Isolation and molecular identification of deteriorating fungi from Cyrus the Great tomb stones

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ABSTRACT

Background and Objective: Biodeterioration is an irreversible damage that is caused by colonization of microorganisms on the surface of different materials. Among all microorganisms, fungi play an important role in deterioration of materials. Fungi can colonize on stone surfaces and by secreting different enzymes, organic and inorganic acids and pigments, can cause bio-weathering and changing not only the substrate materials but the color of stones. Furthermore, fungal mycelia can penetrate into the internal surfaces of stones and change the interior chemical contents of stones. Pasargadae including Cyrus the Great Tomb is entitled by UNESCO as one of the World Heritage Sites. This study was focused on the identification of fungi that were colonized on the tomb limestone monument.

Materials and Methods: Sampling of stone was carried out to identify inhabiting molds and yeasts. Biochemical and microscopic methods were used for isolated strains. In addition, the Polymerase Chain Reaction (PCR) and sequencing of the PCR products were done. Finally, phylogenetic tree was constructed based on the sequences of ITS region.

Results and Conclusion: The common inhabiting fungi which isolated from the tomb limestone belong to Caldosporium sp., Embellisia sp., Cryptococcus sp., Candida sp., Meyerozyma sp., Arthirinium sp., Ulocladium sp., Fusarium sp., Humicola sp. and Pseudozyma sp.. Stereomicroscopic and Scanning Electron Microscope images and XRD, were taken from pieces of stone samples and indicated the severe pattern damages such as pitting, biomineralization, etching and sugaring on the surfaces of stones.

Keywords: Biodeterioration, Fungi, Pasargadae, Cyrus the Great, PCR

INTRODUCTION

Biodeterioration was defined by Hueck (1965, 1968) for the first time as “any undesirable change in the properties of a material caused by the vital activities of organisms”. This phenomenon can cause irreversible impacts on materials by colonization of different microorganisms on the surface of materials (1, 2).

Animals, plants and microorganisms could biodeteriorate and biodegrade different kinds of materials through biophysical and biochemical processes. Inhabitant microorganisms are included bacteria, cyanobacteria, yeast, some algae and many fungi species. Microbial colonization usually launched by photosynthetic microorganisms such as cyanobacteria and algae which produce organic nutrients for other microorganism. Filamentous microorganisms cause biophysical attack by penetrating into materials. Some microorganisms exert organic and inorganic acids and also recessional CO₂ which has crucial role in the biochemical deteriorating (2-4). In addition to these mentioned vital organisms, many outdoor environmental factors such as temperature, UV radiation and moisture can cause deteriorating of the stone (5).

Rocks are naturally-formed materials which composed of one or some mineral materials. Different types of rocks...
include marble stone, sand stone, limestone, quartzite stone, gneiss stone, basalt stone, granite stone and slate stone. Water availability, pH, climatic exposure, nutrient sources, porosity and mineral composition of the stones are the factors affecting microbial colonization. All stone surfaces are susceptible for colonizing lithobiontic organisms (4, 6, 7).

Fungi are one of the most important microorganisms among all microbial communities on the stone surfaces (5). They are the dominant group of soil microorganisms and could survive in low levels of pH. Inhabitant stone fungi can grow on the surface of rocks which grow epitheles or can penetrate in pores of rocks which are called endoliths. Fungi exert pigments which cause discoloration and also exert different kinds of organic acids such as oxalic acid, gluconic acid and lactic acid that chelate magnesium, manganese, iron and calcium ions from the surface of stone and biodeteriorate stones (8).

In this study, the main subject which cause biogenic weathering is fungi. Limestone (CaCO$_3$) has carbonate and calcium sources and rarely has cracks and pores on its surface, consequently euendolithic organisms are colonized on these types of rocks. There are strategies that describe the low speed of these organisms in consuming their substratum (6, 9). Inhibiting fungi on limestone dissolve and transform one mineral into another mineral, also they exert organic acids and ligands which bind to metals in limestone. Metals such as Mn and Fe may oxidize by fungi or reductive attacks may occur and cause biodeterioration of limestone. In fact, fungi cause precipitation of oxalates such as (CaC$_2$O$_4$.H$_2$O) and (CaC$_2$O$_4$.2H$_2$O). Epilithic and endolithic fungi can transform carbonate minerals in limestone (4).

The importance of fungi on biodeterioration and biodegradation has led to the definition of Geomycology. This science involves the effects of fungi in geological processes which are including biodeterioration, exchange of mineral materials and metal aggregation (4, 10). In this study, conventional and molecular methods were used to isolate and identify fungi from Cyrus the Great Tomb Stones. The characterization of biodeteriorants is the first step before any conservation of the monuments.

MATERIALS AND METHODS

The region of sampling. Established by Cyrus the Great in the 6th century BC, Pasargadae is the first dynastic capital of the Achaemenid Empire and the tomb of Cyrus the Great (559-530 BC) was built with large pieces of limestone. Pasargadae stone monument has 1850 altitude and 30°12’00”N, 53°10’46”E longitudes. In the whole year in this area, the mean annual temperature is 17.3 °C. The mean annual precipitation is 298 mm with about 76.2 rainy days per year. Average relative humidity is 49% (11).

Sampling and culturing. Sampling was carried out from all sides of the monument on May 2012. Sterile needle was used for sampling of fungi and cultured onto Potato Dextrose Agar (PDA) and Sabourud Dextrose Agar (SDA). All of the isolates were coded from 1 to 9 and each number demonstrated the steps and sides which the isolates were obtained.

After that, slide culture was done to perform morphological characterization. For this reason, PDA media culture was used and slides were finally dyed with lactophenol cotton blue and used for microscopic observations.

Biochemical tests. For these procedures, four tests were done as described below.

Diazenium Blue B (DBB) was used to differentiate Ascomycota and Basidiomycota (12, 13). In urea hydrolysis test, yeasts were cultured on Christensen’s urea agar (CUA) and incubated in 25 °C for 4 days (14, 15). Corn meal agar prepares is convenient environment to show the ability of mycelia growth of yeasts. For this purpose, Dalman plate technique was used and yeasts were cultured in the middle of plate, and then incubated at 28 °C for a week. Every day, the mycelia or pseudomycelia growth of yeasts was assayed (14).

For carbohydrate fermentation test, 100 microliter of the 10$^7$ CFU/ml fungal suspensions is added to yeast broth medium including Doreham tube. The concentration of sugar was adjusted to 50 mM (14).

Macroscopic study of isolates. In this study, exterior properties of colonies were demonstrated. Furthermore, the colors of the colonies were considered for identifying the fungi.

Microscopic study of isolates. Optical microscope, Stereomicroscope and Scanning Electron Microscope (SEM) were used in this study. Also with X-ray Diffraction experiment, the fluctuation of mineral elements on the stone surface was studied. Mycelium,
spores and budding features in fungi were observed by optical microscope after Watanabe 2002. Inhabitant microorganisms and possible damages on the surface of the stone can be seen by stereomicroscope.

Bacterial and fungal biofilms on the crystal surface of substrate is noticeable by Scanning Electron Microscope (16, 37). Preparation of stone samples needs fixation of samples (17). After doing fixation procedure, stone samples were dried and covered by gold. As a final point, the stone samples were observed by SEM (XL 30 model).

XRD was used to investigate the crystalline compounds of materials. Diffraction pattern of compounds are different (18-21, 37).

**Molecular methods.** On the basis of biochemical tests, macroscopic and microscopic observations, 18 isolates were selected for DNA extraction. Three extraction methods were used. Two of these methods were specified for molds after Liu et al. 2000 and Vinland. The other was Phenol-Chloroform-Isomilalehohol method which is specified for yeasts (36).

**Polymerase chain reaction.** In this study, primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTATTGATATGC) were used for amplification (22, 23). The master mix buffer contained 2.5 μl PCR buffer, 1 μl MgCl2, 0.5 μl dNTP, 0.75 μl ITS1F, 0.75 μl ITS4 and 0.5 μl tαq DNA polymerase, was used in PCR procedure. The PCR products were visualized after electrophoresis and sent for sequencing.

![Fig 2](http://ijm.tums.ac.ir)

**Fig 2.** Black spots on the stone surface indicating to micro-colony fungi.

![Fig 3](http://ijm.tums.ac.ir)

**Fig 3.** (a) Actinomycets, fungal mycelia, bacterial cells and salt crystals. (b) Surface layers of limestone and crystals on it. (c) Penetration of fungal mycelia in the limestone, also sugaring phenomenon on the surface of the limestone can be seen. (d) SEM micrograph shows mycelia of fungi, actinomycetes and crystals. Penetration of fungal mycelia is observed.
RESULTS

In this study, 33 isolates were obtained from different sides of tomb. On the basis of micro and macroscopic observations, Alternaria sp., Cladosporium sp., Ulocladium sp., Fusarium sp., Humicula sp., and Arthrinium sp. were identified (Fig. 1, a-g). This identification was based on data provided in the Atlas of Mycology (38).

DBB, urea hydrolysis, culturing on corn meal agar and fermentation of sugars were performed for yeasts. Of 18 yeasts, 6 showed positive results in DBB and the cultures change their color to red, which means that yeasts with positive result tests belong to Basidiomycota and yeasts with negative test results belong to Ascomycota. Also in urea hydrolysis test, 6 yeasts showed positive results. In this test, after four days, the color of the cultures changes from orange to pink. In corn meal agar test 12 isolated yeasts showed positive results. Positive results mean yeasts have ability to grow in form of mycelium or pseudomycelium. In sugar fermentation test six different sugars were used and in positive results, some bubbles formed in Doreham tubes. It means that some yeasts have an ability to ferment sugars and produce sugar during this function.

Using stereomicroscope, micro-colony fungion surface of stones were observed which created biopitting pattern on it as shown in Fig. 2. SEM micrographs showed clearly spores and fungal mycelia, actinomycetes, algae and lichens. Sugaring, etching, crystallization and biopitting were observed on the stone samples (Fig. 3, a-d).

On the basis of studies, calcium and other ions are absorbed during biodeterioration procedures and stone surfaces are etched and exposed to physico-chemical alterations (24). Increasing in carbon and alteration of calcium level are the evidence of microbial activities on the stone surfaces which was shown in XRD results and led to alteration of the surface.

Before analyzing molecular results, PCR products were prepared and loaded on agarose gel. The sequence results, analyzed just in forward direction (ITS1F), were compared with those in the Genbank/ Mycobank/ nucleotide sequence databases by using the BLAST (blastn) program (http://www.ncbi.nlm.nih.gov), and fungi are classified on the basis of Mycobank (www.mycobank.org) analysis and morphological characterization as shown in Table 1 (25). All of the sequences were aligned using the Clustal W program (26). A phylogenetic tree and neighbor-joining phylogenies were constructed by using the MEGA software package, version 5.0 (27) and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1000 replicates). The trees were shown in Figs 4 and 5, separately. Among ribosomal systronic areas, ITS region is the best area for identifying fungi (28).

All isolated fungi belong to Ascomycota and Basidiomycota groups. Six molds and four yeasts were identified using the methods described in this study. All of the molds belong to Ascomycota. Two yeasts belong to Basidiomycota and two isolates were identified to family level.

DISCUSSION

Scientific studies show that the weather is an important factor in biodeterioration process. Chemical sediments originated from air pollution on the stone surfaces provide convenient food supply for microorganisms. Fungi are one the crucial organisms causing biodeterioration (7, 29-31).

The classical methods for identification of microorganisms is very limited since these methods can identify less than 1% of microbial population. There are different reasons for this finding. Some microorganisms are in their inactive cycles and show limited metabolic activities; therefore they could not be detected by classic methods. In these conditions, molecular methods are thoroughly the best techniques to identify microorganism (32-34).

One of the molecular science and techniques to identify microorganisms is sequencing of small subunits (SSU) of 16S, 18S ribosomal RNA and ITS region (17). In this study sequencing of ITS was successfully done on 17 fungi isolates that showed differences in morphology and microscopic observations with other isolates. ITS is known as the standard region for this aim (28).

Studies show that, general fungi population on the stone surfaces belong to the different genera including Cladosporiumsp., Aureobasidiumsp., Alternaria sp., Trichoderma sp., Penicillium sp., Exophiala sp., Fusarium sp., Phialophora sp., Cryptococcus sp. and Phoma sp. (4, 35) and based on stone type, these population will change. Burford and et al. (2003) reported that the most common fungi genus on limestone surfaces included
Fig 1. Microscopic images of isolated fungi on limestone surfaces: (a) Arthrinium sp. (b) Ulocladium sp. (c) Fusarium sp. (d) Ulocladium sp. (e) Cladosporium sp. (f) Cladosporium sp. (g) Alternaria sp.
Aspergillus sp., Aureobasidium sp., Penicillium sp., Fusarium sp., Cephalosporium sp. and Monilia sp. (4).

Common fungi on the tomb limestone surfaces are Cladosporiumsp., Embellisiasp., Cryptococcus sp., Candida sp., Meyerozyma sp., Arthrinium sp., Ulocladium sp., Montagnulaceae sp., Fusarium sp., Humicola sp., and Pseudozyma sp. The most common fungi belonged to Tremellaceae, Davidiellaceae, Saccharomycescaceae, Chaetomiaceae, Ustilaginaceae, Montagnulaceae, Pleosporaceae, Apiosporaceae and Nectriaeace families. In comparison to the past and current studies, Cladosporiumsp. Candida sp., and Fusariumsp. are mutual fungi which detected on the tomb limestone surfaces, but genus such as Pseudozyma sp., Cryptococcus sp. and Meyerozyma sp. has not been reported before. Also, Arthrinium sp. and Ulocladium sp. was not reported on limestone surfaces yet. These differences lead to conclude that geographical altitude and longitude, weather conditions and relative humidity have crucial impacts on diversity of microorganisms’ population on stone surfaces.

It can be concluded that limestone surfaces of Cyrus the Great of Pasargadae are proper substrates for microbial colonization. In this study, it was focused on isolation and identification of fungi. Detection and characterization of biodeteriorants are necessary before any restoration and conservation treatments. Further investigation should be carried out to find the best methods to remove and control of microbial growth on the stone surfaces of the tomb.

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| Strain    | Received accession from Genbank | Morphology | Best BLAST match and Strain | Identical sites (%) | Accession |
|-----------|--------------------------------|------------|-----------------------------|---------------------|-----------|
| MM 1_1    | KJ361495                        | Yeast      | Cryptococcus friedmannii Strain DBVPG 5303 | 100%                | KC455900  |
| MM 1_2    | KJ361498                        | Cladosporiumsp. | Cladosporiumcladosporioides Strain ML370 | 99%                 | KC692219  |
| MM 2_9_c  | KJ361496                        | Yeast      | Candida albicans Strain ZB044 | 99%                 | GQ280317  |
| MM 2_9_d  | KJ361482                        | Yeast      | Candida albicans Strain ATCC MYA-4901 | 99%                 | KC113639  |
| MM 5_2    | KJ361483                        | Sterile mycelia | Humicola sp. Strain CY186 | 99%                 | HQ608016  |
| MM 5_3    | KJ361484                        | Cladosporium sp. | Cladosporiumossifragi Strain WA0000019048 | 100%                | JX981486  |
| MM 5_4    | KJ361485                        | Yeast      | Pseudozymashanxiensis Strain SN37 | 94%                 | FJ515182  |
| MM 6_1_a  | KJ361486                        | -          | Montagnulaceae sp. Strain 1 SMR-2011 | 99%                 | HQ909081  |
| MM 6_1_b  | KJ361487                        | Ulocladium sp. | Ulocladiumconsortiale Strain UL1 | 99%                 | KC577270  |
| MM 6_3    | KJ361488                        | -          | Montagnulaceae sp. Strain 1 SMR-2011 | 100%                | HQ909081  |
| MM 7_1    | KJ361489                        | Ulocladium sp. | Ulocladiumconsortiale | 100%                | FJ266482  |
| MM 9_1_n  | KJ361491                        | Alternaria sp. | Alternariachlamydosporigena Strain CBS 341.71 | 99%                 | KC584231  |
| MM 9_2    | KJ361492                        | Arthrinium sp. | Arthriniumsacchari Strain A09 | 100%                | HQ115646  |
| MM 20_t   | KJ361493                        | Yeast      | Meyerozymaguilliermondii Strain SACCR 010861 | 100%                | JX427051  |
| MM C_K    | KJ361494                        | Yeast      | Candida tropicalis Strain URM4261 | 100%                | KF031306  |
| MM C_K_n  | KJ361497                        | Yeast      | Candida tropicalis Strain R11 | 97%                 | JQ640572  |
| MM 8_b_3  | KJ361490                        | Fusarium sp. | Fusariumsolani Strain f. sp. eumartii | 99%                 | AB498983  |
Fig 4. Neighbor-joining tree depicting the relationships between yeast isolates, using the ITS. Bootstrap values are given above 50%.
Fig 5. Neighbor-joining tree depicting the relationships between mold isolates, using the ITS. Bootstrap values are given above 50%.
ACKNOWLEDGEMENTS

The authors thank Faezeh Borzooee, Mahnaz Gholipour Shahraki, Simin Ashraf, Maryam Yousefi and Zahra Fallahi for their useful helps in technical and cultural methods. Dr Bazrgar, the research director of the Iranian Cultural Heritage Organization, and Dr Talebian, the director of Parse - Pasargadae Research Foundation, are acknowledged for providing the facility of the site sampling in this study. All of the microbiological experiments done in the national laboratory of industrial microbiology by project of fund research and technology coded by 88001692 number in Alzahra University.

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