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Original article

Isolation and attempts of biomolecular characterization of fungal strains associated to foxing on a 19th century book

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Abstract

The brownish spots known as foxing, commonly found on old paper artefacts contain sometimes structures, which look like filamentous fungi. Attempts to grow these structures *in vitro* have been always unsuccessful so far. In order to study the role of these biological elements in paper decay, it is essential to identify them. This study is aimed at the identification of isolates from these brownish areas by culture-independent approaches using molecular biology techniques. The two Internal Transcribed Spacers and the 5.8 S gene (ITS1-5.8S-ITS2) from the nuclear ribosomal DNA were amplified, cloned and sequenced. Following a preliminary treatment with cellulase from *Trichoderma reesei*, DNA extractions were successfully achieved directly from paper samples. From 22 selected stained spots from a book dating from the 19th century, 8 extracts of genomic DNA were entirely analysed, which yielded 145 sequenced clones. No DNA could be sampled in unstained areas. Multiple alignment of the ITS sequences and comparison with reference sequences published in the NCBI database allowed to identify 14 groups of fungi belonging mainly to the following genera: *Aspergillus*, *Bjerkandera*, *Chaetomium*, *Gloeotinia*, *Penicillium*, *Polyporus*, *Saccharicola*, *Trichoderma* and *Ulocladium*. Some of these fungi are cellulolytic species but are not commonly found as indoor contaminant in storage rooms of graphic documents. The majority of the foxing spots exhibited sequences identified as *Penicillium minioluteum*. *Gloeotinia temulenta* occurred also frequently. Few isolates such as *Aspergillus japonicus*, *Aspergillus oryzae*, *Chaetomium globosum*, *Penicillium citrinum*, *Trichoderma citrinoviride*, *Ulocladium chartarum* and *Ulocladium cucurbitae* were present in only 1 or 2 clones. One of the foxing spots produced only one type of sequence similar to both *Cordyceps sinensis* and *Fusarium lateritium*, which have identical sequences in this rDNA region. This molecular approach, simple and rapid, could provide additional data for further discussion on the origin of the phenomenon of biological foxing.

Keywords: Foxing; Fungi; Molecular characterization; Internal transcribed spacer; Cloning; Nucleotide sequencing

1. Introduction and research aims

Reddish-brown or yellowish-brown patches often occur on books and prints. They are common on paper artefacts from the 16th to the 19th century. However, it is known that even modern papers can exhibit this phenomenon [1,2]. These colored spots are called ''foxing'', as they appear to have the colour of fox fur. Foxing is a serious problem in paper conservation. Stains might migrate through successive pages, causing irreversible damage, which on the long-term would harm the documents' legibility. Furthermore, the number of stained areas increases with time and can ultimately cause severe degradation. Foxing spots are usually found in documents stored in damp and warm environment [1], however the real cause of the deterioration is not well understood despite the numerous studies devoted to it. There is a general consensus that the cause of the formation of foxing is either fungal or mineral or both. Mineral foxing results from the oxidation of metal impurities incorporated in the paper during its manufacture. Biological foxing is caused by the presence of mould that reacts with the paper in a slow process and can be associated with iron salts

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present in the paper. Several studies confirm the presence of fungal elements and spores by microscopic observations [3–5]. But it is difficult to determine whether mould is the causal agent of the degradation or if the mould growth is opportunistic in nature, taking advantage of the weakening of the paper due to chemical factors. Some studies were undertaken to improve the understanding of the chemical and physical processes occurring in foxing stains. The colour development has been explained according to two hypothesis: (1) the combination of fluorogenic and chromogenic compounds with the degradation product of cellulose such as free sugars and amino acids [6]; and (2) a succession of steps beginning with the appearance of the fluorogenic compound, followed by the chromogenic ones, which react with free ketones to give rise to the rust colour [7,8]. The intensity of the colour is not always associated with high concentrations of fungal elements [9]. Using different biochemical and spectrometric methods (IR, XRF, X-ray, etc.) some authors have demonstrated the oxidation of cellulose especially in the presence of iron ions [10,11]. More recent studies on optical reflectance spectroscopic properties of ancient paper have focused on different kinds of degradation including foxing. As a similar reflectance spectrum was observed by measuring foxing spots of various intensities on a single stained sheet, the authors demonstrated the importance of gelatine sizing in the paper discoloration. They also concluded that the paper discoloration and widespread foxing were produced by the same physical-chemical alteration on the paper material [12,13]. Others authors have observed that browning does not appear arbitrarily but often occurs in specific places like those soiled by residue from gelatine sizing for example [14]. Mass spectrometry and spatial resolution EDXS analysis confirmed the presence of iron and suggested that biological activity tends to take place in paper areas containing iron [15]. All these studies describe the nature and level of paper degradation but no mechanism of foxing formation is clearly explained. The exact role of filamentous fungi on foxing and their growth process is still unknown.

We have performed this study in order to gain a better understanding of the role of biological agents in paper discoloration. The first step was to characterize the species present in stained papers of a 19th century book. Some authors published works on the identification of the fungal strains isolated from the spots after culturing in solid media [1,2], but they did not reproduce the phenomenon from the isolates. Other authors report unsuccessful attempts in obtaining growth from swab samples and from direct culture of stained papers. Consequently, we decided to use culture-independent approaches for identifying the biological elements in foxing. Molecular biology techniques were used to carry out this research. The analyses were based on DNA sequences data from the specific ITS region (Internal Transcribed Spacer) of rDNA regions including the two spacer regions ITS1, ITS2 and the highly conserved 5.8 S ribosomal gene, which lies between the two spacers. These regions are widely used for the identification of organisms and for phylogeny studies because the nucleotide variability of ITS is adequate to distinguish species from different genera. Additionally, the high copy number of the rDNA gene allows amplification with very small amounts of DNA. Indeed, the microscopic observations of the stained spots showed that the number of fungal elements was often very weak.

This paper presents the first study on communities of strains within stained spots of old papers, based on data from ITS1-5.8 S-ITS2 rDNA PCR (polymerase chain reaction), cloning and sequencing after DNA extraction. The study was limited on a selection of spots from one page of a 19th century stained book.

2. Experimental section

2.1. Sampling

The samples were taken from a book of Dr Eduard Zeller entitled ‘‘Philosophie der Griechen, eine untersuchung’’, printed in Germany and published in 1852. Fibre analysis showed that the paper was made of linen and hemp. Storage conditions prior its arrival in the laboratory, were unknown. Since 1995, the book has been kept in the dark at a relative humidity of 40% and a temperature ranging from 20 to 32 °C according to season variations. The pages showed several foxing spots, at least 50 patches per page. For the present experiment, a page with a large quantity of brown spots was chosen (Fig. 1). The spots were numbered from 101 to 197 and 22 of them, from various locations in the page, were selected and sampled for analysis purposes. The observation under the optical microscope of the different spots showed clearly the presence of elements resembling to mycelia or/and spores. Some samples were also examined directly (no pre-treatment, no preparation) with JEOL JSM 5410 LV (low vacuum) scanning electron microscope (SEM). The 22 studied spots were named: FOX112, FOX113, FOX123, FOX129, FOX162, FOX165, FOX167, FOX170, FOX174, FOX175, FOX178, FOX179, FOX180, FOX181, FOX182, FOX183, FOX184, FOX185, FOX186, FOX187, FOX188 and FOX189. Six unstained areas from the same page were also submitted to analysis as control.

Before the DNA extraction, fungal elements were released from paper by treating the samples with cellulase from *Trichoderma reesei*, to digest the cellulose fibres. Each stained and unstained areas were cut out from the document and the pieces of paper were placed each in a 2 mL Eppendorf tube. A volume of 500 μL of cellulase (20 U/mL) was added and the samples were incubated overnight at 37 °C.

2.2. DNA extraction

Genomic DNA was extracted using CTAB (cethyltrimethylammonium bromide) micropreparation method. Briefly, 400 μL of cold lysis buffer (with sodium dodecylsulfate at 30%) were added to the samples and crushed with a plastic micropestle. Five microliter of proteinase K 800 U/mL (MBI Fermentas, France) was added immediately and the mixture was incubated at 60 °C for 30 min. The extraction was continued by addition of 112 μL of 5 M NaCl and 102 μL of 10% CTAB and vortexed. After an incubation period of 10 min at