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Short communication

# Fungal diversity in ancient documents. A case study on the Archive of the University of Coimbra

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ABSTRACT

This multidisciplinary research combines knowledge in molecular biology with fungal morphology, aiming at the identification of infecting fungi from historical documents on the Archive of the University of Coimbra. The identification of infecting fungi on several bibliographic documents and support materials was based both on ribosomal DNA loci amplification and sequencing, and morphological identification, using macro- and microscopical traits. A high fungal diversity was found in all types of support: parchment, laid-paper and wood-pulp paper. Fourteen fungal genera were isolated, identified, and kept in culture. The most frequent were *Cladosporium*, *Penicillium* and *Aspergillus*, and other less frequent genera, such as *Alternaria*, *Botrytis*, *Chaetomium*, *Chromelosporium*, *Epicoccum*, *Phlebiopsys* and *Toxicocladosporium* were also present. Within these genera, 20 different species were identified, from which 15 were found only in a single support type. *Cladosporium cladosporioides* and *Penicillium chrysogenum* were the only species present in all support types.



## 1. Introduction

The Archive of the University of Coimbra holds a vast documentation asset produced and received by the University, essential for the analysis of the history of the University of Coimbra since its foundation by King D. Dinis in 1290, but mainly since its definitive establishment in the city of Coimbra in 1537. The oldest document in the Archive is the parchment of "Colegiada de Guimarães", dated from 983, prior to the foundation of Portugal. The past preservation and conservation conditions of some of the Archive collection were not adequate and the documents are now susceptible to biodegradation.

Library documents are generally composites of different materials (natural, semi-synthetic or synthetic compounds), each with different possible responses to environmental changes. This is a setback in understanding the biodegradation processes (Harvey, 1992). The biodeterioration of organic materials is very important as a recycling process, however, in some cases, this process also destroys historical records, resulting in the loss of valuable information (Cappitelli and Sorlini, 2005).

Microorganisms are important biodegrading agents. The presence of spores or vegetative cells on the surface of documents may indicate a possible degradation in the future. Fungi are considered as serious degrading agents of bibliographic documents, particularly cellulolytic fungi (Fabbri et al., 1997). One of the major concerns regarding fungal colonization is the change in the document aesthetics, either by discoloration by weak acids produced by fungi, or by the accumulation of pigments that may stain its support in a phenomenon referred to as foxing (Arai, 2000). Some foxing stains occur as reddish and/or brownish marks on the paper support, and are thought to have both biotic and abiotic origins, from the metabolic activity of microorganisms, to metal or ink oxidation (Meynell and Newsam, 1978; Arai, 2000). However, the majority of foxing stains are supposedly due to the effect of microorganisms, or their metabolites (Montemarini-Corte et al., 2003). Foxing may occur on other non-cellulosic materials, as long as an optimum environment for "foxing-causing fungi" is present, because cellulose is not always an essential nutrient for fungal growth (Arai, 2000).

Some fungi involved in the deterioration of library material may be dangerous to library professionals and users, due to the production of mycotoxins. They enter the body via inhalation of toxicogenic spores and direct dermal contact, and can cause several diseases (Bennet and Kilch, 2003) from which, airway infections, mycosis, immune system issues, and asthma are examples (Nielsen,

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2003). Zyska (1997) reported 84 genera and 234 species of filamentous fungi isolated from library material (different supports), 19% of which, could be a source of different health effects.

Our main goal was to assess the diversity of infecting fungi, in documents from the Archive of the University of Coimbra. Thirty documents, made of three different types of support (parchment, laid-paper and wood-pulp paper) were analyzed. Parchment, made of treated animal leather, was sampled from a 13th century document and from the parchment cover of an 18th century book. The second support type, laid-paper, is a hand-made paper made of cotton, hemp and linen fibers, and was sampled from 12 different sources, from the 16th to 18th century. Wood-pulp paper, industrially produced from wood-pulp processing, was sampled from 6 documents from the 19th and 20th centuries.

For routine use, the molecular approach gives more reliable results than traditional morphological analysis (Scharbereiter-Gurtner et al., 2001). Therefore the identification of infecting fungi was assessed by fungal genomic DNA analysis, using PCR (Polymerase Chain Reaction) and DNA sequencing, complemented with microscopy and other morphological analysis techniques.

## 2. Materials and methods

### 2.1. Sample isolation and culture

Several contaminated documents, made of different materials were initially selected from the Archive of the University of Coimbra, according to biodeterioration signs, such as paper colouring/discolouring, presence of microfungal structures or any other observable texture changes. Only areas without any text, or close to the book bindings were sampled using a scalpel to scratch the paper surface or to remove a small portion, whenever biodegradation symptoms were observed. Around 150 small samples (max. 0.5 $cm^2$) were retrieved from 30 different documents, and stored in sterile Petri dishes until further processing.

The sample's texture, colouring and surface pigmentation were quite diverse: white, pink, purple, brown, black and green (among others) were found, and this diversity suggested different origins for the observed deterioration. All sample manipulations were made aseptically with previously sterilized material, in order to prevent cross contaminations.

Paper fragments were then incubated between 25 °C and 28 °C, on several culture media, such as malt extract agar (MEA) and potato dextrose agar (PDA), with streptomycin (0.5 g/L) to prevent bacterial growth.

### 2.2. Molecular and morphological identification

After culturing the samples in the different mediums, colony DNA extraction was performed, using a commercial kit (Nucleon Phytopure Plant DNA Kit, Amersham Pharmacia Biotech). A PCR was performed to amplify the total ITS region of all DNA samples, using primers ITS 4 and ITS 5 (White et al., 1990). The reaction components for the PCR were 5 μl Taq (DNA polymerase) buffer 10× concentrated (Pharmacia Biotech, USA), 1 μl of 10 mM dNTPs (deoxynucleotides) (Pharmacia Biotech, USA), 2 μl of each primer (10 mM) (Pharmacia Biotech, Sweden), 1.5 μl of 50 mM $MgCl_2$ (Sigma, St. Louis, USA), 2 U (enzymatic units) of *Taq* Polymerase (Pharmacia Biotech, Sweden) and 1 μl of template DNA, combined in a final volume of 50 μl for each amplification.

The PCR reactions were done in a Applied Biosystems 9700 Thermocycler (Norwalk, USA), according to the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 51–53 °C for 1 min, extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Negative controls without DNA template were prepared in all amplifications. The PCR products were separated by 1.2% agarose gel electrophoresis (Pharmacia Biotech, Uppsala, Sweden), stained with ethidium bromide (Sigma, St. Louis, USA) and photographed under UV-light on a fluorescent table (Vilber Lourmat, Marne La Valée, France) to confirm the amplified fragment size.

The PCR products were purified and directly sequenced using an ABI 3730 Genetic Analyzer, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), using the primer ITS4. Sequences were analyzed using Geneious software (www.geneious.com), and were ran against NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences, belonging to identified fungal species. The sequences obtained in this study were deposited in GenBank under accession numbers FJ791124–FJ791161 (see Tables 1–3).

To confirm these identifications, upon colony growth and fruiting bodies emergence (if visible), the different cultures were identified to the genus level, according to their macro-morphology and micro-morphology (Barnett and Hunter, 1998; Watanabe, 2002) and, whenever possible, to the species level. This was performed using optical microscope observation with Lactophenol Cotton Blue (Sigma) as staining solution. Different culturing methods were used, from the direct observation of small colony samples in microscope slides to the use of Riddell's chamber (Riddell, 1950) and other variants of this method (Kawato and Shinobu, 1960; Nugent et al., 2006).

## 3. Results

The first step for fungal identification was the sequencing of the total ITS region, followed by genetic alignments with the NCBI's GenBank database to determine the fungal species. Determined sequence similarities ranged from 95.5% to 100.0% (Tables 1–3). This identification was subsequently confirmed by morphological examination of the isolates.

According to both methodologies, 20 species and 14 genera were isolated and identified from the various sample sources. The results of the identification are presented in Tables 1–3, for the three different support types. Overall, all support types presented a high diversity of species. The most common species were

**Table 1**
Fungi isolated from ancient parchment documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate. The * refers to isolates that have only been identified using the molecular approach.

| ID | Original document | Century | Isolated fungi | Accession number | Similarity (%) |
|---|---|---|---|---|---|
| 32 | *Register book of Jorge Botelho, private notary of the Cárquere Monastery* | 18th (1755–1761) | *Phlebiopsis gigantea* | FJ791151 | 96.7 |
| 33 | *Register book of Jorge Botelho, private notary of the Cárquere Monastery* | 18th (1755–1761) | *Penicillium chrysogenum* | FJ791152 | 99.8 |
| 34 | *Register book of Jorge Botelho, private notary of the Cárquere Monastery* | 18th (1755–1761) | *Cladosporium cladosporioides* | FJ791153 | 100.0 |
| 35 | *Register book of Jorge Botelho, private notary of the Cárquere Monastery* | 18th (1755–1761) | *Cladosporium cladosporioides* | FJ791154 | 99.8 |
| 36 | *Registry book of councils from the Santa Cruz Monastery (Coimbra)* | 13th to 14th | *Thanatephorus cucumeris** | FJ791155 | 99.8 |
| 37 | *Registry book of councils from the Santa Cruz Monastery (Coimbra)* | 13th to 14th | *Epicoccum nigrum* | FJ791156 | 99.8 |

**Table 2**
Fungi isolated from ancient laid-paper documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate (omitted ID numbers correspond to isolates that were lost previous to DNA extraction). The * refers to isolates that have only been identified using the molecular approach.

| ID | Original document | Century | Isolated fungi | Accession number | Similarity (%) |
|---|---|---|---|---|---|
| 2 | *Ordination process of João Correia, from Espinhal* | 17th (1690) | *Alternaria alternata* | FJ791124 | 99.5 |
| 3 | *Postil of teachers from the Faculty of Law* | 17th | *Cladosporium cladosporioides* | FJ791125 | 100.0 |
| 4 | *Postil of teachers from the Faculty of Law* | 17th | *Cladosporium cladosporioides* | FJ791126 | 99.8 |
| 5 | *Ordination process of João Correia, from Santo Varão* | 18th (1787) | *Penicillium chrysogenum* | FJ791127 | 100.0 |
| 6 | *Postil of teachers from the Faculty of Law* | 17th (1620) | *Cladosporium cladosporioides* | FJ791128 | 100.0 |
| 8 | *Postil of teachers from the Faculty of Law* | 17th (1620) | *Skeletocutis* sp.* | FJ791129 | 99.0 |
| 9 | *Ordination process of João Correia, from Espinhal* | 17th (1690) | *Penicillium* sp.* | FJ791130 | 97.4 |
| 10 | *Judicial sentence (copy)* | 16th (1574) | *Penicillium helicum* | FJ791131 | 99.2 |
| 11 | *Postil of teachers from the Faculty of Law* | 17th | *Coprinus* sp.* | FJ791132 | 99.3 |
| 12 | *Postil of teachers from the Faculty of Law* | 16th to 17th | *Cladosporium cladosporioides* | FJ791133 | 100.0 |
| 13 | *Ordination process of João Correia, from Espinhal* | 17th (1690) | *Phlebia subserialis* | FJ791134 | 95.5 |
| 14 | *Postil of teachers from the Faculty of Law* | 17th (1620) | *Toxicocladosporium irritans** | FJ791135 | 99.2 |
| 15 | *Judicial sentence (copy)* | 16th (1574) | *Cladosporium cladosporioides* | FJ791136 | 100.0 |
| 16 | *Registry of accounts from the University of Coimbra* | 17th (1650) | *Penicillium chrysogenum* | FJ791137 | 100.0 |
| 17 | *Royal Hospital of Coimbra – registry of rents* | 18th (1757) | *Cladosporium cladosporioides* | FJ791138 | 100.0 |
| 25 | *Postil of teachers from the Faculty of Law* | 17th | *Aspergillus nidulans* | FJ791144 | 99.3 |
| 26 | *Judicial process from the Diocesan court of Coimbra* | 18th (1725) | *Chaetomium globosum* | FJ791145 | 97.0 |
| 27 | *Judicial process from the Diocesan court of Coimbra* | 18th (1725) | *Chaetomium globosum* | FJ791146 | 99.7 |
| 31 | *Ordination process of João Correia, from Condeixa-a-Nova* | 18th (1779) | *Penicillium chrysogenum* | FJ791150 | 100.0 |
| 39 | *Ordination process of João Correia, from Espinhal* | 17th (1690) | *Botrytis cinerea* | FJ791158 | 99.8 |
| 42 | *Postil of teachers from the Faculty of Law* | 17th | *Chromelosporium carneum* | FJ791160 | 99.6 |

*Cladosporium cladosporioides* and *Penicillium chrysogenum*, however other less common species, like *Chromelosporium carneum* and *Toxicocladosporium irritans* were also isolated from these materials (Table 4).

In the laid-paper samples, *C. cladosporioides* was the most recurrent species. The most frequent genera were *Cladosporium*, and *Penicillium*, in almost half the samples. However, less common species such as *T. irritans* and *C. carneum* were found. In wood-pulp paper, *C. carneum*, *Aspergillus versicolor* and *P. chrysogenum* were the most frequent species, and the less common *Chaetomium globosum* was also isolated. Regarding parchment, *C. cladosporioides* was the most common, and *Epicoccum nigrum*, *Thanatephorus cucumeris* and *Phlebiopsis gigantea* were found exclusively in this type of support (Table 4).

## 4. Discussion

The ITS region has been frequently used, and with good results, for the identification and inventory of fungal organisms that contaminate documents and art objects (*e.g.* Michaelsen et al., 2006 or Rakotonirainy et al., 2007). The sequences we obtained from the total ITS region, together with the morphological analysis, allowed the identification of most isolates. These are the first results on the identification of Archive fungi reported in Portugal.

Considering the number of samples, a great variety of species have been isolated and identified. Some of the species were only found in one type of support, which may be explained by production of different enzymes by the various organisms. As example, the cellulase enzyme complex is responsible for the degradation of cellulose from paper fibers (Deacon, 1997). In parchment and leather, keratin is the most abundant structural protein together with collagen and proteases like keratinases and collagenases are responsible for its degradation (Popescu et al., 2008).

Regarding parchment, the majority of the species found were already reported in this support (Zyska, 1997), with *C. cladosporioides* being the most abundant. *E. nigrum* and *T. cucumeris* appeared in parchment samples only. Strzelczyk et al. (1989) state *C. globosum* to be a very active organism in the decay of leather from book bindings, and Sharma and Sharma (1979) described the presence of *Alternaria alternata* in finished leather, but we didn't find these species in this support.

*C. globosum* and *C. carneum* were found in both wood-pulp paper and laid-paper, while some other species were only found in one support type (see Table 4), like *A. alternata* and *Toxicocladosporium*, only found in laid-paper, and *Penicillium canescens*

**Table 3**
Fungi isolated from ancient wood-pulp paper documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate (omitted ID numbers correspond to isolates that were lost previous to DNA extraction).

| ID | Original document | Century | Isolated fungi | Accession number | Similarity (%) |
|---|---|---|---|---|---|
| 19 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Penicillium* sp. | FJ791139 | 100.0 |
| 20 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Cladosporium cladosporioides* | FJ791140 | 100.0 |
| 21 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Penicillium canescens* | FJ791141 | 100.0 |
| 22 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Penicillium chrysogenum* | FJ791142 | 100.0 |
| 23 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Aspergillus fumigatus* | FJ791143 | 100.0 |
| 28 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Chromelosporium carneum* | FJ791147 | 99.6 |
| 29 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Chromelosporium carneum* | FJ791148 | 99.6 |
| 30 | *Letter from Francisco Gomes de Almeida Branquinho, Secretary of the Civil Governor of Coimbra* | 19th (1860) | *Aspergillus versicolor* | FJ791149 | 99.8 |
| 38 | *Petitions to the Rector of the University of Coimbra* | 20th (1937–1939) | *Penicillium chrysogenum* | FJ791157 | 100.0 |
| 40 | *Correspondence of the Coimbra University Rectorat* | 20th (1936) | *Chaetomium globosum* | FJ791159 | 100.0 |
| 44 | *Letter from Francisco Gomes de Almeida Branquinho, Secretary of the Civil Governor of Coimbra* | 19th (1860) | *Aspergillus versicolor* | FJ791161 | 98.6 |

**Table 4**
Frequency table for the isolated fungal species in all support types. The * refers to isolates that have only been identified using the molecular approach.

| Species | Parchment | Laid-paper | Wood-pulp paper | Total |
|---|---|---|---|---|
| *Cladosporium cladosporioides* | 6 | 1 | 2 | 9 |
| *Penicillium chrysogenum* | 3 | 2 | 1 | 6 |
| *Chaetomium globosum* | 2 | 1 | – | 3 |
| *Chromelosporium carneum* | 1 | 2 | – | 3 |
| *Aspergillus versicolor* | – | 2 | – | 2 |
| *Alternaria alternata* | 1 | – | – | 1 |
| *Aspergillus fumigatus* | – | 1 | – | 1 |
| *Aspergillus nidulans* | 1 | – | – | 1 |
| *Botrytis cinerea* | 1 | – | – | 1 |
| *Coprinus* sp.* | 1 | – | – | 1 |
| *Epicoccum nigrum* | – | – | 1 | 1 |
| *Penicillium canescens* | – | 1 | – | 1 |
| *Penicillium helicum* | 1 | – | – | 1 |
| *Penicillium* sp.* | 1 | – | – | 1 |
| *Penicillium* sp.* | – | 1 | – | 1 |
| *Phlebia subserialis* | 1 | – | – | 1 |
| *Phlebiopsis gigantea* | – | – | 1 | 1 |
| *Skeletocutis* sp.* | 1 | – | – | 1 |
| *Thanatephorus cucumeris* | – | – | 1 | 1 |
| *Toxicocladosporium irritans** | 1 | – | – | 1 |

and *A. versicolor*, only found in wood-pulp paper. Less frequent genera such as *Botrytis*, *Chaetomium*, *Chromelosporium*, *Epicoccum* and *Phlebiopsys* have also been described in different supports and in various countries (Zyska, 1997; Szczepanowska and Cavaliere, 2000; Montemarini-Corte et al., 2003).

Overall, the high frequency of *C. cladosporioides*, *Aspergillus* spp. and *Penicillium* spp. is in agreement with other works (Zyska, 1997; Hyvärinen et al., 2002; Montemarini-Corte et al., 2003; Nielsen, 2003; Da Silva et al., 2006). These are almost ubiquitous taxa, and can produce numerous mitospores and conidia that are easily dispersed by air (Abrusci et al., 2005). However, for a parallel research, we sampled the air from the Archive, to test if air dispersion of airborne fungal spores or propagules occurs. The first analysis showed little diversity, with *Aspergillus fumigatus* clearly being the most frequent (data not shown), and in the current work, it was found in only one wood-pulp sample. At least in this case, the fungal diversity found in the documents is probably due to other reasons, such as the storing conditions these documents were subjected to in the past.

Apart from the degradation of the library material, most of these organisms (*e.g. Penicillium*, *Aspergillus* and *Alternaria* spp.) can also cause adverse human health effects in both Archive workers and users. A good example of this, found in one of the laid-paper samples is *T. irritans*, that produces ample amounts of volatile metabolites, which cause a skin rash within minutes of opening an inoculated dish for microscopic examination (Crous et al., 2007). It is important for library workers and users to be aware of this problem so that adequate care is taken when handling ancient documents.

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