Egyptian Journal of Archaeological and Restoration Studies "EJARS"
An International peer-reviewed journal published bi-annually

Volume 4, Issue 2, December - 2014: pp: 105-112 www. ejars.sohag-univ.edu.eg

Original article

APPLICATION OF DNA TECHNIQUES FOR IDENTIFICATION OF FUNGAL COMMUNITIES COLONIZING BOOK OF EGYPT VOLUMES V PLANCHES ANTIQUITIES

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Received 3/10/2014 Accepted 16/11/2014

Abstract

This study represents both a study case about the fungi associated with a description of Egypt volumes V Planches Antiquities in the Central library of Islamic manuscripts in Cairo, were used for identification of isolates from these brownish area from a description of Egypt The plate method with a DNA was used for identification of fungi. The two internal transcribed spacers and the 5.8S gene (ITS1-5.8S- ITS2) from the nuclear ribosomal DNA were amplified. The results show that the most dominant fungi isolated from the examined old paper samples belong to Aspergillus spp. (niger – flavus – fumigates and – terreus). In the present study, culture-independent molecular methods were applied to identify fungi communities colonizing paper samples. Some of these fungi are cellulolytic species. Pure fungal strains were used to constitute a marker for further comparative investigations of a description of Egypt volumes V Planches Antiquities.

Keywords: *Manuscripts, DNA, Fungal strains, Investigations, Diagnosis*

1. Introduction

Fungal deterioration can lead to very fast deterioration of organic libraries materials such as paper, wood, etc. [1]. The ability of paper to absorb and retain moisture from the surrounding environment in the libraries, coupled with their organic components makes them highly susceptible to fungal deterioration. There are many factors causing deterioration to a description of Egypt volumes V Planches Antiquities. More liable to fungal deterioration, e.g. they are good nutrient sources. Progressive changes of properties of paper materials are common done during natural ageing and they may cause deterioration to the object. To become more susceptible to fungal deterioration, fungal deterioration of a description of Egypt volume V Planches Antiquities. Is a serious conservation problem in Egypt? This is due to improper environmental conditions promoting fungal growth and changes in the nature of the papers. Due to the surrounding environment, a description of Egypt volumes V Planches Antiquities in Egypt are more acidic, (pH 5) which makes the conditions for fungal growth more favorable. High humidity accompanied by a lack of ventilation in storage libraries in Egyptian also enhances fungal growth on paper objects. In some cases, contaminated conservation materials can cause fungal infection of conserved paper

objects [2]. Fungal deterioration of old paper causes changes in the properties of papers such as loss of strength, general durability, discoloration, and appearance. In addition, many fungi contain produced colored substances that can cause stains and spots on papers objects. And Biodeterioration fungi often cause degradation of objects such as paintings, stone, wood, paper, masonry, leather, parchment, glass, metal and cinematographic films [3]. 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 colonization on works of art [4]. 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) [5]. 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 colonizing 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 colonizing art objects, using non-destructive sampling, or sampling that needs only small amounts of material. There are several works describing bacterial communities involved in the degradation of art objects [6]. 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. 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. With classical cultivation or microscopic methods. The cellulolytic *Aspergillus spp.* [7], could be detected on paper material. Other fungal strains are frequently found as contaminants in archives and libraries; such as Paecilomycesvariotii, Myrotheciumverrucaria, Stachybotrysatra, and Fungi Imperfecti (Deuteromycetes) [8]. Molecular approaches have been developed for the assessment of microbial diversity in complex communities methods based on DNA analysis can reveal fungal diversity in ecosystems, and offer the potential benefits of highly sensitive and rapid detection [9]. 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. 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 RNA genes. As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions [10]. 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 colonizing 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 optimize 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. In addition, pure fungal strains were used to constitute marker for further comparative investigations of a description of Egypt volumes V Planches Antiquities.

2. Materials and Methods

2.1. *Fungi strains and culture medium*

Fungal strain used in this study had been previously isolated from a sample of book description of Egypt volume V Planches Antiquities affected by foxing, fig. (1-a, b). Aspergillus spp, were used as reference strains in the different analyses. All the fungi were routinely cultured and maintained on potato dextrose agar (PDA) medium containing per liter 15g. Dextrose, 200g. Potatoes extract and 15g. Agar.

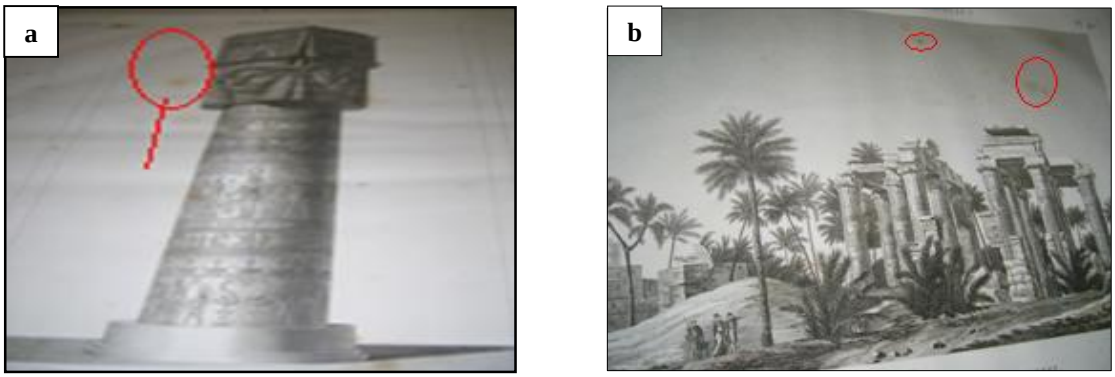

Figure (1-a, b) the brown spot on the book description of Egypt volume V Planches Antiquities.

2.2. *Fungal strains and growth conditions*

The following fungal strains, provided by the laboratories of plant pathology dept., Faculty of Agriculture, Alexandria Univ. Randomly amplified polymorphic DNA (RAPD) were used as marker strains: *Aspergillus spp*. In addition to the aforementioned strains, fungal strains considered to be frequently associated with library material deterioration had been utilized 20 years ago to inoculate paper strips. For DNA extraction, the strains utilized for the inoculation of paper samples were grown on PDA for 7 days at 25± 1˚C. The spore suspensions were obtained by gently scraping the surface of the 7-day-old cultures with a swab, washing with 30ml of H_2O containing 0.02 % Tween 80 and filtering through a sterile cotton cloth to remove impurities, fig. (2).

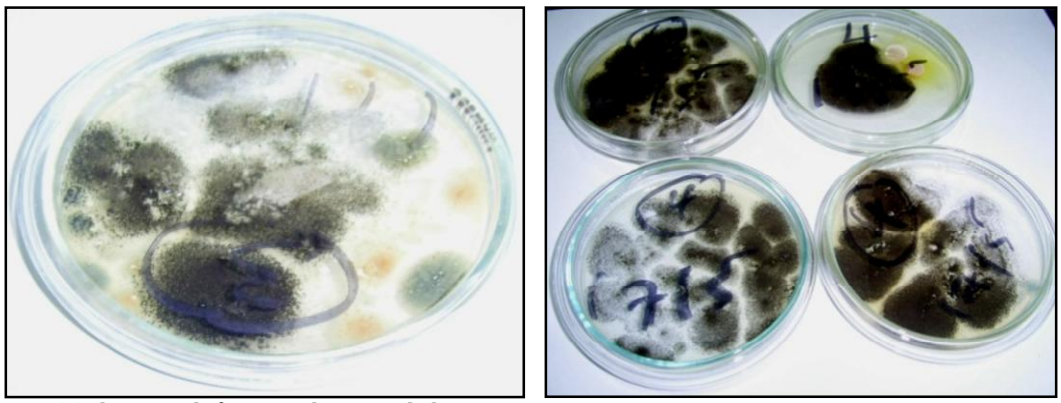

Figure (2) the growth fungi in the Petri dishes.

2.3. *Fungi's cells preparation for DNA amplification*

Pure cultures of fungi were grown overnight at 30± 1˚C on PDA medium to the final cell concentration of about 108 ml. /1. Two milliliters sample of each culture were centrifuged (14,000 rev min/1, 41˚C, 15 min.) and the resulting pellet was re-suspended in 2 ml. of sterile distilled water. The cell suspension was then diluted to 105–106 cells ml/1 for direct amplification of DNA, as described by Oppong, et al, 2003 [11]. In addition, DNA was extracted according to the methodology on 2 ml. of overnight cultures [12].

2.4. *Extraction of genomic DNA*

The total genomic DNA was isolated from fresh mycelia taken from the surface of plate cultures. The method is based on that of using modified cetyltrimethyl ammonium bromide (CTAB) in the presence of X-mercaptoe-thanol [13]. In which, 0.20 g. of urediniospores were transferred to an Eppendorf tube, frozen at –80 ˚C and freeze–dried. samples were mixed with 1 ml. of CTAB buffer (2 % CTAB, 100 mM. \ EDTA and 1.4 M NaCl), then grinded using mortar and pestle and transferred to sampled tubes and vortexed then incubated at 60-˚C for 30 minutes. Cold chloroform– isoamyl alcohol (24:1 vol./ vol.) solution was add, mixed and centrifuged by Hermle, 2230 M, BHG at 3622 g. for minutes. After centrifugation, the aqueous upper phase (supernatant) was transferred to anew Eppendorf tube and supplemented with cold isopropanol, mixed and left in the freezer at –20˚C for 30 min. to precipitate the DNA. Samples were centrifuged at 8765 g. for 15 minutes, the supernatant was removed and the precipitated DNA was carefully washed with 0.5 ml of cold ethanol 70% and air dried then redissolved in 20 ml of TE buffer (10 mM Tris–HCL, pH 8.0 and 1 mM EDTA) .

2.5. *DNA purification and quantification*

DNA purity and quantity were determined via UV spectrophotometer measurement (Jen way model 6305). The UV absorbance was used to check the purity of a DNA preparation. For a pure sample of DNA the ratio of absorbencies at 260nm and 280nm (A260/A280) is 1.8. This is because protein absorbs maximum UV light at A280. Ratio of less than 1.8 is indicative of protein contamination. The A260 / A230 ratio of DNA should be roughly equal to its A260/A280 ratio. Lower ratio may indicate contamination by organic compounds (e.g. phenol, alcohol, or carbohydrates). Absorbance at A260 is converted into DNA concentration by using the following equation. OD260 of sample X dilution factor X50mg/ml (1OD) = mg/ml DNA. The DNA samples were diluted for final concentration of 50 mg/ml. and stored at -20 -˚C until usage.

2.6. *RAPD-PCR amplification*

Five arbitrary decamer primers shown in tab. (1) were used for randomly amplified polymorphic DNA (RAPD) polymerase chain reaction master mix (Dream Taq Green PCR master mix (2X), Thermo Scientific was used for PCR reaction, containing all necessary reagents i.e. dNTPs 0.4 mM each, 2X Taq polymerase and 4 mm. $MgCl_2$ except primers and DNA template. A total volume of 25 ml. PCR reaction contained 12.5 ml. of Master Mix, 6 ml. of primer (100pmol), 4 ml. of urediniospores DNA and supplemented with 2 ml. of 5 mm. $MgCl_2$ and 0.5 ml. of Taq polymerase to 25 ml. total volume. The PCR conditions for all primer sets programmed 94 ˚C for 3 min followed by 40 cycles of 1 min at 94 ˚C, 2 min at melting temperature minus 5 ˚C and 2 min at 72 ˚C followed by 10 min at 72 ˚C were used for DNA amplification. Melting temperature (Tm) for each primer was calculated using the following equation: " Tm = 4(G +C) + 2 (A + T)" [14].

Table (1) sequences and melting temperature of arbitrary universal pairs primers used for RAPD analysis of Aspergillus spp

RAPD	*Sequence of primer 5- to 3-*	*Melting temperature C*
ITS4	5-TCCTCCGCTTATTGATATGC-3	32
ITS5	5- GGAAGTAAAAGTCGTAACAAGG-3	34

2.7. *Gel electrophoresis*

Amplification products of PCR (12.5 ml. each sample) were electrop-horesed at 72v for about 20 min. in 1.5 % agarros gel stained with Ethidium-

bromide. The DNA ladder (100 bp. DNA ladder H3 RTU, Nippon Genetics Europe GmbH) was used (5 ml.) to determine the molecular size of the DNA bands. The DNA patterns were visualized using UV-transilluminat or (Herolab UVT 2020, Kurzwellig) and photographed. The buffers and gels were prepared as follows: *TBE running buffer (0.5 x), Tris bas 54.0 g. Boric acid 27.5 g. EDTA (pH 8.0) 20 ml of 0.5 M H_2O (distilled) up to 1 liter. *Gel preparation (1.5 %), Agar rose 0.45 g., TBE buffer (0.5 x) 0.30 ml., Ethidium bromide (10 mg. /ml.) 2 ml. *Loading buffer (100 ml.), Bromophenol blue 0.25 g., sucrose 40.0 g., H_2O (distilled) up to 100 ml.

2.8. *Data analysis*

All isolates were tested at least twice with each primer to confirm the DNA banding pattern. For each primer generated a single polymorphic band that was scored as either present was designated as type 1 or absent was type (M). Each *Aspergillus spp* (niger - flavus - fumigatus - terreus) was isolated and assigned molecular phenotype based on the banding pattern of the primers, fig. (3).

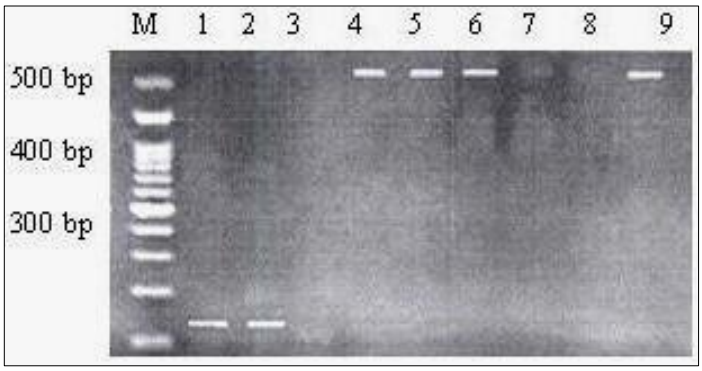

Figure (3) randomly amplified polymorphic DNA (RAPD) PCR pattern of the ITS regions in genomic DNA extracts from stains.

2.9. *SEM microscopy*

SEM photomicrographs of paper material with and without fungal colonization were taken using microstructure of these material samples. These samples were investigated under Joel Scanning Electron Microscope *JSM-5300* according to the method of described by Arai, 2000 [5] the results are shown in figs. (4- a, b, c, d).

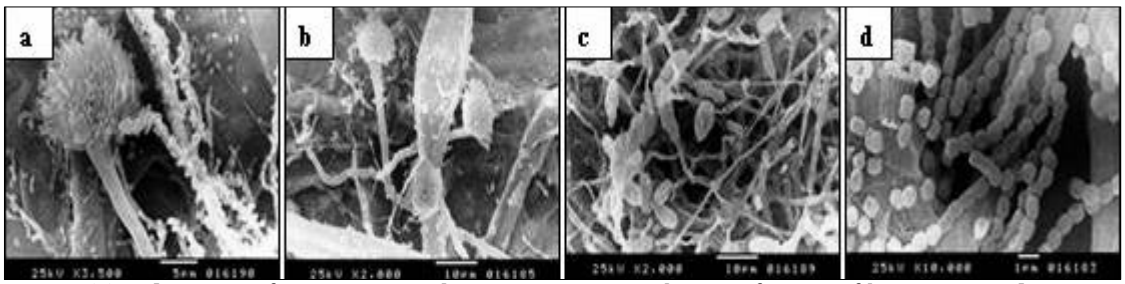

Figure (4) **a**, **b**. images from scanning electron microscope showing fungi on fiber paper, **c**, **d**. images from scanning electron microscope showing spores of fungi on fiber paper.

3. Results

In our Laboratory, some fungal strains were isolated from foxed paper samples, among which the strain C exhibited high cellulolytic activity. This strain was, aerobic and motile rod shaped fungi of about 0.5 mm. in diameter and 2.5 mm. in length, which in older liquid cultures showed many speculating cells, and then refractive spores. Preliminary attempts to define the taxonomic position

of this strain resulted in a doubtful identification as *aspergillus sp*. Cellulose degradation may be an important effect of microbiological infections capable to induce various alterations on paper substrates. Therefore, the knowledge of the taxonomic position and the derivative pathway of the fungi species may be of great importance to study and prevent paper degradation processes, as well as to know the physio-chemical factors and the environmental conditions that favor or allow paper degradation. Molecular variation is among the prevalent. However, these and other fungi cannot be considered the causal agent of foxing, even if they performed rivative activities in finished paper. DNA extraction efficiency was tested fungal-specific PCR amplification of the minimal content of fungal DNA on the inoculated paper, The fungal specific primer pairs ITS 4 – ITS 5 allowed DNA of pure fungal strains to be amplified using these specific primer sets, To compare both methods, DNA extracts from all samples were used to amplify both the ITS 1 and ITS 2 regions, shown to be all PCR-amplifiable using either the ITS 1 or ITS 2, the primer sets showed in tab. (2).

Table (2) showing *Aspergillus* species fungi on old paper

Aspergillus species	
Aspergillus niger	*Aspergillus flavus*
Aspergillus fumigates	*Aspergillus terreus*

4. Discussion

Through the previous study it could be argued that foxing is a term used to describe a variety of damage and discoloration in paper used in books and manuscripts. Studies of foxing were undertaken by Iiams & Beckwith, 1935 [15], and have been carried on from various points of view, but the causes of this paper deterioration are not yet completely understood. Physical, chemical, and environmental factors in addition to microbiological agents are thought to be involved in the appearance of foxing. Because of the sub-multiple factors, it is very difficult to experimentally reproduce the phenomenon. However, Arai et al., 1990 induced the formation of brown spots on inoculating some fungi on paper under appropriate environmental conditi-ons and then it is usually considered that xerophilic fungi are the main cause of foxing [16]. Many fungal species have been long isolated from foxed papers by many authors such as Arai, 1984 [17] and recently Corte et al. 2003, [18]. They proved that isolated microorganisms from differently aged foxing papers and ancient maps are about 58 % fungi, 30 % yeasts and 12 % bacteria, but only the fungal species were further characterized. In this work, samples from fungal spots on old paper were removed and DNA was isolated from these spots. ITS regions were amplified and the PCR products were cloned, sequenced and compared to existing ITS sequences of a large database of fungal species. In this addition the study focused on establishing a reliable and fast molecular strategy to investigate complex fungal communities on paper, the major challenge of this study was to recover DNA directly extracted from paper materials suitable for PCR amplification of fungal-specific sequences. Moreover, two different DNA extraction protocols routinely were compared in our laboratory for direct DNA extraction from old paper, the results showed, depending on the DNA extraction method that a further purification step could be needed and that fungal DNA extraction was more effective from paper**.**

Acknowledgments

This work was supported by the laboratories of plant pathology department, faculty of agriculture, Alexandria university of Egypt. And give thanks for each of Prof. Dr. Abdel-

Elmageed M. kamara, Prof. of plant pathology, plant pathology department, faculty of agriculture, Alexandria university of Egypt, and Prof. Dr. Mohammed S. Abou Elseoud, Prof. of plant pathology, plant pathology department, faculty of agriculture, Alexandria university of Egypt.

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