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

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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.

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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