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ORIGINAL ARTICLE

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

Parisa Mohammadi*, NasimMaghboli-Balasjin

Faculty of Biological Sciences, Alzahra University, Vanak, Tehran, Iran

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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, phylogenic tree was constructed basde 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 CO_2 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

*Corresponding author: Parisa Mohammadi,
Address: Faculty of Biological sciences, Alzahra University, 1993893973, Tehran, Iran.
Tel: +98-21 85692714
E-mail: p.mohammadi@alzahra.ac.ir

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 called epiliths 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 dissolute 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 ($CaC_2O_4.H_2O$) and ($CaC_2O_4.2H_2O$). 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 30º12´00˝N, 53º10´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.

Diazonium 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 ºC 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 ºC for a week. Every day, the mycelia or psuedomycelia 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,

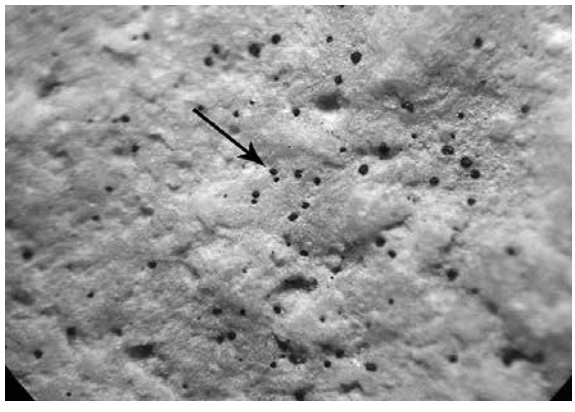


Fig 2. Black spots on the stone surface indicating to micro-colony fungi.

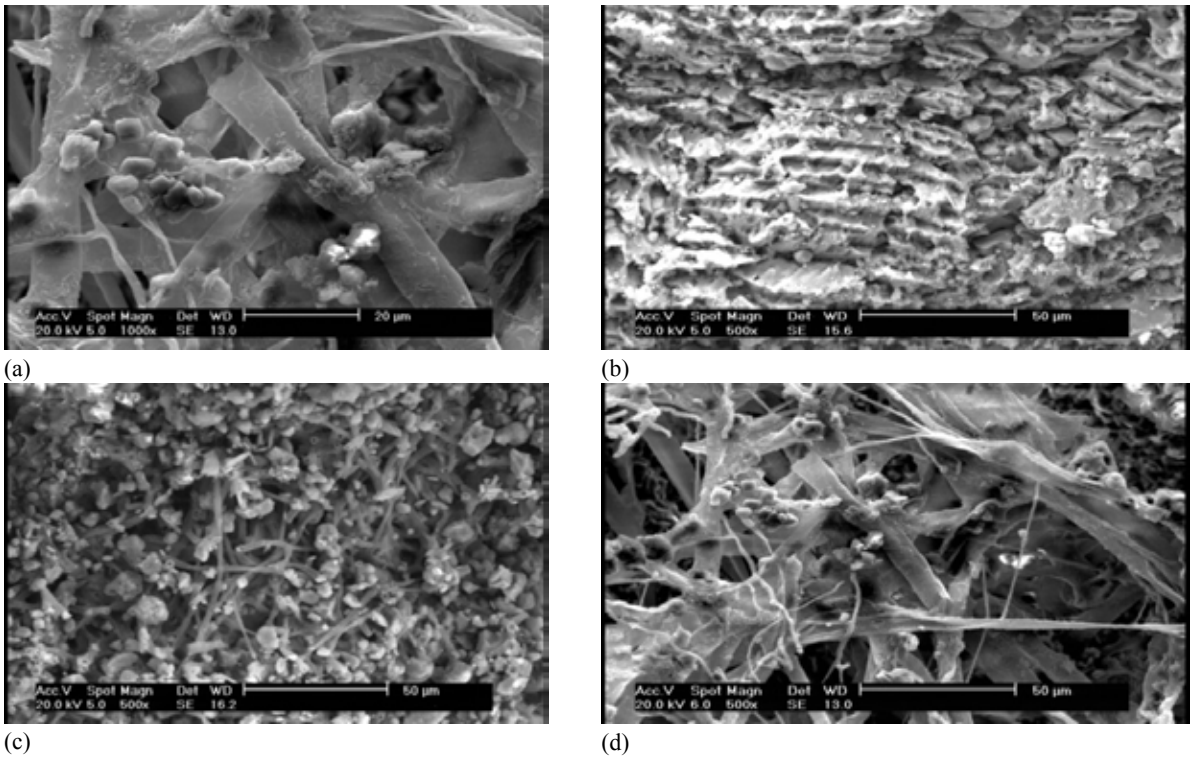
spores and budding features in fungi were observed by optical microscope after Watanabe 2002. Inhabitant microorganisms and possible damages on the surface of the stone can be seen by stereomicroscope.

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-Isoamilalchohol method which is specified for yeasts (36).

Polymerase chain reaction. In this study, primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) 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 *taq* DNA polymerase, was used in PCR procedure. The PCR products were visualized after electrophoresis and sent for sequencing.



(a) (b) (c) (d)

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 limeston 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.

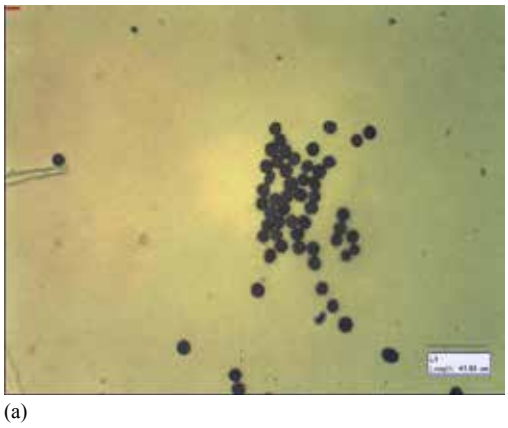
DISCUSSION

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).

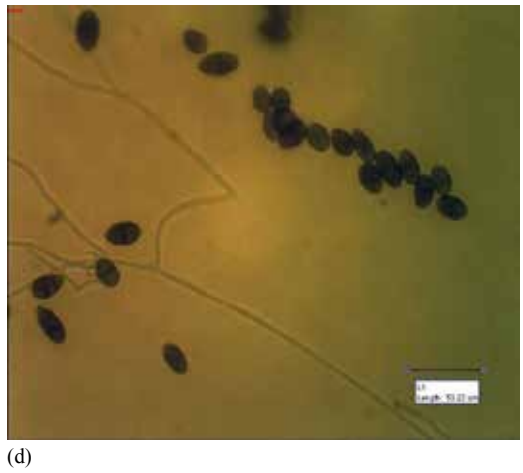
Studies show that, general fungi population on the stone surfaces belong to the different genera including *Cladosporium*sp., *Aureobasidium*sp., *Alternaria*sp., *Trichoderma*sp., *Penicillium*sp., *Exophiala*sp., *Fusarium*sp., *Phialophora*sp., *Cryptococcus* sp. and *Phoma* sp. (4, 35) and based on stone type, these population will change. Burford and *et al.* (2003) reported that the most common fungi genus on limestone surfaces included



(a)

(b)

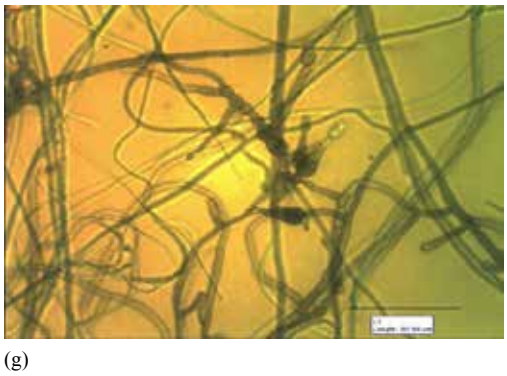
(c)



(d)

(e)

(f)



(g)

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.

Table 1. Molecular characterizations of isolates

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	*Cladosporium*sp.	*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.	*Cladosporiumossifraqi*Strain WA0000019048	100%	JX981486
MM 5_4	KJ361485	Yeast	*Pseudozymashanxiensis* Strain SN37	94%	FJ515182
MM 6_1_a	KJ361486	-	Montagnulaceae sp. Strain I SMR-2011	99%	HQ909081
MM 6_1_b	KJ361487	*Ulocladium* sp.	*Ulocladiumconsortiale* Strain UL1	99%	KC577270
MM 6_3	KJ361488	-	Montagnulaceae sp. Strain I 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

Aspergillus sp*., Aureobasidium* sp*., Penicillium* sp*., Fusarium* sp*., Cephalosporium* sp. and *Monilia* sp. (4).

Common fungi on the tomb limestone surfaces are *Caldosporium*sp., *Embellisia*sp., *Cryptococcus* sp., *Candida* sp., *Meyerozyma* sp., *Arthirinium* sp., *Ulocladium* sp., *Montagnulaceae* sp., *Fusarium* sp., *Humicola* sp., and *Pseudozyma* sp. The most common fungi belonged to Tremellaceae, Davidiellaceae, Saccharomycetaceae, Chaetomiaceae, Ustilaginaceae, Montagnulaceae, Pleosporaceae, Apiosporaceae and Nectriaceae families. In comparison to the past and current studies, *Caldosporium*sp. *Candida* sp., and *Fusarium*sp. 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, *Arthirinium* 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.

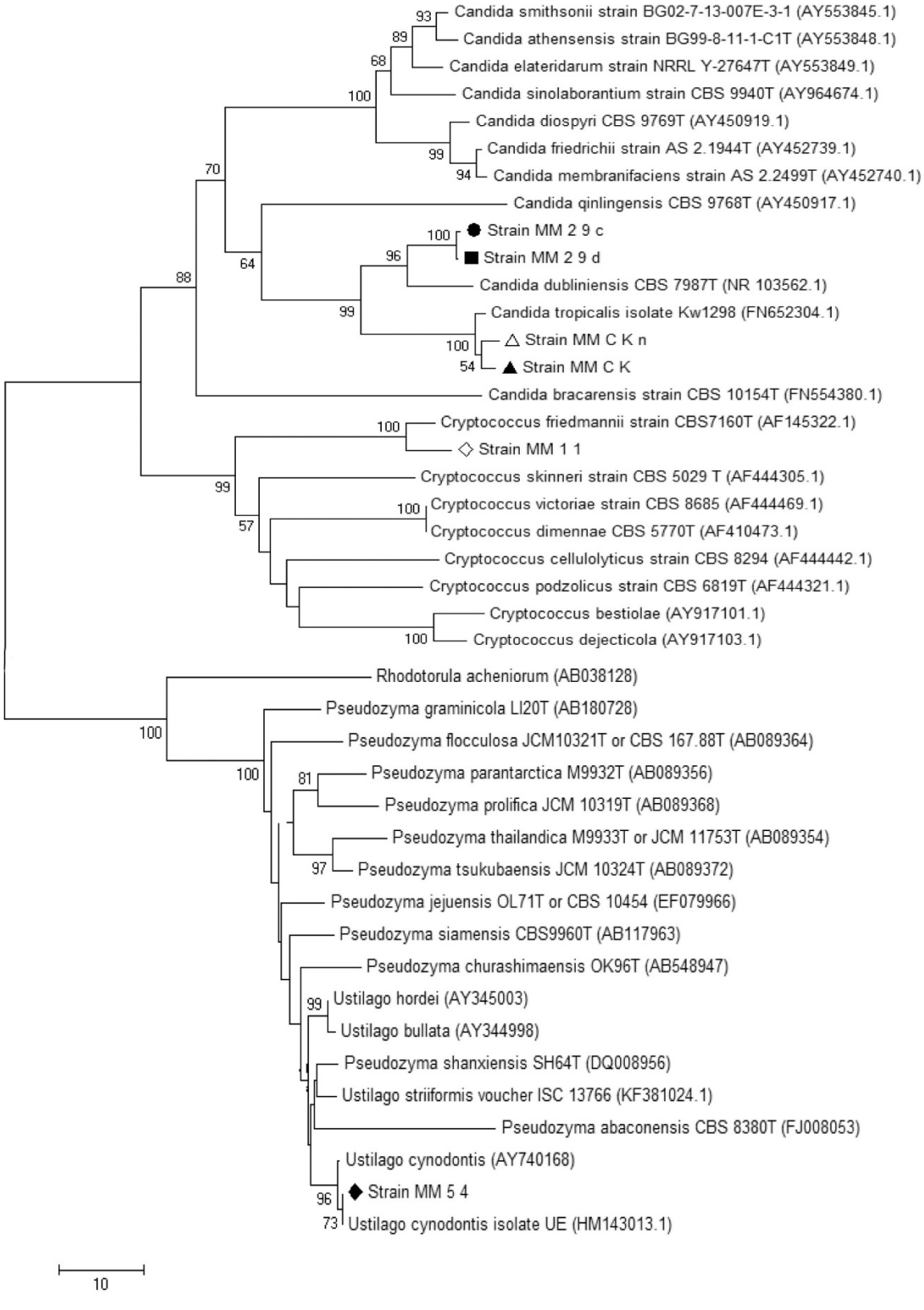


Fig 4. Neighbor-joining tree depicting the relationships between yeast isolates, using the ITS. Bootstrap values are given above 50%.

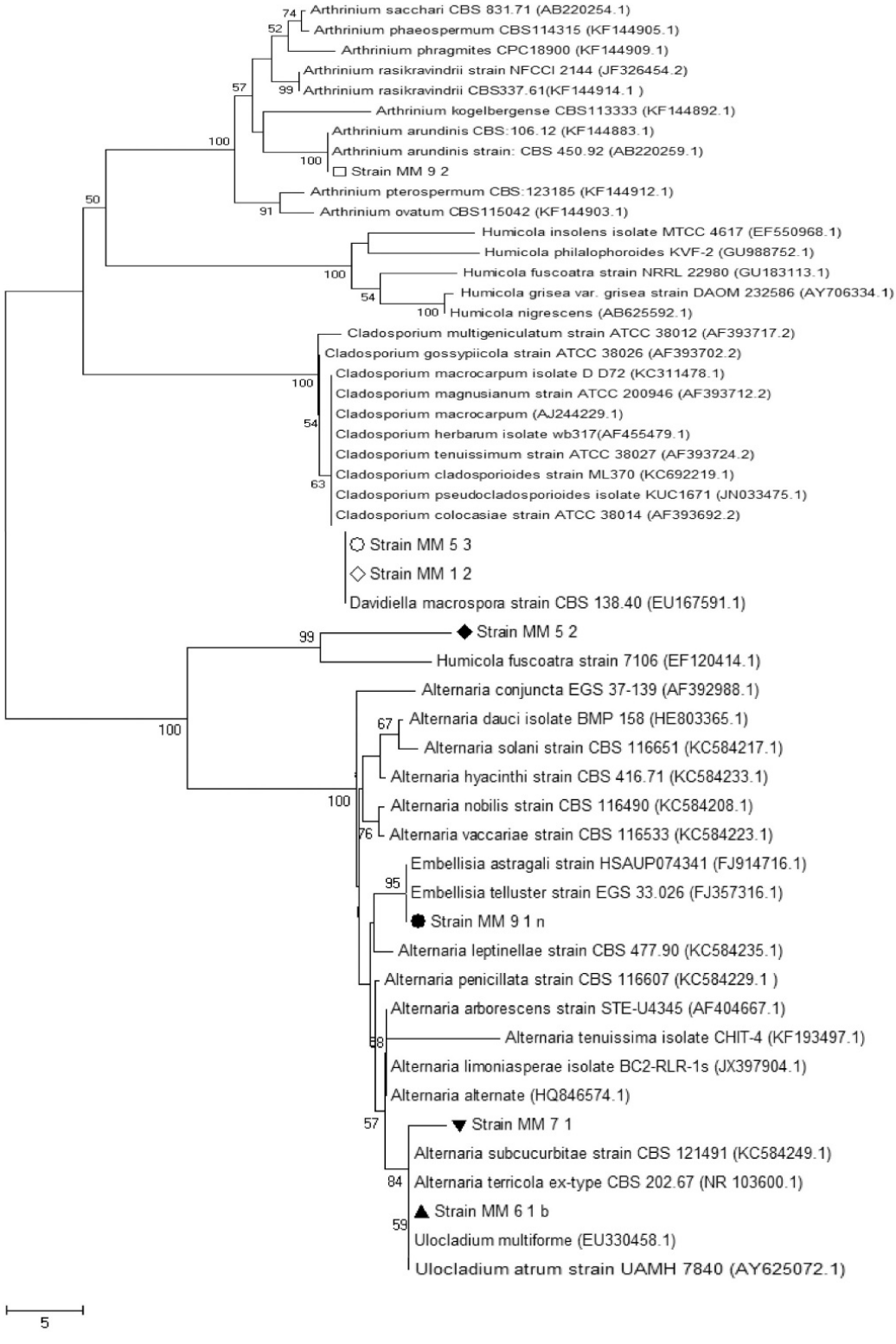


Fig 5. Neighbor-joining tree depicting the relationships between mold isolates, using the ITS. Bootstrap values are given above 50%.

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REFERENCES

1. Allsopp D, Seal KJ, Gaylarde C (2004). Introduction to biodeterioration. 2nd ed.Cambridge University Press. New York, USA.
2. Cutler N, Viles H. Eukaryotic Microorganisms and Stone Biodeterioration. *Geomicrobiology Journal* 2010; 27: 630-647.
3. Pinzari F, Pasquariello G, DeMico A. Biodeterioration of Paper: A SEM Study of Fungal Spoilage Reproduced Under Controlled Conditions. *Macromol Symp* 2006; 238: 57-66.
4. Burford EP, Kierans, M, Gadd, GM. Geomycology: Fungal growth in mineral substrata. *Mycologist* 2003; 17: 98-107.
5. Páramo-Aguilera L, Ortega-Morales B, Narváez-Zapata J. Culturable fungi associated with urban stone surfaces in Mexico City. *Electronic J Biotechnol* 2012; ISSN: 0717-3458.
6. Hoppert M, Flies C, Pohl W, Gunzel B, Schneider J. Colonization strategies of lithobiontic microorganisms on carbonate rocks. *Environ Geology* 2004; 46: 421-428.
7. WarscheidTh, Braams J. Biodeterioration of Stone: a review. *International Biodeterioration and Biodegredation* 2000; 46, 343-368.
8. Scheerer S, Ortega-Morales O, Gaylarde C (2009). Microbial Deterioration of Stone Monuments-An Updated Overview. In: *Advances inApplied Microbiology Vol.66.* Ed, AI Laskin, S Sariaslani,GM Gadd. Academic Press.1st Ed. San Diego, USA, pp. 97-139.
9. Concha-Lozano N, Gaudon P, Pages J, Billerbeck G, Lafon D, Eterradossi O. Protective effect of endolithic fungal hyphae on oolitic limestone buildings. *Journal of Cultural Heritage* 2012; 13:120-127.
10. Onofri S, Selbmann L, de Hoog GS, Grube M, Barreca D, Ruisi S, Zucconi L. Evolution and adaption of fungi at boundries of life. *Advances in Space Research* 2007; 40: 1657-1664.
11. http://www.farsmet.ir/Default.aspx
12. Soudi, MR(1999). Fermentation Processes, Theory and Applications. Alzahra University Press. Tehran, Iran.
13. Land GA, Horstmeier CD, Roberts GD, Foxworth JH. Rapid urea broth test for Yeasts. *J Clin Microbiol* 1978; 7:584-588.
14. Kurtzman CP, Fell JW, Boekhout T (1998). The yeasts: a taxonomic study. 4th ed. Elsevier. San Diego, USA.
15. Kurtzman, CP, Fell JW (2011). The yeasts: a taxonomic study. 5thed. Elsevier. San Diego, USA.
16. Mohammadi P, Krumbein WE. Biodeterioration of ancient stone materials from the Persepolis monuments (Iran). *Aerobiologia* 2008; 24: 27-33.
17. Dakal TC, Arora PK. Evaluation of potential of molecular and physical techniques in studying biodeterioration. *Rev Environ Sci Biotechnol* 2012; 11: 71-104.
18. Bertrand L, Vantelon D, Pantos E. Novel interface for cultural heritage at Soleil. *Applied Physics* 2006; 83: 225-228.
19. Cuzman OA, Ventura S, Sili C, Mascalchi C, Turchetti T, D'Acqui LP, Tiano P. Biodiversity of phototrophic biofilms dwelling on monumental fountains. *Microb Ecol* 2010; 60: 81-95.
20. Albertano P, Urzì C. Structural interactions among epilithic cyanobacteria and heterotrophic microorganisms in Roman hypogeal. *Microb Ecol* 1999; 38: 244-252.
21. Uda M, Demortier G, Nakai I (2005). X-rays for Archaeology. Springer Science & Business Media. Amsterdam, Netherlands.
22. Brown TA (2010). Gene Cloning and DNA Analysis: An Introduction. 6th ed. John Wiley & Sons.Kuala Lumpur, Malaysia.
23. Primrose SB, Twyman RM, Old RW (2001).Principles of Gene Manipulation. 6th ed. University of California Press. London, UK.
24. Griffin P S, Indictor N, Koestler RJ. The Biodeterioration of Stone: a Review of Deterioration Mechanisms, Conservation Case Histories, and Treatment. *Int Biodeterior* 1991; 28: 187-207.
25. Zhang Zh, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences, J *Comput Biol* 2000; 7(1-2): 203-14.
26. Thompson JD, Higgins DG, Gibson TJ. CLUSTALW: improving the sensi tivity of progressive multiple

sequence alignment through sequence weighting, position-specific gap penalties and weigh matrix choice. *Nucleic Acids Res* 1994; 22: 4673-4680.

27. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24: 1596-1599.
28. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W. Fungal Barcoding Consortium Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *PNAS*2012; 109: 6241-6246.
29. Saiz-Jimenez C. Biodeteriorationvs Biodegradation: the Role of Microorganisms in the Removal of Pollutants Deposited on Historic Buildings. *Int Biodeterior Biodegredation* 1997; 40: 225-232.
30. Sharma K, Lanjewar S. Biodeterioration of ancient monument (Devarbija) of Chattisgarh by fungi. *J Phytol* 2010; 2(11): 47-49.
31. Polo A, Cappitelli F, Brusetti L, Principi P, Villa F, Giacomucci L, Ranalli G, Sorlini C. Feasibility of Removing Surface Deposits on Stone Using Biological and Chemical Remediation Methods. *Microb Ecol* 2010; 60:1-14.
32. González JM, Saiz-Jiménez C. Application of molecular nucleic acid-based techniques for the study of microbial communities in monuments and artworks. *Int Microbiol* 2005; 8: 189-194.
33. Ascaso C, Wierzchos J, Souza-Egipsy V, Rios A, Delgado Rodrigues J. In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon). *Int Biodeterior Biodegradation* 2002; 49: 1-12.
34. Piñar G, Lubitz W (2004). Molecular techniques: aplication to the analysis of microbial communities colonising art works and to the monitoring of changes. case study: Wall Paintings of the Castle of Herberstein. In:*European Research on Cultural Heritage. State-of-the-Art Studies. Vol.2*. Ed, M Drdácký. Academy of Sciences of the Czech Republic, 1st Ed. Prague, Czech Republic, pp. 421-432.
35. DorniedenTh, Gorbushina AA, Krumbein WE (2000). Patina- physical and chemical interactions of sub-aerial biofilms with objects of Art, In: *Of Microbes and Art: The Role of Microbial Communities in the Degradation and Protection of Cultural Heritage*. Ed, O Ciferri, P Tiano, G Mastromei. Springer Science & Business Media, 1st ed. New York, USA, pp. 105-120.
36. Liu D, Coloe S, Baird R, Pedersen J. Rapid mini-preparation of fungal DNA for PCR. *J Clin Microbiol*2000; 38: 471.
37. Herrera LK, Videla HA. Surface analysis and materials characterization for the study of biodeterioration and weathering effects on cultural property. *Int Biodeterior Biodegradation* 2009; 6: 813-822.
38. Watanabe T (2010). Pictorial Atlas of Soil and Seed Fungi. 3rd ed. CRC Press. No3ew York, USA.