



Molecular Identification of deteriorating *Aspergillus* spp. from sandstone monuments

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Abstract: Biodeterioration is irreversible damage that is caused by the colonization of bio communities on the surface of sandstone monuments. Out of all bio communities, fungi play the most vital role in the deterioration of sandstone. Fungi can colonize on the surface of sandstone and by releasing many types of inorganic and organic acids, pigments and enzymes, can changing not only the surface of the materials but the color of the sandstone. Moreover, mycelia of fungi can penetrate into the internal surface of sandstone and change the interior chemical contents of sandstone. This study was focused on the identification of fungi that were colonized on the sandstone monument. The samples were collected from different monument places built during different time periods and analyzed using cellophane tape methods and swabbing & serial dilution methods. A sampling of stone was carried out to identify inhibiting microorganisms. Biochemical and microscopic methods were used for isolated strains of samples. In addition, the Polymerase Chain Reaction (PCR) and sequencing of the PCR products were done. Finally, the phylogenetic tree was constructed base on the sequences of ITS region. We identify high microbial diversity in fungal groups. The fungal commodity structure differed among the samples. The inhabiting fungi which isolated from sandstone monuments belong to high microbial diversity fungal groups. The result revealed that the strain exhibited a high level of 18S rRNA similarity with *Aspergillus costaricensis*, *Aspergillus luchuensis* was identified as a close relative to *Aspergillus*.

Keywords: Biodeterioration; fungi; *Aspergillus* spp.; PCR

Introduction

Biological agent's microfloras in co-relation, ranging from synergistic to antagonistic, have critical importance, in stone deterioration. They can cause diverse damages on the sandstone surface, by formation of biofilms, chemical reactions with substrate, physical penetration into the substrate as well as pigments production. (El-Derby *et al.*, 2016 and Gupta *et al.*, 2013). During the recent decades there has been a general concern about the deterioration of heritage monuments in India. The knowledge and understanding of the role of microbes in biodeterioration of historic monuments and artworks extended by the geomicrobiology (Dakal and cameotra 2012). Biodeterioration

by biocommunities is predominately noticed not only on cultural heritage, stone artifacts such as historical monuments but on modern materials, private collections, museums and buildings (Lan *et al.*, 2010; Abd El-Ghany, 2013; Gu *et al.*, 2013). Prokaryotic and eukaryotic both microorganisms cause the deterioration of sandstone monuments.

Fungal population on the stone surfaces belongs to the different genera including *Cladosporium* sp. *Aureobasidium* sp. *Alternaria* sp. *Trichoderma* sp. *Penicillium* sp. *Exophila* sp. *Fusarium* sp. *phialophora* sp. *Cryptococcus* sp. and *Phoma* sp.

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and based on stone type these population will change (Mohammadi and maghbolibasjin, 2014). This study was focused on the molecular identification of fungi that were colonized on the sandstone monument.

Materials and Methods

Study area and Sampling

We selected samples of sandstone were collected from five localities such as Red Fort (Agra), Akbar Tomb (Agra), Fatehpur Sikri (Fatehpur), Mariam Tomb (Agra) and Etma Ud Daula (Agra) from India.

Isolation of microflora

After a caution examination seeable degradation and alterations were graphed and the samples were collected for mycological analyses by swabbing surfaces with sterile cotton swabs with the help of sterilized tools (scalpes, rushes, swab and cellophane tape) (Othman AS. 2015) and are preserved at 4°C until the time of analysis in the laboratory.

Swabbing and serial dilution method (Nigam, 1965): In this method the surface of deteriorated sandstone sample was swabbed by sterilized moist cotton and shaken in 10 ml of sterilized distilled water. Serial dilutions 10^{-2} , 10^{-3} to 10^{-7} were made by pipetting measured volumes (1ml) into additional dilution blanks (having 9ml sterile water). Finally, 1 ml aliquots of various dilutions were added 20 ml of the sterile, cool molten (45°C) media (Czapeck-dox agar/ rose Bengal agar for fungi and Nutrient agar for bacteria). The dilution 10^{-2} to 10^{-5} were selected for enumeration of fungi and 10^{-4} to 10^{-7} for bacteria. Upon solidification, the plat was incubated at 25°C for fungi and $35\pm 1^\circ\text{C}$ (for bacteria) for 3-7 days and 24-72 hours respectively. Only with the difference incubation conditions, 30-35°C temperature, light of 60W tungsten, 15-20 days and grown in Beneck's broth priegsheim and modified Knop's broth

Molecular and morphological identification of fungus:

DNA isolation, PCR using universal primers for the type of organism, purification of the PCR amplicons, cycle sequencing reactions, purification and run them on an automated capillary-based Sanger DNA Sequencing system. At every step, there is in-house quality check to ensure success of the sequencing reactions. Post sequencing, fragments are manually checked and only good quality sequences are used to form contigs, which are then matched in well-curated databases for assigning closest neighbor.

Results

Investigation is taken from five localities of different monuments resulted to analyze the effect on stone due to many Physical, chemical and biological factors. The alterations shown in the form of black patina, rusting of stone exfoliation.

Before analysis of molecular results, Polymerase Chain Reaction products were prepared and loaded on agarose gel. The sequence results, analyzed just in frontward path (ITS), 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. A phylogenetic tree and neighbor-joining phylogenies were constructed by using the MEGA software package and bootstrapping was used to estimation the trustworthiness of the phylogenetic reconstructions. The trees were shown in Figure 3. Among ribosomal systronic areas, ITS region is the best area for identifying fungi (Schoch *et al.*).

The result revealed the isolation and identification of fungal strain that relate to *Aspergillus*. These results are similar to those reported by Mohammad *et al.*, (2014) similar results were

showed by Seth *et al.*, (2016)., The presence of *Aspergillus sp.* that include *Aspergillus costaricaensis* and *Aspergillus luchuensis*.

Identification:

From 5 monuments about 2 fungus were isolated, purified and identified as, *Aspergillus costaricaensis* and *Aspergillus luchuensis* sp. 1 and sp 2. The identification test for 5 most common fungus sp. Is given in table - A - *costaricaensis* isolates (gene bank accession no. MH862988.1) (Table A). The second fungal strain exhibit level of 18S rRNA similarity (98.16%) with *Aspergillus*

costaricaensis isolates (Gene bank Accession no. MH862988.1) next fungal strain showed high level of 18S rRNA similarity (99.07%) *Aspergillus costaricaensis* and *Aspergillus luchuensis* isolates (gene bank accession no. MH862988.1, NR_135449.1). Forth one detected 18S rRNA similarity (98.64%) with *Aspergillus costaricaensis* isolates (gene bank accession no. MH862988.1) Another strain revealed a high level of 18S rRNA similarity (99.2%) with *Aspergillus costaricaensis* and *Aspergillus luchuensis* isolates (gene accession no. MH862988.1, NR_135449.1).

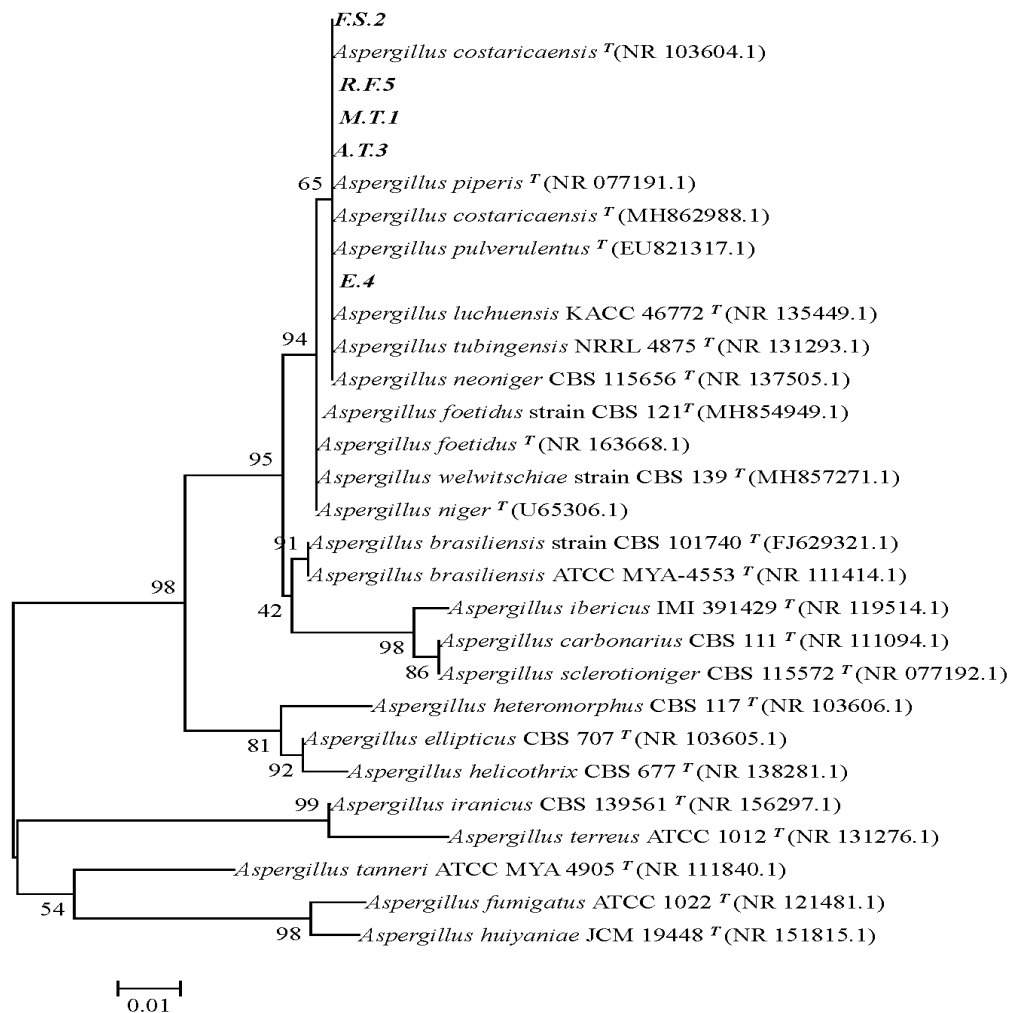


Figure 3. Phylogenetic tree showing the relationship of closely relative species constructed using the neighbor-joining method and based on 18s rRNA genes sequences. Isolate is closely related to *Aspergillus*

Table A: Identification of fungus

PRN	Strain No.	Closest Neighbour	Accession No	%Similarity
A_MAR_19_112	M.T.1	<i>Aspergillus costaricensis</i> CBS 115574	MH862988.1	98.60
A_MAR_19_113	F.S.2	<i>Aspergillus costaricensis</i> CBS 115574	MH862988.1	98.16
A_MAR_19_114	A.T.3	<i>Aspergillus costaricensis</i> CBS 115574	MH862988.1	99.07
A_MAR_19_114	A.T.3	<i>Aspergillus luchuensis</i> KACC 46772	NR_135449.1	99.07
A_MAR_19_115	E.4	<i>Aspergillus costaricensis</i> CBS 115574	MH862988.1	98.64
A_MAR_19_116	R.F.5	<i>Aspergillus costaricensis</i> CBS 115574	MH862988.1	99.22
A_MAR_19_116	R.F.5	<i>Aspergillus luchuensis</i> KACC 46772	NR_135449.1	99.22

Table B. Nucleotide sequence of 18S rRNA gene of *Aspergillus sp.*

Fungi name	Sequence
<i>Aspergillus costaricensis</i> CBS 115574	CGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCACCTCCATCCGT GTCTATTATAACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCC GGGGGGGCGCCTTTGCCCGGGCCCGTGCCCGCCGAGACCCCAA CACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAAT CAGTAAAACCTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG AACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAA TCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC ATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTG GGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCAGAAAGGCAGCGGCG GCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGT AGGATTGGCCGGCGCCTGCCGACGTTTCCAACCATTTCCAGGtGACC TCGGATCAGTAGGGATAACCGCTGAACTTAAGCATATCAT
<i>Aspergillus costaricensis</i> CBS 115574	CTGCGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCACCCCATC CGTGTCTATTATAACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCC GCCGGGGGGGCGCCTTTGCCCGGGCCCGTGCCCGCCGAGACC CCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATG CAATCAGTAAAACCTTCAACAATGGATCTCTTGGTTCCGGCATCGAT GAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTC GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGG GGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGT GTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCAGAAAGGCA GCGGCGGACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACAT GCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTCCAACCATTTCCAG GTGACCTCGATCAGTA

<i>Aspergillus costaricensis</i> CBS 115574	<p>AAGGATCATTACCGAGTGC GGGTCCTTTGGGCCCAACCTCCCATCCG TGCTATTATACCCIGTTGCTTCGGCGGGCCCGCCGCTTGTTCGGCCGC CGGGGGGGCGCCTTTGCCCCCGGGCCCGTGCCCGCCGGAGACCCC AACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCA ATCAGTTAAAACCTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGA AGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGT GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGG GGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGT GTTGGGTCCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAG CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTACATG CTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTCCAACCATTTTCCAG GTGACCTCGATCAG</p>
<i>Aspergillus luchuensis</i> KACC 46772	<p>CGGCGGGCCCGCCGAAGCAACAGGGTATAATAGACACGGATGGGA GGTTGGGCCCAAAGGACCCGCACTCGGTAATGATCTTTCTGTAGGTG AACCTGCGGAAGGATCATTACCGAGTGC GGGTCCTTTGGGCCCAACC TCCATCCGIGTCTATTATACCCIGTTGCTTCGGCGGGCCCGCCGCTTG TCGGCCCGGGGGGGCGCCTTTGCCCCCGGGCCCGTGCCCGCCGG AGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATT GAATGCAATCAGTTAAAACCTTTCAACAATGGATCTCTTGGTTCCGGC ATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAG AATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGT ATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCC GGCTTGTGTGTTGGGTCCCGTCCCCCTCTCCGGGGGGACGGGCCCG AAAGGCAGCGGCGGCACCGCGTCCGATCCTCRAGCGTATGGGGCTTT GTCACATGtCTCTGTAgAGATTGGCCGGCGCCcTGCCGACGTTTtCCAA CCATTTTTtCCAGGTTGACCCTCGGATCAGGTAGGGATAACCCGCTGAA CTTAAGCCATAA</p>
<i>Aspergillus costaricensis</i> CBS 115574	<p>GGAAATGGTTGAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGC ATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCG CTGCCCTTCGGGCCCGTCCCCCGGAGAGGGGGACGGCGACCCAAC ACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGC CCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGA TTCCTGAATTCTGCAATTCACATTAGTTATCGCATTTTCGCTGCGTTCT TCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTAACTGA TTGCATTCAATCAACTCAGACTGCACGCTTTCAGACAGTGTTCGTGTT GGGGTCTCCGGCGGGCACGGGCCCGGGGGCAAAGGCGCCCCCCCCG GCGGCCGACAAGCGGCGGGCCCGCCGAAGCAACAGGGTATAATAG ACACGGATGGAGGTGGCCCAAAGGACCCCGCaCTCGGTAATGATCCTT CC</p>

Discussion

Biological infections and the intensity of permeability, environmental conditions of the biodeterioration processes are strongly affected by water availability. This is determined by Material specific parameters, like porosity and site and exposure of the object (Warscheid and Braams 2000).

In molecular technique to identification of biocommunities is sequencing of small subunits of 16s, 18S ribosomal RNA and its region. In this study sequencing of ITS was successfully done on 5 fungi. ITS is known as the standard region for this study.

Studies revealed that, general fungi population on the stone surfaces belong to the different genera including *Aspergillus costaricensis* and *Aspergillus luchuensis*. Seth *et al.*, (2016) recorded 8 species of *Aspergillus* viz. *Aspergillus luchuensis*, *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus varicolor*, *Aspergillus awamori* and *Aspergillus niveus*. Abdel Ghany *et al.*, (2019) revealed the presence of numerous fungal spp. including *Aspergillus niger*, *A. fumigatus*, *A. sulphureus* and *A. flavus*. Furthermore, the fungal swabs cultures from False door contain the same fungal species of *aspergilli*, with the adding up to other genera including *Alternaria alternata*, *Alternaria sp.* and *Cladosporium herbarum*.

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