDS-based methods. Therefore, field-collected and even dehydrated coriaceous and woody basidiomata, possessing a high content of secondary metabolites and/or polysaccharides, could be promptly used to obtain fungal DNA for use in a variety of molecular assays, avoiding all laborious work involved in culture collections.

The following conclusions may thus be considered as general recommendations: (i) Both methods can be used for DNA extraction from frozen field-collected basidiomata, since samples do not exhibit high polysaccharide content (gelatinous when fresh). (ii) For DNA extraction from basidiomata of herbarium specimens and frozen field-collected specimens with a high polysaccharide content, CTAB is definitely the method of choice.

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