ELSEVIER

International Biodeterioration & Biodegradation 58 (2006) 133–141

INTERNATIONAL BIODETERIORATION & BIODEGRADATION

www.elsevier.com/locate/ibiod

Application of molecular techniques for identification of fungal communities colonising paper material

Astrid Michaelsen[a,*], Flavia Pinzari[b], Katrin Ripka[a], Werner Lubitz[a], Guadalupe Piñar[a]

[a]*Department of Medicinal Chemistry, University of Vienna, Althanstr. 14, A-1090, Vienna, Austria*
[b]*Istituto Centrale per la Patologia del Libro, Laboratoria di Biologia, Via Milano 76, I- 00184, Rome, Italy*

Received 2 September 2005; accepted 21 June 2006
Available online 12 September 2006

Abstract

Archives and libraries all over the world suffer from biodeterioration of writings caused by microorganisms, especially fungi. With traditionally used culture-dependent methods, only a small amount of effectively colonising organisms is detected. Restoration and maintenance of written cultural heritage is therefore problematic due to incomplete knowledge of the deterioration agents.

In the present study, culture-independent molecular methods were applied to identify fungal communities colonising paper samples of different composition and age. Nucleic-acid-based strategies targeting the internally transcribed spacer (ITS) regions, which are nested in the nuclear rDNA repeats, were selected to investigate the fungal diversity on paper. The ITS regions possess a high variation among taxonomically distinct fungal species and even within the species.

With this aim, several molecular biological methods were optimised for working with paper materials. Here, we introduce a DNA extraction protocol, which allowed the direct extraction of PCR-amplifiable DNA from samples derived from different kinds of paper. The DNA extracts were used to amplify either the ITS1 or ITS2 region by using different fungi-specific primer sets. The ITS-amplified regions were subsequently analysed by denaturing gradient gel electrophoresis (DGGE). Conditions for DGGE analysis, gradient, voltage, and running time, were established to accurately discriminate different fungal species in complex communities. Pure fungal strains were used to constitute a marker for further comparative investigations of historic papers.

Keywords: Objects of art; DGGE; Internal spacer regions; PCR; Fungal communities

1. Introduction

In addition to presenting environmental concerns, microorganisms are responsible for the biodeterioration of cultural objects in the areas of art and writing. They often cause degradation of objects such as paintings, stone, wood, paper, masonry, leather, parchment, glass and metal, and cinematographic films (Cappitelli and Sorlini, 2005; Abrusci et al., 2005). The chemical and structural nature of the substratum, as well as environmental conditions such as moisture, temperature, pH, and light, are the significant parameters affecting quantity and quality of microbial colonisation on works of art (Saiz-Jimenez, 1993).

One type of deterioration found on cultural properties made of paper, and on books, is called foxing, the brown spots that appear to be caused by airborne fungi (Arai, 2000). It has also been suggested that contamination can occur during paper-making or book preparation, in addition to being caused by airborne fungi. Archives and libraries from all over the world suffer from biodeterioration phenomena, but with the traditionally used culture-dependent methods, only a small amount of effectively colonising microorganisms is detected and a relatively large amount of sample is needed. Restoration and maintenance of written cultural heritage is therefore problematic because of the incomplete knowledge of deteriorative organisms or agents. For correct conservation, it is important to identify the complete microbial community colonising art objects, using non-destructive sampling, or sampling that needs only small amounts of material.

*Corresponding author. Tel.: +43 1 4277 55116; fax: +43 1 4277 55120.
E-mail address: astrid.michaelsen@univie.ac.at (A. Michaelsen).

0964-8305/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibiod.2006.06.019

There are several works describing bacterial communities involved in the degradation of art objects (Schabereiter-Gurtner et al., 2001). However, the fungal flora responsible for the biodeterioration of such objects is not yet well described. There are very few works focusing on the investigation of the fungal flora responsible for the biodeterioration of paper materials by applying molecular techniques (Di Bonaventura et al., 2003a, b).

It has been estimated that only about 5% of fungal species involved have been accurately described owing to culture limitations, misidentifications in culture collections, and unexplored habitats (Hawksworth and Rossman, 1997). With classical cultivation or microscopic methods, species such as the cellulolytic *Chaetomium* spp., *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp. or *Trichoderma* spp. (Szczepanowska and Cavaliere, 2000; Corte et al., 2003), could be detected on paper material. Other strains frequently found as contaminants in archives and libraries are *Paecilomyces variotii, Myrothecium verrucaria*, *Stachybotrys atra,* and Fungi Imperfecti (Deuteromycetes) (Florian and Manning, 2000).

Molecular approaches have been developed for the assessment of microbial diversity in complex communities (Gonzalez and Saiz-Jimenez, 2005). Methods based on DNA analysis can reveal fungal diversity in ecosystems, and offer the potential benefits of highly sensitive and rapid detection (Saad et al., 2004). The molecular identification of fungi to species level has been based mostly on the use of variable ribosomal-DNA (rDNA) internally transcribed spacer (ITS) regions. The non-coding ITS region consisting of ITS1, the 5.8S rDNA and ITS2, should produce a highly sensitive assay as the target sequence for amplification, because of its high copy number in the fungal genome as part of tandemly repeated nuclear rDNA. These regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the rRNA genes. As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions (Anderson et al., 2003; Lord et al., 2002). Additionally, the DNA sequences in the ITS region are highly variable and might serve as markers for taxonomically more distant groups.

In this study, culture-independent molecular methods were applied to investigate fungal communities colonising paper material of different age and quality for the first time. Pure fungal strains were used to artificially infect different kinds of paper, which were subsequently used to compare and optimise established molecular biological methods for working with this kind of material.

Here we introduce a DNA extraction protocol, which allowed the direct extraction of PCR-amplifiable DNA from samples derived from different kinds of paper. The DNA extracts were used to amplify either the ITS1 or ITS2 regions, which were subsequently analysed by denaturing gradient gel electrophoresis (DGGE). Conditions for PCR, such as the use of different fungi-specific primer sets, as well as for DGGE analysis, such as gradient, voltage, and running time, were established to accurately discriminate different fungal species in complex communities. In addition, pure fungal strains were used to constitute a marker for further comparative investigations of historic papers.

2. Materials and methods

2.1. Preparation of samples

The optimisation of DNA extraction protocols to obtain PCR-amplifiable DNA from paper materials was performed with the following set of materials: (a) pure fungal colonies obtained from strains known to be involved in paper, wood, and cellulose biodeterioration; (b) samples of model-paper inoculated with suspensions of spores from single fungal strains or mixtures in known proportions; (c) paper samples with naturally occurring fungal infections; (d) paper samples with 20-year-old inocula and naturally occurring infections, selected in order to verify the chemical and physical changes in fungal nucleic acids on paper during time, and the effects on molecular diagnosis.

2.1.1. Fungal strains and growth conditions

The following four fungal strains, provided by the Institut für Holzforschung, Vienna, Austria, were used as marker strains: *Alternaria alternata* (BAM Berlin DSM 62010-VdL-RL06), *Aspergillus versicolor* (EMPA St.Gallen Nr.517), *Chaetomium globosum* (Messehalle Hannover) and *Cladosporium cladosporioides* (BAM Berlin DSM 62121-VdL-RL06). In addition to the aforementioned strains, fungal strains considered to be frequently associated with library material deterioration had been utilised 20years ago to inoculate paper strips, and this was repeated more recently. *Aspergillus terreus* Thom (strain no. 3) was obtained from the ATCC culture collection, *Aspergillus hollandicus* (Anam.: of *Eurotium amstelodami*, (Mangin) Thom and Church) and *Eurotium chevalieri* L. Mangin from the culture collection of the Istituto Centrale per la Patologia del Libro (ICPL), Rome, Italy, and were isolated from deteriorated paper materials. Additionally, *Penicillium rubrum*, *Byssoclamys fulva,* and *Aspergillus niger,* from the same collection, were used for inoculation 20 years ago.

For DNA extraction, the strains were grown on malt-peptone agar at 27 °C until vigorous growth was observed, prior to their harvest. The strains utilised for the inoculation of paper samples were grown on MEA (malt extract agar) for 7 days at 25 °C. The spore suspensions were obtained by gently scraping the surface of the 7-day-old cultures with a swab, washing it with 30 mL of H_2O containing 0.02% Tween 80 (Merck-Schuchardt, Hohenbrunn, Germany) and filtering through a sterile cotton cloth to remove impurities.

2.1.2. Artificial inoculation of paper material

Preparation and inoculation of paper materials was performed at the ICPL. Three types of paper with different composition, depending on the origin of the fibres and chemical treatments undergone during the manufacturing process, were used in this study (Table 1). Whatman paper is considered as a model because of its standard composition and manufacturing and its high level of availability in laboratories all over the world, although it is not used as writing material.

Paper strips of 2 × 6 cm of the three different paper types were exposed to UV light for 45 min on both sides to eliminate airborne fungal and bacterial cells from the surface prior to inoculation. The spore density used for inoculation of paper strips was defined for each fungal strain by optical microscopy in a Thoma Chamber. A defined volume of each spore suspension was diluted with Sabouraud Broth (DIFCO, Becton Dickinson, Heidelberg, Germany) in order to perform the inoculation with a comparable number of spores. Paper samples with single-strain inocula contained 3500 spores/100 µL. Mixtures with different proportions of spores were used to inoculate paper strips. The strips were inoculated with

Table 1
Paper types with fungal infections

Paper type	Composition	pH	Inoculation	Natural alterations
Whatman 1CHR paper for chromatography Cat. No. 3001 917	Pure cellulose	5.4	Single: *Aspergillus hollandicus* *Aspergillus terreus* *Eurotium chevalieri* *Mixed: 1:1:1* 3:1:1 3:3:1 20 y ago: *Chaetomium globosum* *Aspergillus niger* *Penicillium rubrum* *Byssoclamys fulva*	Recent 20 years ago
"Freelife vellum" from Fedrigoni Mills (Italy), long-life type (according to ISO CD 9706)	80% recycled fibres, 15% cellulose chlorine free, 5% cotton fibres	7.2	Single (as above)	Recent
"Old mill" ivory from Fedrigoni Mills (Italy), uncoated long-life type	100% cellulose fibres	6.9	Single (as above)	Recent

100 μL of broth with fungal spores distributed in 8 dots, and were incubated for 10 days at 27 °C and 75% relative humidity (saturated NaCl solution) each in 50 mL polystyrene vials. The incubation was performed in a thermostatic cell, and a sensor was used to register the relative humidity (Hygrolog-D Rotronic AY-Swiss).

2.2. DNA extraction from paper material

Two different methods were tested for the extraction of fungal DNA from paper materials of different age and quality: (A) DNA was extracted directly from paper by using the FastDNA Spin Kit for Soil (Qbiogene, Illkirch, France). The protocol of the manufacturer was slightly modified. About 10–20 mg (0.5–1 cm^2) paper was placed in the Multimix2 tissue tube with the appropriate buffer and then processed on a bead-beater twice for 1 min, with 1 min intervals on ice. The PPS reagent and the binding matrix solution were applied to the supernatant; the suspension was transferred to the provided spin filter and centrifuged at 13,000 rpm for 1 min following the instructions of the manufacturer. DNA was washed twice with 500 μL of the SEWS-M solution and eluted from the binding matrix with 100–150 μL DNase/Pyrogene Free Water. (B) DNA extraction was done following the protocol previously described by Schabereiter-Gurtner et al. (2001). Briefly, this method combines enzymatic (lysozyme and proteinase K) and mechanical steps (freeze and thaw cycles) in the presence of cetyltrimethylammonium bromide (CTAB).

DNA crude extracts were used directly for PCR amplification analysis or were further purified prior to PCR amplification with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The final elution step was repeated three times with 80 μL of 80 °C preheated ddH_2O (Sigma Aldrich, St. Louis, USA).

2.3. DNA extraction from pure fungal cultures

DNA was isolated from fresh mycelia taken from the surface of plate cultures. The method is based on that of Jasalavich et al. (2000), using CTAB in the presence of ß-mercaptoethanol. The mycelia was further processed mechanically with glass beads (0.1–2 mm) in a bead-beater and with two cycles of freeze–thawing (2 min at 95 °C, 2 min at −20 °C, 2 min at 95 °C), followed by organic extractions and isopropanol precipitation of DNA. The DNA yield was visualised in a 1% agarose gel.

2.4. PCR amplification of extracted DNA

All PCR reactions were performed in a Robocycler (Stratagene, La Jolla, USA) using PCR Master Mix (Promega, Mannheim, Germany). Fragments of about 300 bp in size, corresponding to either the ITS1 or ITS2 region of the ribosomal-DNA ITS region, were first amplified with the primer pairs ITS1 forward and ITS2 reverse, or ITS3 and ITS4, according to White et al. (1990). Primer pair EF3RCN (Lord et al., 2002) with the reverse primer ITS4 (as above) was also tested. All reactions were carried out in a 25-μL volume containing 12.5 pmol of each primer and 1.5–2.5 μL DNA as template, following the manufacturer's instructions for PCR Master Mix. The thermocycling program was as follows: 5 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 1 min extension at 72 °C. Five minutes at 72 °C were used as a final extension step.

For DGGE analysis, a nested PCR was performed in a total volume of 100 μL (2 × 50 μL reaction size), each with 3.5 μL of PCR product from the first amplification as template DNA. The same forward and reverse primers were used, with a 37-base GC-clamp attached to either 5′-end of the forward primer, to stabilise the melting behaviour of the DNA fragments (Muyzer et al., 1993). The cycling scheme was as follows: 5 min denaturation at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 58 °C for ITS1-GC primer or 62 °C for ITS2-GC primer, 1 min extension at 72 °C with a final extension step at 72 °C. PCR products were analysed by electrophoresis in a 2% (w/v) agarose gel.

2.5. Denaturing gradient gel electrophoresis (DGGE)

For the genetic fingerprint of the amplified ITS regions, 50–100 μL of PCR products containing the GC-clamp were precipitated with 96% ethanol at −20 °C overnight, resuspended in 15 μL double-distilled H_2O, and separated by DGGE. Gel electrophoresis was performed as previously described (Muyzer et al., 1993) in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) with 8% (w/v) acrylamide gels containing a gradient of denaturants in a D-GENE-System (Bio-Rad, Munich, Germany). The gradient of denaturants, as well as running conditions, was optimised in this study and is given in the results section. After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with a UVP documentation system (Bio-Rad Quantity-One, Munich, Germany).

2.6. Preparation of a reference marker for DGGE analysis

A marker containing ITS1-GC or ITS2-GC PCR products, derived with primers ITS1-GC/ITS2 and ITS3-GC/ITS4 from seven different fungal strains, was designed to allow comparative analyses of DGGE patterns from different gels, and as a quality control of the gel run. One hundred μL PCR product from each species was pooled, precipitated in 96% ethanol at −20 °C, and then resuspended in 50 μL ddH_2O. Fifteen μL were used as marker for DGGE analysis. The reference species used were: *A. alternata*, *C. globosum*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *A. terreus,* and *A. versicolor*.

2.7. SEM microscopy

SEM pictures of paper material with and without fungal colonisation were taken by a LEO 1450 VP from the Carl-Zeiss Electron Microscopy Group (Jena, Germany).

3. Results

3.1. Optimisation of DNA extraction protocols from fungal-infected paper samples and evaluation of their efficiency by PCR amplification

From paper material, DNA was extracted following two different protocols—the DNA extraction protocol described by Schabereiter-Gurtner et al. (2001) and the commercial kit FastDNA Spin Kit for Soil with modifications, as described in the materials and methods section. A further purification step using the QIAamp Viral RNA mini kit was carried out with the crude DNA extracts obtained from both DNA extraction methods to compare the PCR-amplifiability after a second purification step.

Both protocols (with and without the extra purification step) were tested for all three different types of paper (see Table 1). Each batch of paper samples consisted of several inoculated paper strips containing different mixture proportions of the selected fungal spores. In addition, each batch of paper samples included an incubated, non-inoculated paper as negative control.

DNA extraction efficiency was tested via fungal-specific PCR amplification of the minimal content of fungal DNA on the inoculated paper. The fungal specific primer pairs EF3RCN/ITS4, ITS1/ITS2, and ITS3/ITS4 allowed DNA of pure fungal strains to be amplified using these specific primer sets, whereas bacterial DNA from different phylogenetic groups, used as control for fungal specificity of the primer sets, was not amplified. No amplification was observed in any of the non-inoculated paper strip controls, excluding contamination during extraction procedure, as well as the amplification of plant material.

By using the DNA extraction protocol described by Schabereiter-Gurtner et al. (2001), only paper C gave amplifiable material without a further purification step. PCR products from papers A and B could be obtained only when this DNA extraction protocol was applied in combination with the QIAamp Viral RNA mini kit (data not shown). In contrast, the bead-beating homogenisation of the FastDNA Spin Kit proved to be more efficient for fungal DNA extraction from infected paper material. It resulted in pure DNA for direct use in PCR analysis, without the need for a second purification step, for all three different kinds of paper materials tested, independently of the primer set used. When this protocol was used in combination with the QIAamp Viral RNA mini kit as a second purification step, no differences in PCR amplifiability were observed from the first eluent of purified and non-purified DNA. A decrease in the intensity of PCR products obtained from the consecutive second and third eluents, according to their presumed DNA content, was observed, as shown in Fig. 1. Consequently, the FastDNA Spin Kit for Soil was selected for further experiments without the need of an extra time-consuming purification step. For DGGE analysis, a nested PCR with primers containing a GC-clamp was carried out, as mentioned in the materials and methods section. Some differences could be observed, depending on the primer pair used. Primer pair ITS1-GC/ITS2, amplifying the ITS1 region, produced specific PCR products, whereas amplification using the primer pair ITS3-GC/ITS4, for the ITS2 region, resulted in unspecific PCR products, which could not be eliminated by changing PCR conditions, such as number of cycles, annealing temperature, or amount of template (data not shown).

3.2. Optimisation of conditions for DGGE analysis

To establish the optimal DGGE running conditions for the fungal DNA fragments, a "time travel" experiment was arranged, in which the marker mixture of PCR products derived from genomic DNA of seven different fungal species, i.e., *A. alternata*, *C. globosum*, *C. cladosporioides*, *E. chevalieri*, *A. terreus*, *A. hollandicus*, and *A. versicolor*, was applied to a denaturing gradient gel containing a gradient of 20–60% of denaturants and run with a voltage of 200 V. The marker was loaded every 30 min over a total time of 6.5 h to get a variety of time points in one gel. The optimal running time of the gel was determined as the time necessary to achieve the migration of the seven DNA fragments as independent single bands. As shown in Fig. 2, the fragments could not be separated as single bands for the first 60 min. After this time, they started to separate from each other due to the denaturing conditions within the gel; the molecules were partially melted and slowed down. After ~7 h the maximum resolution of the three fragments was achieved for both ITS1 and ITS2 region analysis. In parallel, a time travel experiment keeping a low voltage of 100 V for 14 h was tested (data not shown). Results showed a better resolution of bands as when using 200 V and 7 h; therefore we used these conditions for further experiments.

For optimisation of the denaturing gradient, the concentration of denaturants was varied in subsequent experiments to obtain more accurate fungal fingerprints. Gradients of 30–50% and 30–60% were set for DGGE analysis of the ITS1 and ITS2 region, respectively.

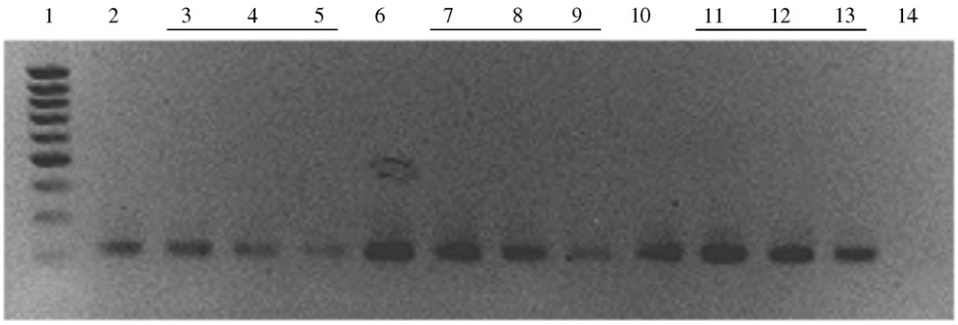

Fig. 1. PCR amplification of DNA extracted from infected papers by using the FastDNA Spin Kit for Soil with and without a further purification step with the Qiagen Viral Kit. PCR was performed with the primer pair ITS1/ITS2. Lane 1: 1kb marker; lane 2: paper A not purified; lanes 3–5: purified paper A eluents 1–3; lane 6: paper B not purified; lanes 7–9: purified paper B eluents 1–3; lane 10: paper C not purified; lanes 11–13: purified paper C eluents 1–3; lane 14: PCR negative control.

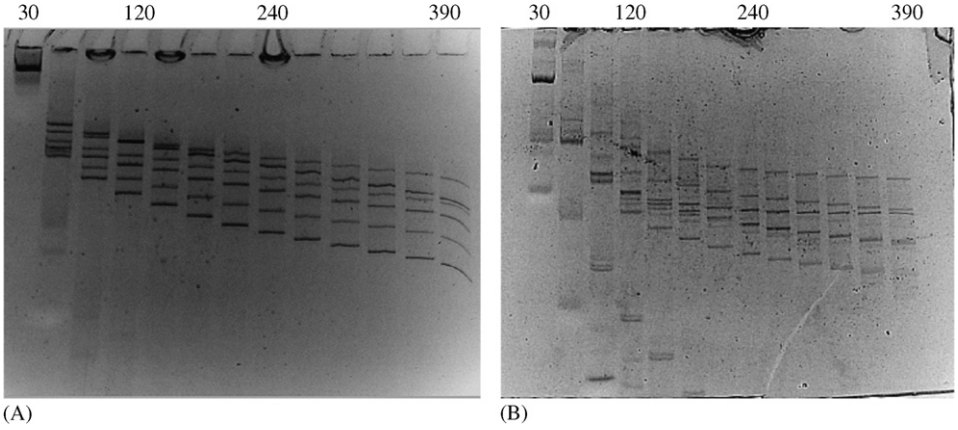

Fig. 2. Time travel experiment for the ITS1 and ITS2 regions. Ethidium bromide-stained DGGE separation pattern of fungal specific PCR fragments derived from the ITS1 (A) or the ITS2 (B) region. A mixture of PCR products obtained from *Alternaria alternata*, *Chaetomium globosum*, *Cladosporum cladosporioides*, *Eurotium chevalieri*, *Aspergillus terreus*, *Aspergillus hollandicus*, and *Aspergillus versicolor* was applied every 30 min over a total of 6.5 h. Both gels were run at 200 V with a gradient of 20–60% urea and formamide.

3.3. Creation of a fungal DNA reference marker for DGGE analysis

Seven different fungal strains (see Section 2.6) were selected for the creation of a fungal DNA reference marker for DGGE analysis. Three of them, namely *A. terreus*, *A. hollandicus*, and *E. chevalieri*, were the strains used to inoculate the paper strips. DNA extraction from pure fungal cultures was performed using the CTAB method, as described in the materials and methods section. PCR-amplifiable DNA could be extracted from all seven fungal strains without further need for purification or dilution (data not shown).

The extracted DNA was subsequently amplified with primers ITS1-GC/ITS2 and ITS3-GC/ITS4 and the resulting ITS-amplified fragments were used to create an internal reference marker for DGGE analysis of the ITS1 and ITS2 region, respectively (Fig. 3). DGGE of PCR fragments amplifying the ITS1 region resulted in reproducible clear single bands for each fungal strain (Fig. 3A). In contrast, DGGE analysis of PCR fragments amplifying the ITS2 region led to multiple bands for single strains and, therefore, to an overestimation of fungal strains (Fig. 3B). A discrimination between *A. hollandicus* and *E. chevalieri* strains was not possible in any case. Even by changing running conditions and/or denaturing gradients, no differences in the melting behaviour of these two fungal strains could be observed.

To correlate the consecutive order of the amplified fungal ITS regions in our marker with their phylogenetic identity, the marker and the DNA of the individual fungal strains were run together in one gel (Fig. 3). DGGE revealed the consecutive order of the fungal strains in our marker as being: *A. alternata*, *C. globosum*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *A. terreus*, and *A. versicolor* for the ITS1 region DGGE marker, and *A. alternata*, *A. terreus*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *C. globosum*, and *A. versicolor* for the ITS2 region marker. The creation of this marker allowed the identification of the inoculated strains in our samples, as well as the comparative analysis of DGGE patterns obtained from different gels.

3.4. DGGE analysis of naturally altered paper samples

Once the molecular strategy for working with fungal strains on paper materials was optimised, the next step was

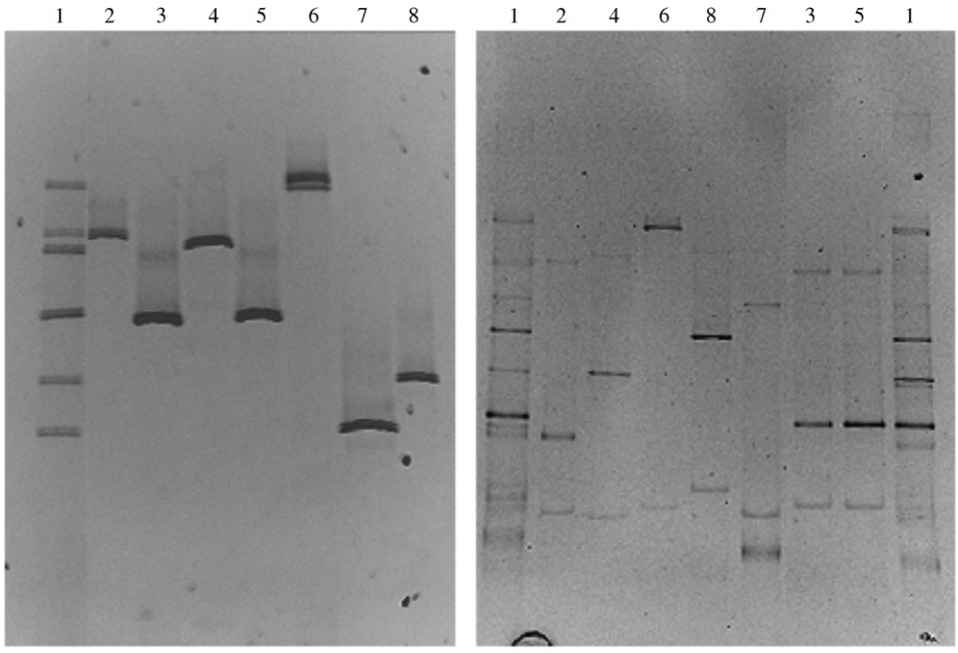

Fig. 3. Fungal DNA marker for DGGE analysis: (A) DNA amplified with primer pair ITS1-GC/ITS2. (B) DNA amplified with primer pair ITS3-GC/ITS4. Gels were run for 14 h at 100 V in gels containing a denaturing gradient of 30–50% for the ITS1 region or 30–60% for the ITS2 region. Nomenclature for both A and B. Lane 1: marker: mixture of all seven fungal strains; lane 2: *Chaetomium globo*sum; lane 3: *Aspergillus hollandic*us; lane 4: *Cladosporum cladosporioides*; lane 5: *Eurotium chevalieri*; lane 6: *Alternata alterna*ria; lane 7: *Aspergillus versicolor*; lane 8: *Aspergillus terreus*.

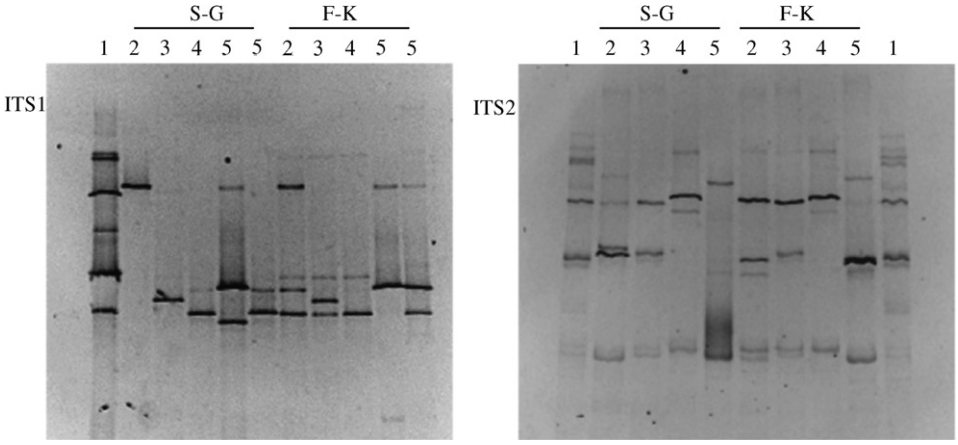

Fig. 4. DGGE analysis of different naturally infected paper types: a comparison between two types of DNA extraction methods and amplified-ITS target regions: (A) fingerprints of the amplified-ITS1 region on a gel with a denaturing gradient of 30–50% urea and formamide. (B) Fingerprints of the amplified-ITS2 region on a gel with a denaturing gradient of 30–60%. Both gels were run over 14 h with 100 V. Nomenclature counts for both. S–G indicates the extraction of DNA according to Schabereiter-Gurtner et al. (2001) whereas F–K stands for the use of the FastDNA Spin Kit for Soil DNA preparation. Lane 1: marker mixture; lane 2: paper A; lane 3: paper B; lane 4: paper C; lane 5: paper A naturally infected 20 years ago.

to test the established methodology for paper samples naturally infected or artificially inoculated 20 years ago (see Table 1). DNA extraction from infected paper samples was performed by using the two protocols tested in this study, as described in the materials and methods section.

To compare both methods, DNA extracts from all samples were used to amplify both the ITS1 and ITS2 regions, shown to be all PCR-amplifiable using either the ITS1 or ITS2 primer sets. Further DGGE analyses in 30–50% denaturing gels for ITS1-GC and 30–60% denaturing gels for ITS2-GC were carried out as mentioned above. Fig. 4 shows the DGGE fingerprints of paper samples naturally infected with fungal strains. DGGE profiles were different depending on the DNA extraction method. Using the DNA extraction protocol developed by Schabereiter-Gurtner et al. (2001), some dominant bands were completely absent, indicating that this lysis method was not suitable for fungal spores. In addition, as already observed for the fungal DNA fragments contained in our DGGE marker, only the fungal amplified ITS1 region fragments led to reproducible single bands on the DGGE fingerprints (Fig. 4A), whereas fingerprints obtained from

fungal amplified ITS2 region fragments produced no clear band patterns, with many weak bands (Fig. 4B). This let us to conclude that further experiments to investigate complex fungal communities on naturally infected paper samples will be based on the combination of the FastDNA Spin Kit for Soil DNA extraction protocol, followed by PCR amplification of the ITS1 region and the subsequent analysis of the resulting fragments by DGGE.

4. Discussion

Paper has been the main material for recording cultural achievements all over the world, and its microbial degradation is one of the most unappreciated and serious reasons for damage to library and archival materials. Fungal communities have been investigated with PCR-DGGE in different ecological systems such as grass, wheat, wood, soil (Smit et al., 1999; van Elsas et al., 2000; Vainio and Hantula, 2000), and historical church window glass (Schabereiter-Gurtner et al., 2001). Furthermore, the study of fungal communities colonising objects of art made of paper by using PCR amplification of the ITS of the nuclear ribosomal RNA genes has been already described (Di Bonaventura et al., 2003a, b). In this work, samples from fungal spots on Tiffany's drawings were removed and DNA was isolated from these fungal spots. ITS regions were amplified and the PCR products were cloned, sequenced and compared to existing ITS sequences of a large database of fungal species.

In this study, we focused on establishing a reliable and fast molecular strategy to investigate complex fungal communities on paper-based art objects. The major challenge of this study was to recover DNA directly extracted from paper materials suitable for PCR amplification of fungal-specific sequences. We compared two different DNA extraction protocols routinely used in our laboratory for direct DNA extraction from art objects, i.e., wall paintings, stone-works and glass, as well as from soil samples. The modified DNA extraction protocol described in the materials and methods section, based on the commercial FastDNA Spin Kit for soil, has the advantage that it is at least five times faster than previous methods described for DNA extraction from soil samples. The protocol includes an extra purification step for removing PCR inhibitors, which results in very pure, colourless DNA, suitable for cloning and PCR amplification. The protocol used for DNA extraction of art samples described by Schabereiter-Gurtner et al. (2001) has been developed on the basis of an existing protocol from Zhou et al. (1996). This modified extraction protocol, in combination with a method to purify the DNA, allowed successful DNA extraction from small sample amounts. In most extraction protocols, the DNA used for further DGGE analysis is recovered by phenol-chloroform extraction and precipitation by alcohol. These two steps raise the risk of contamination during the extraction procedure and impede extraction of DNA from tiny sample amounts. This problem could be minimised by passing the cell lysate through silica-gel membranes. We chose the QIAamp Viral RNA Mini Kit (Qiagen) because, according to the manufacturer, it removes PCR inhibitors. Furthermore, it contained carrier RNA, which facilitates the recovery of small amounts of DNA. From each sample, three DNA eluents were tested for PCR-amplifiable DNA using primers targeting the ITS1 and ITS2 regions.

Our results showed that, for pure fungal strains existing predominantly as mycelia, a combination of enzymatic and mechanical steps for DNA extraction, as performed in the protocol described by Schabereiter-Gurtner et al. (2001), is a useful tool. However, for paper materials containing mycelia as well as spores, it proved to be ineffective in spore lysis. As already observed by Saad et al. (2004) for the analysis of fungal communities in biofilms on paint coatings, DNA yield and quality varies greatly depending on the complexity of the microbial community and the DNA extraction protocols employed, particularly when dealing with fungal spores instead of mycelia. Spore characteristics may affect the procedure of cell wall disruption, which then directly affects the final quality of DNA released (Vainio and Hantula, 2000). In contrast, the optimised commercial FastDNA Spin Kit for Soil protocol showed the best results for the DNA extraction from fungal strains, even those containing thick cell walls, as well as from spores (Kuske et al., 1998; Hughes et al., 2001). Furthermore, this method had the advantage of being less time-consuming and needing no extra purification step, resulting in a convenient method to extract fungal DNA from communities colonising paper material. Also, a small amount of sample (about 5–10 mg) produced an amplifiable amount of DNA, which fulfils the requirement that objects of cultural heritage not be further destroyed.

For PCR analysis, the purity of the extracted DNA was the main factor. Cellulose from native wood, used for paper production from the beginning of the 19th century, exhibits a lower degree of polymerisation (up to 10,000), while the one from native cotton can reach up to 15,000. In addition to cellulose fibres, paper can contain hemicellulose (wood polyoses), lignin, and additives such as fillers, pigments, and metal ions (Fellers et al., 1991). All these components can act as inhibitors of PCR reactions, and therefore their efficient removal contributes to optimal results in DNA extraction from paper materials, as well as for the PCR amplification of the extracted DNA. Our results showed that, depending on the DNA extraction method used, a further purification step could be needed (as per the protocol described by Schabereiter-Gurtner et al. (2001)) or not (as per the FastDNA Spin Kit for Soil) to obtain reproducible PCR products from the crude DNA extracts. Furthermore, it was possible to observe differences in the amplification of the extracted DNA depending on the paper type. The three paper types tested in this study were of different qualities, depending on their composition, coating, and pH value. Our results showed that fungal DNA extraction was more effective from paper type C

("Old mill" ivory from Fedrigoni Mills, Italy), an uncoated long-life type made of 100% cellulose fibres, than from paper type A (Whatman paper), or type B (Freelife vellum), which is also a long-life type but made of 80% recycled fibres, 15% cellulose chlorine free and 5% cotton fibres and of a more alkaline surface pH. It can be concluded that paper made from cellulose only leads to the best results in PCR amplifiable DNA due to its greater purity.

DGGE analysis carried out with amplified DNA extracted from pure fungal strains cultures, as well as from artificial and natural infected paper samples, revealed differences when targeting either the ITS1 or ITS2 region between the rRNA coding regions. When using the ITS2 region, multiple bands were visualised in the fingerprints derived from single fungal strains. This fact can lead to an overestimation of bands when working with unknown natural complex microbial communities. In contrast, the fingerprints obtained from the amplification of the ITS1 region showed unique bands for pure fungal strains displaying a one-fungi–one-band pattern in DGGE. Therefore, the amplification of this region was chosen for further DGGE analysis. Sequence variation within the ITS1 region is reported to be higher than in ITS2 for fungi such as *Aspergillus* and *Penicillium* (Gaskell et al., 1997).

Analysis of naturally infected paper samples corroborated the results obtained with paper samples artificially infected with pure fungal strains. Once more, the choice of DNA extraction had an influence on the resulting fungal community composition, as measured by DGGE analysis. Not only was the presence/absence of DNA bands affected, but their relative abundance in the total fingerprints was affected as well; this was also found by de Lipthay et al. (2004), Kozdroj and van Elsas, 2000, and Westergaard et al. (2001) for soil communities.

The molecular strategy described here also proved successful for the analysis of a 20-year-old paper. After this time, it is not expected to have any more viable material. This fact is also important because it allows the detection not only of viable fungi, but also of formerly active fungi, which could hence be responsible for the biodeterioration, as in the case of foxing spots. Florian and Manning (2000) proved that irregular fox spots in a 145-year-old book were caused by no longer viable fungal species, especially *E. amstelodami*, found randomly distributed on a page and throughout the book, and *Eurotium rubrum* and *Eurotium repens* in combination with ink, suggesting that irregular fox spots are an inherent feature of the book paper of the 19th century. Kirkpatrick et al. (1989) showed that modern paper can contain up to 10% of fungal hyphae because lignin digesting fungi are used in paper pulp for biological bleaching.

In conclusion, the bead-beating homogenisation of samples showed best results in fungal DNA extraction from paper materials of different compositions and ages. It rendered a pure DNA able to be directly amplified without the need for any extra purification step. By using this DNA extraction protocol, we expect to obtain the highest diversity of fungal sequences to be recovered from natural samples, as reported for bacteria (de Lipthay et al., 2004). However, the problem still remains that no single method of cell lysis is appropriate for all fungi potentially colonising paper-made art objects, and, therefore, the need for further modifications cannot be ruled out. The PCR amplification of the ITS1 region, followed by fingerprinting discrimination of fungal sequences in DGGE analysis is a reproducible and powerful technique for the visualisation not only of viable fungi, but also of formerly active fungi, which could have had an important role in paper biodeterioration processes. If the detection of only viable microorganisms is desirable, this could be overcome by using RNA assays. The molecular strategy presented here could be combined with the construction of clone libraries and sequencing of resulting clones to obtain a detailed phylogenetic identification of the individual members of the fungal communities present on paper.

Acknowledgements

This work and Astrid Michaelsen were supported by the Austrian Science Fund (FWF) within the framework of project P17328-B12. We are grateful to Mag. Andrea Steitz from the Holzforschung Austria for providing pure fungal material.

References

Abrusci, C., Martin-Gonzalez, A., Del Amo, A., Catalina, F., Collado, J., Platas, G., 2005. Isolation and identification of bacteria and fungi from cinematographic films. International Biodeterioration & Biodegradation 56, 58–68.

Anderson, I.C., Campbell, C.D., Prosser, J.I., 2003. Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. Applied and Environmental Microbiology 5, 36–47.

Arai, H., 2000. Foxing caused by fungi: twenty-five years of study. International Biodeterioration & Biodegradation 46, 181–188.

Cappitelli, F., Sorlini, C., 2005. From papyrus to compact disc: the microbial deterioration of documentary heritage. Critical Reviews in Microbiology 31, 1–10.

Corte, A.M., Ferroni, A., Salvo, V.S., 2003. Isolation of fungal species from test samples and maps damaged by foxing, and correlation between these species and the environment. International Biodeterioration & Biodegradation 51, 167–173.

Di Bonaventura, M.P., De Salle, R., Bonacum, J., Koestler, R.J., 2003a. Phylogenetic trees, fungal infections and the Tiffany's drawings. In: Saiz-Jimenez, C. (Ed.), Molecular Biology and Cultural Heritage. A.A. Balkema Pub., Lisse/Abindon/Exton (PA)/Tokyo, pp. 131–135.

Di Bonaventura, M.P., DeSalle, R., Eveleigh, D.E., Baldwin, A., Koestler, R.J., 2003b. Studies of fungal infestations of Tiffany's Drawings: limits and advantages of classical and molecular techniques. In: Koestler, R.J., Koestler, V.R., Charola, A.E., Nieto-Fernandez, F.E. (Eds.), Art, Biology, and Conservation: Biodeterioration of Works of Art. The Metropolitan Museum of Art. Yale UniversityPress, New York, New Haven, pp. 94–109.

van Elsas, D., Duarte, G.F., Keijzer-Wolters, A., Smit, E., 2000. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. Journal of Microbiological Methods 43, 133–151.

Fellers, G., Wegener, Papparstekik, 1991. Institutionen för papparstekik. Stockholm.

Florian, M.-L.E., Manning, L., 2000. SEM analysis of irregular fungal spots in an 1854 book: population dynamics and species identification. International Biodeterioration & Biodegradation 46, 205–220.

Gaskell, G.J., Carter, D.A., Britton, W.J., Tovey, E.R., Benyon, F.H., Lovborg, U., 1997. Analysis of the internal transcribed spacer regions of ribosomal DNA in common airborne allergenic fungi. Electrophoresis 18, 1567–1569.

Gonzalez, J.M., Saiz-Jimenez, C., 2005. Application of molecular nucleic acid-based techniques for the study of microbial communities in monuments and artworks. International Microbiology 8, 189–194.

Hawksworth, D.L., Rossman, A.Y., 1997. Where are all the undescribed fungi? Phytopathology 87, 888–891.

Hughes, K.W., Peterson, R.H., Johnson, J.E., Moncalvo, J.M., Vilgalys, R., Redhaed, S.A., Thomas, T., McGhee, L.L., 2001. Intragenic phylogeny of Collybia str. based on sequnces of ribosomal ITS and LSU regions. Mycological Research 105, 164–172.

Jasalavich, C.A., Ostrofsky, A., Jellison, J., 2000. detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. Applied and Environmental Microbiology 66, 4725–4734.

Kirkpatrick, N., Reid, I.D., Ziomek, E., Ho, C., Paice, M.G., 1989. Relationship between fungal biomass production and the brightening of hardwood kraft pulp by Coriolus versicolor. Applied and Environmental Microbiology 55, 1147–1152.

Kozdroj, J., van Elsas, J.D., 2000. Application of polymerase chain reaction-denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community fingerprinting. Biology and Fertility of Soils 31, 372–378.

Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K., Jackson, P.J., 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Applied and Environmental Microbiology 64, 2463–2472.

de Lipthay, J.R., Enzinger, C., Johnsen, K., Aamand, J., Sørensen, S., 2004. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. Soil Biology and Biochemistry 36, 1607–1614.

Lord, N.S., Kaplan, C.W., Shank, P., Kitts, C.L., Elrod, S.L., 2002. Assessment of fungal diversity using terminal restriction fragment pattern analysis: comparison of 18S and ITS ribosomal regions. FEMS Microbiology Ecology 42, 327–337.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis: analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Saad, D.S., Kinsey, G.C., Kim, S., Gaylarde, C.C., 2004. Extraction of genomic DNA from filamentous fungi in biofilms on water-based paint coatings. International Biodeterioration & Biodegradation 54, 99–103.

Saiz-Jimenez, C., 1993. Deposition of airborne organic pollutants on historic buildings. Atmospheric Environment 27B, 77–85.

Schabereiter-Gurtner, C., Piñar, G., Lubitz, W., Rölleke, S., 2001. An advanced strategy to identify bacterial communities on art objects. Journal of Microbiological Methods 45, 77–87.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J.D., Wernars, K., 1999. Analysis of fungal diversity in the wheat rhizophere by sequencing of cloned PCR-amplfied genes encoding 18S rRNA and temperature gradient gel electrophoresis. Applied and Environmental Microbiology 65, 2614–2621.

Szczepanowska, H., Cavaliere, A.R., 2000. Fungal deterioration of 18th and 19th century documents: a case study of the Tilghman Family Collection, Wye House, Easton, Maryland. International Biodeterioration & Biodegradation 46, 245–249.

Vainio, E.J., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycological Research 104, 927–936.

Westergaard, K., Müller, A.K., Christensen, S., Bloem, J., Sørensen, S.J., 2001. Effects of tylosin as a disturbance on the soil microbial community. Soil Biology and Biochemistry 33, 2061–2071.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

Zhou, J., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology 62, 316–322.