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Original paper

Molecular characterization based on** Internal Transcribed Spacer (ITS) **marker sequence of fungal strains isolated from heritage ethnographic textiles

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Abstract

Fungi play an important role in the biotransformation process in nature. Through their rich enzymatic equipment there are able to colonize and degrade a broad range of substrates, inorganic and organic. Though positive in most economical areas, their ecological behavior is harmfull for heritage goods, especially made of organic polymers. Heritage textiles are a special field where the action of microfungi can be highly damaging, since most of the textiles collections worldwide are made of natural fibres, such as hemp, flax, cotton, wool or silk. In the present study 16 strains of filamentous fungi were isolated from different heritage ethnographical textiles, part of the collection of The National Museum of Romanian Peasant. After the phenotypical culture-based characterization, a molecular approach was developed and applied in order to identify the genetic fingerprint of each strain. The molecular characterization was done based on the ITS (*Internal Transcribed Spacer*) marker, since in the last decade it is the widely used sequence for taxonomy and molecular phylogeny for fungi and other taxa. Our results showed a predominance of *Penicillium* species and also *Alternaria* sp. and *Cladosporium* sp. Molecular methods combined with classical methods for species identification give complementary data and an accurate image of the fungal biodeterioration process on heritage textiles.

Keywords Internal Transcribed Spacer (ITS), DNA extraction, PCR-ITS, Heritage textiles, Ethnographical textiles, *Penicillium* sp.

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Introduction

The microorganisms consider our cultural heritage nothing more than a simple substrate. Microbiota degrading heritage textile goods can represent a serious problem for art collections (CAPODICASA & al [1]; STERFLINGER [2]; LÓPEZ-MIRAS & al [3]; DYDA & al [4]). Textiles made from natural fibres are generally more susceptible to biodeterioration than are the synthetic fibres (PAUL [5]). This is because their porous hydrophilic structure retains water, oxygen and nutrients, providing perfect environments for microorganisms growth. Products such as starch, protein derivatives, fats and oils used in finishing of textiles can also promote microbial growth (BORYO [6]). Microorganisms may attack the entire substrate, that is the textiles fibres or may attack only one components of the substrate, such as plasticizer contained there in, or grow on dirt that has accumulated on the surface of a product. Nevertheless, even mild surface growth can make a fabric look unattractive by the appearance of unwanted pigmentation. Heavy infestation which results in rotting and breakdown of the fibres and subsequent physical changes such as loss of strength or flexibility may cause the fabric to fail in service. The material is chemically attacked by the action of extracellular enzymes produced by the microorganisms for the purpose of obtaining food. Plants fibres such as cotton, flax (*Linum usitatissimum*), jute and hemp (*Canabis sativa*) are very susceptible to attack by cellulolytic (cellulose digesting) fungi. Indeed, the complete degradation of cellulose can be effected by enzymes, produced by the fungi and known as cellulases (BORYO [6]).

Several microbiological and molecular techniques and strategies were developed for the investigation of such 'art-deteriorating' microbial communities (PIÑAR & al [7]; MONTANARI & al [8]). A culture-dependent approach usually included the cultivation of filamentous fungi on agar plates and their identification by the traditional microscopic observation (SIMONOVICOVA & al [9]) or by the amplification of a ribosomal DNA target, such as 18S rDNA, 28S rDNA or *Internal Transcribed Spacer* (PANGALLO & al [10]; SHARMA & al [11]; MESQUITA & al [12]).

Our aim wasthe phenotypical and molecular characterization of cultivated microfungi isolated from the surfaces of textile goods of Cultural Heritage within the Romanian Peasant Museum in Bucharest. The molecular approach gives us more reliable results than traditional morphological analysis. Therefore the identification of fungi was assessed by sequencing the total ITS region followed by genetic alignments with the NCBI GenBank database to determine the fungal species.

Materials and Methods

1. The isolation methods of filamentous microfungi from the surface textile cultural goods made of natural fibers

Sample collection and isolation of fungi for this study: sterile cotton swabs were used to collect the samples from the surface of different textile cultural goods made of natural fibres in the National Museum of the Romanian Peasant in Bucharest, Romania. After carefully buffing the textile surfaces, the swab was seeded on solid media or immersed in a sterile liquid medium. Petri dishes seeded with samples taken from the surface of heritage textiles and liquid media tubes were transported to the laboratory and incubated at 28°C ± 2°C for 14-21 days. Subsequently, each microfungi colony grown on a Petri plate was individually transferred to a new plate with solid culture medium containing Sabouraud Dextrose Agar (SDA); Gélose Sabouraud Glucosée Chloramphénicol (SDC); Sabouraud liquid broth (SAB B-T); PDA (Potato Dextrose Agar); Czapek-Dox; Yeast Peptone Glucose Extract (YPG) and incubated under the same conditions in order to highlight morphological and growth characteristics. The microfungal isolates obtained (n=16) were stored in the refrigerator at 4°C for phenotypic characterization. The developed fungal isolates were isolated in pure cultures for further identification to the species level.

2. Molecular and morphological identification

After culturing the samples in the different liquid culture medium, the DNA was extracted from the fungal mycelium, using two commercial kits: REDExtract-N-AmpTM Plant PCR Kit (Sigma-Aldrich) and FastDNATM SPIN Kit for Soil (MP Biomedicals).

The DNA extraction using the first kit was done directly from the mycelium, following the protocol steps, without any other preliminary phase. The extraction using the second kit was done after two preliminary treatments: the mycelium was grownded in liquid nitrogen and the homogenized in FastPrep-24TM 5G (MP, Biomedicals, USA), in one cycle at 6.0 m/sec. for 40 seconds. DNA integrity was also checked by 1% agarose gel-electrophoresis.

A PCR was performed to amplify the total ITS region of all DNA samples, using primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (WHITE & al [13]), using a Thermal Cycler machine Corbett (Table 1, 2). The amplification products of simplex PCR reaction were visualized by electrophoresis on a 1% agarose gel, stained with ethidium bromide (10 µg/ml) and identified based on their size using specific molecular weight markers (100 bp, Mid Range DNA Ladder).

Table 1. Reaction components used in the PCR reactions

Concentration						Final volume
primer	$MgCl_2$	dNTP	DNA Taq-pol	Reaction buffer	DNA	
0,5 µM	1,2 mM	2 µM	0,2 U	1x	10x	20 µl

Table. 2. Amplification programs of PCR reactions

The gene and the size of the amplicon	Amplification program				
	Denaturation	No. of cycles	Initial denaturation	Anealing	Extension
ITS=600bp	95°C for 5 min	35	95°C, 1 min	55°C, 60 sec	72°C, 8 min

The PCR products were directly sequenced by Dexter Laboratory using both primers. Sequences were analyzed using NCBI BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences, belonging to identified fungal species.

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 & HUNTER [14]; WATANABE [15]) and, whenever possible, to the species level. The colony surface examination was performed using the stereo microscope Stereo Discovery.V8 (Carl Zeiss), equipped with the color digital camera for microscopy, AxioCam ICc1 and the AxioVision software for image acquisition and analysis. The characteristics of the reproductive structures of isolated filamentous fungi were highlighted by staining with lactophenol cotton blue solution on the AxioImager.A2 (Carl Zeiss) optical microscope with AxioCamMRc microscopy professional digital camera and image acquisition and analysis software ZEN Imaging 2012.

From the total number of fungal isolates obtained during October 2015 and June 2016, 16 considered representative from the point of view of the morphological characteristics of the culture on solid medium were phenotypically characterized and of these 13 isolates were also characterized at molecular level.

Results and Conclusions

From the total number of obtained isolates, 16 considered representative by morphological characteristics of the culture on solid medium and by direct microscopic examination, were phenotypically characterized and of these 13 isolates were further characterized at molecular level. The morphological and molecular identification of fungi from the surface of natural textile cultural goods in the National Museum of the Romanian Peasant in Bucharest, showed the presence of different fungal species (Table 3, Figure 3). The fungal contamination of surface of natural heritage textiles was investigated using morphological methods (Figure 1, 2). The macroscopical features of the *Penicillium chrysogenum* observed on Czapek's and PDA media have shown that the obverse colony's had radial, irregular, gray, fluffy, and the reversed orange-colored into the center (Figure 1a, b).

The macro and microscopical features of the *Aspergillus* sp. were observed on Czapek's and PDA media (Figure 2a). *Aspergillus*sp appeared as irregular furrowed green colonies on Czapek's media with yellow spores. Conidial heads are loosely columnar to radiate with subglobose vesicles (Figure 2b).

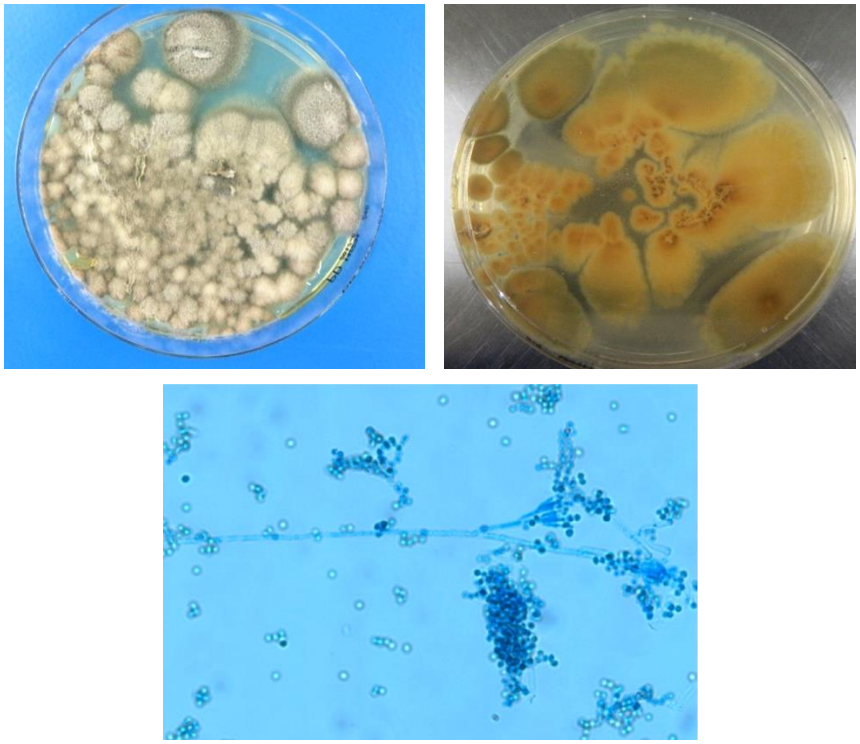

Figure 1. a, b- Macro and **c-** Microscopical (40X) features of *Penicillium chrysogenum* on Czapek's medium.

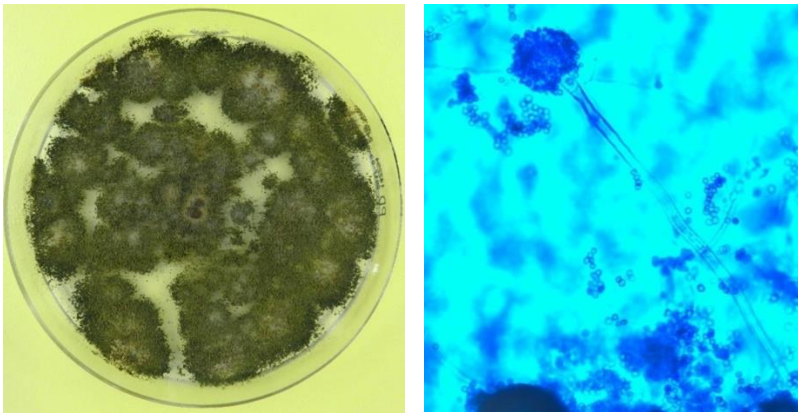

Figure 2. a- Macro and **b-** Microscopical features (20X) of *Aspergillus sp.*Czapek's medium.

For the molecular identification, ITS, which of high degree of variation than other genic regions of rDNA was used. The ITS region of rDNA has been considered as a barcode for most of the fungi (SCHOCH & al [16]). The ITS sequence is situated in the 18S-5.8S-26S region, and is formed by three fragments: the ITS1, which separates the gene for small ribosomal unit from the 5,8S gene, the ITS2 localized between 5,8S and large ribosomal unit and also the exon 5,8 S which is highly conserved. The two fragments ITS1 and ITS2 are cleaved during ribosomal ARN maturation (POCZAI & Hyvönen [17]; GARDES & BRUNS [18]).

The size of the fragment within this region is between 400-600 bp; and the resulted fragments in our results fall in this range (Figure 3). According to both methodologies 16 species and five genera of fungi were isolated and identified from eleven sample sources (Table 3). Of these fungi, the genus *Penicillium* (12/16, 75%) was the most prevalent, followed by *Epicoccum, Cladosporium, Alternaria, Aspergillus* (1/16, 6.25% each). Our developed method could successfully amplify a multi-copy (ITS-rDNA) gene, which confirmed that the extracted DNA could be used for molecular identification of investigated filamentous fungi recovered from the surface of natural textile cultural

goods in the National Museum of the Romanian Peasant in Bucharest.

Other authors have observed the prevalence of the species belonging to *Penicillium* genus in the biodeterioration of Heritage Textile made of natural fibres. This genus is followed by *Aspergillus* and *Cladosporium*. Between *Penicillium* species, *P. chrysogenum* was most often isolated from textiles in different Museum in Slovenia, with both cellulosic and keratin fibrous composition. *Penicillium* and *Aspergillus* are xerophilic fungi and are able to develop at low temperature and humidity (KAVKLER & al [19]; BŁYSKAL [20])

In a survey regarding the biodeterioration of Heritage Textiles in the Egyptian and Coptic Museums in Egypt, *Aspergillus* with 14 species and *Penicillium* with 10 species were the most common fungi. Other fungi involved were species of the genus *Chaetomium*, *Alternaria* and *Trichoderma*. *P. chrysogensum* and *P. citrinum were* most frequent from the *Penicillium* species (ABDEL-KAREM [21]).

This study represent the first attempt oriented to the molecular identification of culturable fungal strains isolated from different heritage ethnographical textiles made of natural fibres, part of the collection from the National Museum of Romanian Peasant in Bucharest, capital city of Romania. Recently, in the context of the biodegradation research of museum items, our interest was focused on phenotypical and molecular identification of filamentous fungi responsible for degradative characteristics.

Table 3. Fungi isolated from the surfaces of textile goods of cultural heritage: original source, estimated data, GenBank accesion number and similarity with existing NCBI sequences.

Laboratory code	Source	Century	Isolated species	Accesion number	Similarity
15	Pillow, made of flax and cotton (Transilvania)	½ XX	*Penicillium crustosum*	KY906188.1	100%
27	Head cover made of hemp (Transilvania)	½ XX	*P. chrysogenum*	KX421460.1	100%
5	Towel made of hemp (Transilvania)	½ XX	*P. oxalicum*	LT797552.1	100%
7	Towel made of hemp (Transilvania)	½ XX	*P. oxalicum*	LT797552.1	100%
8	Rug made of wool (Basarabia)	½ XIX	*P. citrinum*	KX507083.1	100%
13	Towel made of cotton (Muntenia)	2/2 XIX	*Epicoccum nigrum*	KR023621.1	100%
21	Rug made of hemp and wool (Banat)	2/2 XIX	*P. oxalicum*	LT797552.1	100%
25	Women shirt made of cotton (Munteina)	¼ XX	*P. oxalicum*	LT797552.1	100%
44	Rug made of wool (Basarabia)	2/2 XIX	*Penicillium chrysogenum*	KY465761.1	100%
45	Towel made of cotton (Bucovina)	¾ XIX	*Cladosporium cladosporioides*	MF327241.1	99%
47	Women dress made of cotton (Muntenia)	XIX	*Penicillium polonicum*	KY643770.1	99%
49	Shirt made of cotton (Maramureș)	½ XX	*Penicillium chrysogenum*	KY465761.1	100%
51	Shirt made of cotton (Maramureș)	½ XX	*P. oxalicum*	LT797552.1	100%
2	Women dress made of cotton (Muntenia)	XIX	*Alternaria sp.*	Non-sequenced	-
4	Rug made of wool (Basarabia)	2/2 XIX	*Penicillium sp.*	Non-sequenced	-
14	Shirt made of hemp (Banat)	¼ XX	*Aspergillus sp.*	Non-sequenced	-

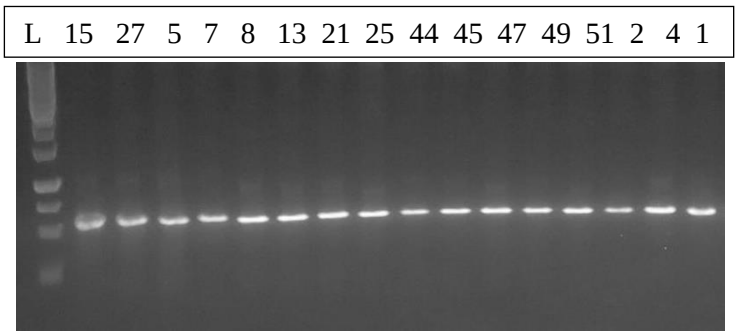

Figure 3. Electrophoresis gel for PCR-ITS amplicons: the figure shows that all the isolates were positives for ITS genetic marker.

The phenotypical and molecular characterization of the microfungi isolated from different ethnographic textiles revealed the presence of sixteen species, mostly belonging to *Penicillium* genus. The species of this genus were frequently detected in the biodeterioration studies of textiles by different authors. The species of *Penicillium* can occur in the museum environment and are able to persist as spores in the air, at low humidity and temperature. *Cladosporium*, *Aspergillus* and *Alternaria* were also identified by the complementary research strategy, phenotypical and molecular, and they are saprofitic fungi able to degrade natural biopolymers.

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