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Diversity of fungal species in ancient parchments collections of the Archive of the University of Coimbra

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ABSTRACT

In order to study the fungal diversity in different parchment collections from the "Arquivo da Universidade de Coimbra", eighty different documents, belonging to five different collections, were screened for the presence of fungal species. Molecular methods complemented with morphological identification were applied to identify all fungal organisms. In total, 230 isolates, belonging to 22 different genera and 42 different species, were obtained. The most common genera were *Alternaria*, *Cladosporium*, *Epicoccum* and *Penicillium* and the most frequent species were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *Penicillium glabrum* and *Penicillium spinulosum*. Shannon–Wiener index was calculated for fungal diversity, presenting a low species diversity in all parchments collections. Also a Linear Model Regression analysis was calculated between the age of the documents, the number of species and number of isolates, confirming that time is significantly associated with species diversity; older collections generally presented a higher number of fungal isolates.

1. Introduction

Ancient written documents made of parchment were an important communication mean to mankind and have, for that reason, a priceless historical value to our culture heritage. These were the main writing material until the middle ages, but since the advent of paper, parchment has mainly been used for distinguished purposes (Pinzari et al., 2012).

Parchment is made of treated animal skin with collagen as its main component. Collagen is susceptible to deterioration by several microorganisms, serving both as energy and carbon source (Troiano et al., 2014). Fungi have a particularly relevant role in the biodeterioration of parchment as well as other archive materials (Cappitelli and Sorlini, 2005; Sterflinger, 2010). As result of this biodeterioration, parchment loses some of its original properties, becoming deformed, with possible occurrence of white films, text fading and spots (Tiano, 2002). This last phenomenon, usually referred as "foxing", occurs because of the enzymatic activity of fungi in the substrate digestion process, producing different coloured spots (brown, dark or reddish). Along with this chemical action, fungal hyphae can also penetrate the fibre structures of parchment and induce mechanical damage in the document (Arai, 2000; Szczepanowska and Cavaliere, 2000; Montemarini-Corte et al., 2003; Sterflinger and Pinzary, 2012). For this reason, the identification of fungal species in Archives and Library items (including parchment documents) has been crucial to determine the capability of fungi to damage these items, but also the environmental conditions required for their establishment in the support (Mesquita et al., 2009; Sterflinger and Pinzary, 2012), which makes biodeterioration one of the main problems in document preservation (Guiamet et al., 2011).

The Archive of the University of Coimbra (AUC) holds a wide asset of documents either produced or received by the university since its founding by King D. Dinis in 1290, documents that reflect the history of the university in a unique way (Vasconcelos, 1991). The AUC is a modern construction, built in the 1940's, and its staff works to prevent the deterioration of this valuable written heritage (Nunes et al., 2013). The different materials used in these documents enhance the likelihood of different ecological niches combined with the propitious conditions for the development of fungi

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(Hyvärinen et al., 2002). The AUC hosts a great collection of parchment documents that is already facing biodeterioration effects (Mesquita et al., 2009).

The use of molecular techniques in the identification of biodeterioration-related fungal species in documents and other culture heritage items is important to the understanding of fungal infection and its further consequences for the material (Sterflinger, 2010). The sequencing of the total ITS region is a still a very strong molecular tool for fungal species identification (Martin and Rygiewicz, 2005; Michaelsen et al., 2006; Schoch et al., 2012).

The research on the deterioration of parchment by surface-associated microorganisms is still limited to very few works (Kraková et al., 2012; Pinzari et al., 2012; Troiano et al., 2014; Piñar et al., 2015a,b). Understanding the cultural relevance of parchment, concerning the biodeterioration of most of these documents and taking into account the previous works done by Mesquita et al. (2009), Trovão et al. (2013) and Nunes et al. (2013) in the AUC, our main objective was to study the fungal diversity in different parchment collections stored within this site and to demonstrate that time has an important role in the fungal colonization and deterioration of documents. For this purpose, eighty documents, belonging to five collections from five different sources were object of analysis, regarding the presence of contaminant fungal organisms. The analysed documents belong to the collections of: "Colegeada de Guimarães", "Mosteiro dos Pedrosos", "Cabido da Sé Velha", "S. João dos Longos Vales" and the other from the University of Coimbra itself. The referred collections date from different centuries (i.e. X to XIX) and in that way represent different timestamps from our history, the oldest belong to the "Colegeada de Guimarães" (century X), prior to the foundation of Portugal. From contracts to diplomas, a really impressive variety can be found within these stored materials. To our knowledge, this is the first study on a broad range of parchment documents, where nearly eighty different documents were screened for fungal species, as biodeterioration promoters.

2. Materials and methods

2.1. Sampling

The samples were retrieved from eighty parchment documents, belonging to five different collections, that were deposited in the AUC (Table 1) and some examples are presented in Fig. 1. Biodeterioration symptoms on parchment, such as spots or areas with fading text, were selected as targets for analysis.

Two sampling methods were applied to each document: in the written parts of the documents, sterile cotton swabs were used to collect fungal samples whereas in the borders of the documents, samples were collected with the use of adhesive tape. After this, samples were isolated in Malt Extract Agar (MEA, Difco™) with streptomycin (0.5 g/L) to prevent bacterial growth. The different colonies were isolated to axenic cultures and incubated for five days at 28 ± 1 °C for further molecular and morphological analysis.

2.2. Molecular and morphological identification

When the colonies reached enough mass for DNA extraction, mycelia were scraped from the agar surfaces using sterile scalpels. Collected material was subjected to total DNA extraction using an ABI Prism™ 6100 Nucleic Acid PrepStation, according to the manufacturer's standards.

After extraction, the ITS region was amplified by PCR, using primers ITS4 and ITS1F (White et al., 1990; Gardes and Bruns, 1996). For that purpose, PCR mixes were prepared with 12.5 μl of Jump Start Taq DNA Polymerase with $MgCl_2$ (Sigma D9307), 0.5 μl of each primer (10 mM), 10.5 μl of ultra-pure water, and 1 μl of template DNA, for a final reaction volume of 25 μl.

The PCR reactions were performed using an ABI GeneAmp PCR System 9700, with the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Visual confirmation of the overall amplification of the ITS region was performed using agarose gel electrophoresis (1.2%) stained with Gel Red (Biotium) and photographed in an image capture device (Bio Rad Gel Doc XR™).

Amplification products were sequenced using an ABI 3730 genetic analyser, with the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were analysed and ran in NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. For similarity values higher than 95%, the molecular identification was considered a valid match, although thoroughly confirmed by morphological traits according to Watanabe (2002) and Seifert et al. (2011). Sequences published in this study were deposited in GenBank under the accession numbers described in Table 1.

2.3. Statistical analysis

Statistical analysis was performed calculating the Shannon–Wiener index ($H' = -\sum Pi \ln (Pi)$) and the species evenness ($E = H'/\ln (S)$), for each collection of documents. The Shannon–Wiener index is used to assess diversity in categorical data. It analyzes entropy, treating the species distribution and the size of a population as a probability, and is used to determine biodiversity values taking into account the number of species, the dominant species, and their distribution (Frosini, 2006). The Shannon–Wiener index values have to be within the range of ($0 < H < \ln (S)$) and species evenness between average values of ($0 < E < 1$).

A Linear Model Regression Analysis was also calculated using Past 3.0 software. The goal of the regression model was to determine the relation between the age of each document and the number of species, but also between the date of the documents and the number of isolates.

3. Results

Table 1 displays the identification results for the isolated fungal species retrieved from the sampled parchment documents from all six collections as well as the corresponding GenBank accession numbers and the similarity values to the sequences deposited in NCBI databases. A total of 230 fungal isolates, corresponding to 22 different genera and 42 different species were obtained, identified and kept in culture (Fig. 2). In documents 23 and 29 from "S. João dos Longos Vales" and in documents 63, 66, 67, 68 and 71 of the "Universidade de Coimbra" collection, no fungal isolates were obtained, even after repeated attempts.

Overall, the most common genera were *Alternaria*, *Cladosporium*, *Epicoccum* and *Penicillium*. The most frequent species overall were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *Penicillium glabrum* and *Penicillium spinulosum* (Fig. 2). The most frequent species in the "Mosteiro de Pedrosos" collection were *A. alternata*, *C. cladosporioides* and *E. nigrum*. The species *Fusarium oxysporum*, *Nigrospora oryzae*, *Nigrospora sphaerica*, *Phanerochaete sordida* and *Phlebiopsis gigantea* were isolated from this collection exclusively (Fig. 2-A). In "Colegeada de Guimarães" the most frequent species were *A. alternata*, *C. cladosporioides*, *P. glabrum* and *P. spinulosum*. *Aspergillus versicolor* and *Penicillium oxalicum* were isolated from this collection only (Fig. 2-B). The most abundant species in the