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ORIGINAL ARTICLE **OPEN ACCESS**

Molecular Identification and Myco-ecological Study of Fungus from Sandstone Monuments

Swati Shakya[1], Neelam Tripathi[1], Seema Bhadauria[2]
1-Department of Biotechnology: Sri Satya Sai University of Technology and Medical Science, Sehore Bhopal (M.P.)
2-Department of Botany, RBS College, Agra
Email: shakyaswati@yahoo.com

ABSTRACT
Cultural heritage monuments may be degraded and discolored by growth and activity of living organisms. Biocommunities form biofilms on surfaces of sandstone, with resulting damage in structure and aesthetic. The organisms involved are bacteria fungi, archaea, algae, and lichens. Among all biocommunities ability in production of organic acids and pigments have crucial role in discoloration and degradation of different types of sandstone in historical monuments. This investigation focuses on myco-ecological analyses of microbial bio-film from different sites and the identification of fungi that were colonized on the sandstone monument. . The five (05) fungal organisms with specific distribution on sandstone monuments were isolated. Microorganisms from Ascomycotina as well as Deuteromycotina were more frequent. The most frequent isolated fungus from these sandstone monuments are Aspergillus costaricaensis, Aspergillus luchuensis, Aspergillus aflatoxiformans isolate DTO 228-G2, Rhizopus sexualis var.americanus, Rhizopus oryzae CBS 111.07 was identified as a close relative to Aspergillus was common in almost all the sandstone structures of this monument. The RAPD PCR techniques seems to be practical and well organized for routine use in high resolution microbial analysis by giving assemblage comparisons through sequence information proving or fingerprinting. The relative frequency and frequency of these fungal species correlated with deteriorated historical sandstone monuments site contributes beneficial data for future studies.
Keywords: Degradation; deterioration; sandstone monuments; myco-ecological; biofilm

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INTRODUCTION

Deterioration of sandstone monuments including, physical, chemical factors, and microbial growth has been reported in many research to understanding the deterioration agents [1,2]. The colonization of sandstone monuments by biocommunities depends on environmental factors such as nutrient sources, water availability, pH and climatic exposure, and they can cause diverse damages on the sandstone surface, such as: chemical reactions with substrate, formation of biofilms, physical penetration into the substrate as well as pigments production [3] Fungi are known as one of the most harmful group of organism associated to biodeterioration of sandstone surfaces. The ability of fungi to colonize the substrate is greatly enhanced by different factor among which the most important are the environmental factors water availability and nutrients, special orientation and bio receptivity of artifact surface. Stone being inorganic in nature does not favor the growth of fungi. However, the presences of organic residues on stone encourage their growth. Fungi are believed to be potential contributors to decay of limestone, silicate minerals (mica and orthoclase), and iron and magnesium bearing minerals, but the extent of total decay attributed solely to them is undetermined.

Apart from that, their occurrence manifest as colour change and black spots on rock surface. The word 'patina' is also used for densely coloured layer covering the original architectural or sculptural surface. The chemo-organotrophic fungal microbiota contributes many stable and lasting organic pigments to the rock surfaces The study of biocommunities in monuments is usually accomplished by using standard culture methods . However , It is believed that only a small percent age of extant microorganism have been discovered, and that cultured methods are in adequate for studying

biocommunities composition[4]. Biocommunities in outdoor stone monuments represents a complex ecosystem including bacteria, algae, fungi, lichens. Biocommunities colonize sand stonework whenever the conditions of temperature, nutrition, moisture, and light are favorable. When a microorganisms colonization is observable, the conservator should substantiate at which expanse it deteriorate the materials and know the non-biogenic agents that take part in the degradation[5]. Many causes have same effects, act in interact, or synergy in quantitatively fluctuating relations. Thus, the significant of biological impact to the entire deterioration process should be estimate very carefully. Observing microorganisms on cultural heritage objects does not automatically assume that they actually change the physical properties or chemical composition of the sandstone [6].

A survey of the art and architecture of the Indian subcontinent, indicates the stones chosen for building, sculpture and ornamentation, were often associated with prominent geographic and geological features of the local areas. The scraps of vindhya Mountains are the prominent feature of the central Indian states. These hills comprise of sandstone and quartzite that provided the structural and sculptural materials used through to this day for building[7]. There are three types of rock stones i.e Ignous rocks, sedimentary rocks and metamorphic rocks, which are used in building constructions. However the study is based on sandstone constructions which are colonized by the microorganisms.

MATERIAL AND METHODS

The present paper deals with various methods used in analysis of biodeterioration of sandstone monument. The methodologies involve the identification of algae, fungi, bacteria and angiosperm.

Sampling

Samples of sandstone were collected from eleven localities: Red Fort (Agra), Akbar Tomb (Agra), Fatehpur Sikri (Fatehpur), Mariam Tomb (Agra),Etma Ud Daula (Agra), St. Johns(Agra), Kailash Temple(Agra), 64 Khamba(Agra), Ochha Temple(Jhansi), Khas Mahal (Fatehpur Sikri and some unidentified monuments. Under the observation visible degradation and alteration were mapped and after that the samples were collected. Sandstone sample were taken for mycological analyses by swabbing surfaces with sterile cotton swabs. The samples were then stored at 4°C.

Isolation of microflora

The samples were collected with the help of sterilized tools (scalpes, rushes, swab and cellophone tape) these are preserved at 4°C until the time of analysis in the laboratory. In the present study isolated was performed directly from the monuments and from collected deterioration sandstone samples.

Historical monument

Scrapping method: The area sampled exhibited black and Browm sports distributed on sandstone surface. These samples were taken from the surface of the stone using sterile scalpels and lancets and scrapping of the surface material to a depth of 1-3 mm, and then transported to the laboratory in sterile vials.

Cellophane tape method: The sampling of fungal growth directly from the affected sandstone wall with the help of stickly tape. The sticky tape directly removes the powdered stone together with fungul fruiting bodies. In this way, direct identification of fungi becomes much easier. These samples were then cultured in the laboratory for further investigation with the help of microscope.

Swabbing and serial dilution method: 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}……10^{-7} were made by pipetting measured volumes (1ml) into additional dilution blanks (having 9ml sterile water). Finally 1 ml aliquots of various dilution 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 plate were incubated at 25°C for fungi and 35±1°C (for bacteria) for 3-7 days and 24 -72 hours respectively.

The convenient techniques used for bacteria and fungi were applicable to microscopic algae too. Only with the difference incubation conditions, 30-35°C temperature, light of 60W tungustun, 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 as the tentative identification of your submitted organism.

Investigating the Inter-species Diversity by RAPD-PCR Techniques

The RAPD-PCR reaction for investing the inter-species diversity was carried out fungus specimens extracted from sand stone monument using five different primer.

Table 1. The isolated fungi

Isolated Fungi/Moss	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	F%	RF%	d=S/√N
Aspergillus Costaricaensis CBS 115574	+	+	+	+	+	-	-	-	-	-	-	45.45	38.46	
Aspergillus Luchuensis KACC 46772	-	-	+	-	+	-	-	-	-	-	-	18.18	15.38	
Aspergillus Aflatoxiformans isolate DTO 228-G2	-	-	-	-	-	+	+	-	-	-	-	18.18	15.38	0.50
Rhizopus Sexualis Var. Americanus	-	-	-	-	-	-	-	+	+	-	+	27.27	23.07	
Rhizopus Oryzae CBS 112.07	-	-	-	-	-	-	-	-	-	+	-	9.09	7.69	
Total												118.17	99.98	

Calculations

Various myco-ecological characters have been calculated using the following formulae:

$$\mathbf{F=100\times\frac{N_1}{N_2}}$$

Where

F = total frequency of organism in %,

N_1 = number of samples in which specific organism occurred

N_2 = total number of samples examined

$$\mathbf{RF= 100\times\frac{F_i}{F}}$$

where

RF = relative frequency in %

F_i = frequency (in %) of individual organism

$$\mathbf{d = \frac{S}{\sqrt{N}}}$$

where

d = variety richness index

S = total number of species.

N = value of total relative frequencies of all fungal

RESULTS AND DISCUSSION

Investigation 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. The present paper stated that microbial deterioration of archeological sandstone sample which is taken from five localities. The result revealed the isolation and molecular identification of fungal strain that relate to Aspergillus. These results are similar to those reported by [8] similar results were showed by [9].The presence of *Aspergillus sp.* that include *Aspergillus costaricaensis* and *Aspergillus luchuensis.*

Fig .1 Isolated fungi

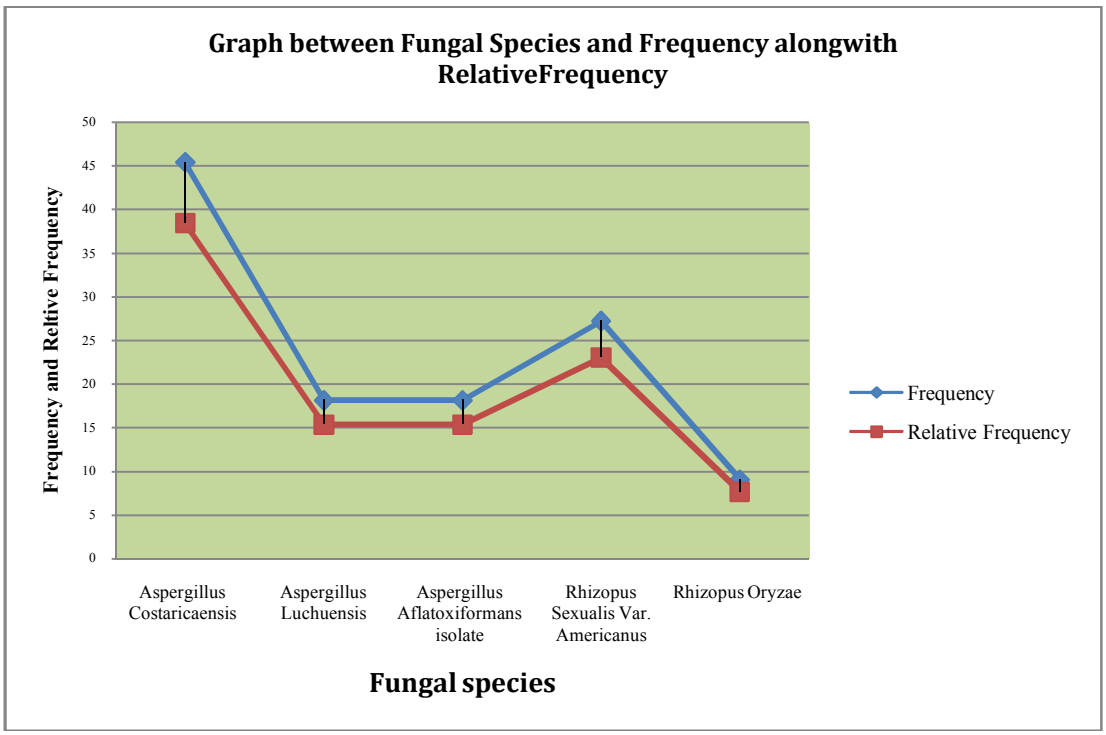

Fig.2 Frequency and Relative frequency of the fungal species

Aspergillus coastaricaensis shows maximum frequency followed by *Rhizopus Oryzae and Aspergillus Aflatoxiformans*. Some of the fungal species are confined to particular area. These confinements of fungal species depend on environmental conditions of the area, which varies from geographical area to area. In the present study *Aspergillus* species are the most common species found in the sites. The Red and black colour of the stone surfaces is not only due to dematiceous fungi but very frequently it is due to the endolithic phototrophic microorganisms like cyanobacteria and algae. Dark pigments (in case of Fungi) protect algal cells against Ultra Violate (UV) rays besides other stress factors.

Value of index of variety richness i.e. 0.50 revealed that the studied fungal community was significant. In each fungal community all the species are not equally important. There are relatively very few of these fungal species, as determine the nature of the community. These few species exert a major controlling influence on the community and also play important role in deterioration of various substrates.

The Composition of fungal organism varies depends upon nature of host, degree of competition between the fungal organisms and the environmental conditions. The frequency and relative frequency are directly or indirectly correlated with meteorological data and climatic conditions.

Table-2 Primer Details

No.	Oligo Name	Sequence (5`à 3`)	Tm (°C)	GC-Content
1	OPA2	TGCCGAGCTG	34	70%
2	OPA3	GGGTCCAAAG	32	60%
3	OPA4	AATCGGGCTG	25	60%
4	OPA5	AGGGGTCTTG	32	60%
5	OPA6	GGTCCCTGAC	27	70%
6	OPA10	GTGATCGCAG	25	60%
7	OPB4	GGACTGGAGT	25	60%
8	OPB5	TGCGCCCTTC	27	70%
9	OPB6	TGCTCTGCCC	27	70%
10	OPB10	CTGCTGGGAC	27	70%

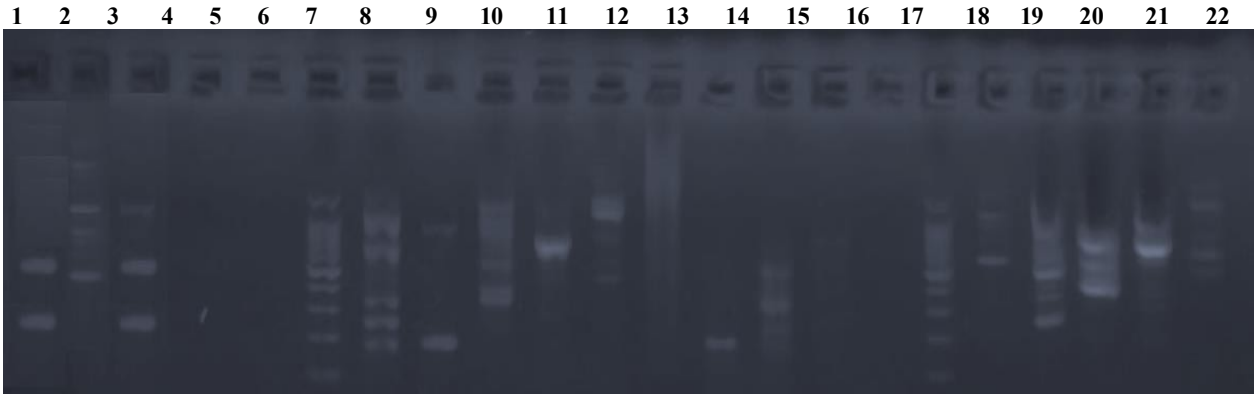

Fig-5: Gel Electrophoresis

Table-3 Gel Description

1. IST-OPB1	12. IIIOT-OPB10
2. IST-OPA2	13. IIIOT-OPA2
3. IST-OPA3	14. IIIOT-OPA3
4. IST-OPA4	15. IIIOT-OPA4
5. IST-OPA5	16. IIIOT-OPA5
6. Ladder(100bp)	17. Ladder(100)
7. IIKT-OPB10	18. IVJM-OPA6
8. IIKT-OPA2	19. IVJM-OPA2
9. IIKT-OPA3	20. IVJM-OPA3
10. IIKT-OPA4	21. IVJM-OPA4
11. IIKT-OPA5	22. IVJM-OPA5

UPGMA- Result

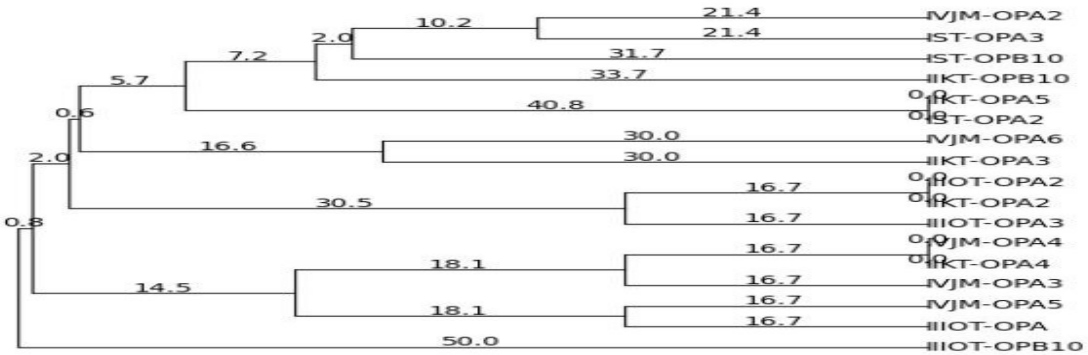

Fig-6 UPGMA Clustering of isolated Based on Similarity Coefficient from RAPD Markers.

Neighbor Joining Result

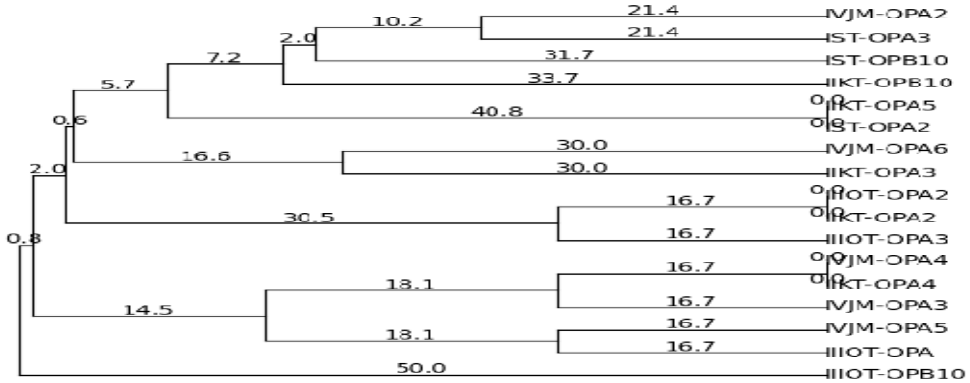

Fig-7

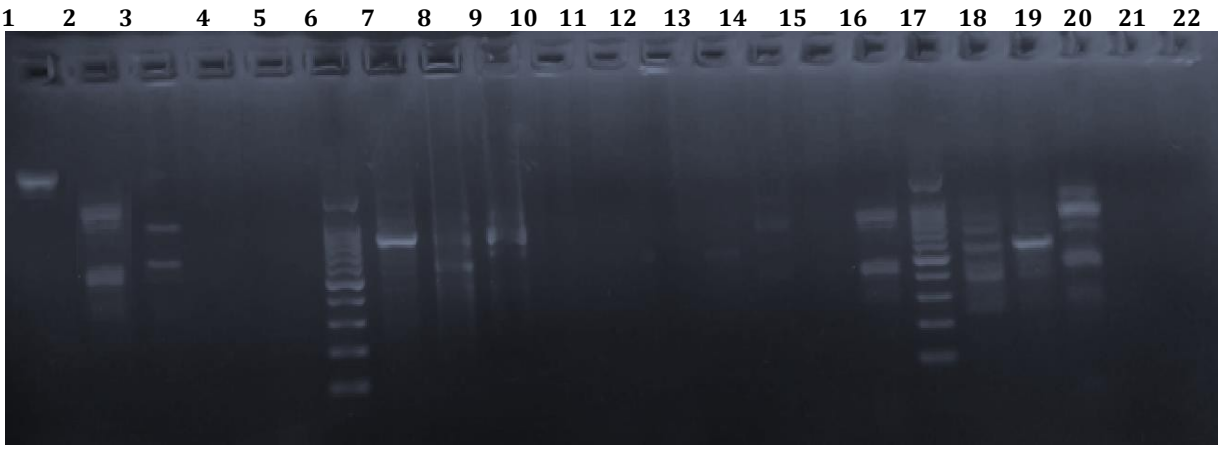

Fig-8

Table-4 Gel Description:

1. V64K-OPA2	12. E4-OPA2
2. V64K-OPA3	13. E4-OPA3
3. V64K-OPA4	14. E4-OPA4
4. V64K-OPA5	15. E4-OPA5
5. V64K-OPB10	16. E4-OPA10
6. Ladder(100bp)	17. Ladder(100)
7. VIKHM-OPA2	18. FS2-OPA2
8. VIKHM-OPA3	19. FS2-OPA3
9. VIKHM-OPA4	20. FS2-OPA4
10. VIKHM-OPB3	21. FS2-OPA5
11. VIKHM-OPA10	22. FS2-OPB10

UPGMA- Result

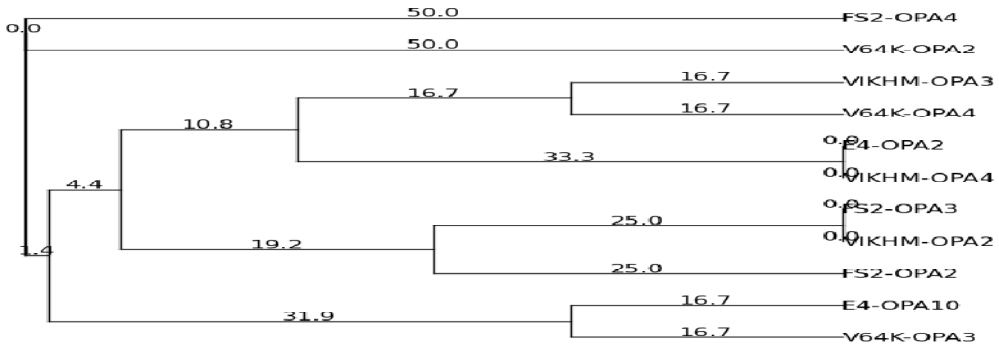

Fig-9 UPGMA Clustering of isolated Based on Similarity Coefficient from RAPD Markers.

Neighbor Joining Result

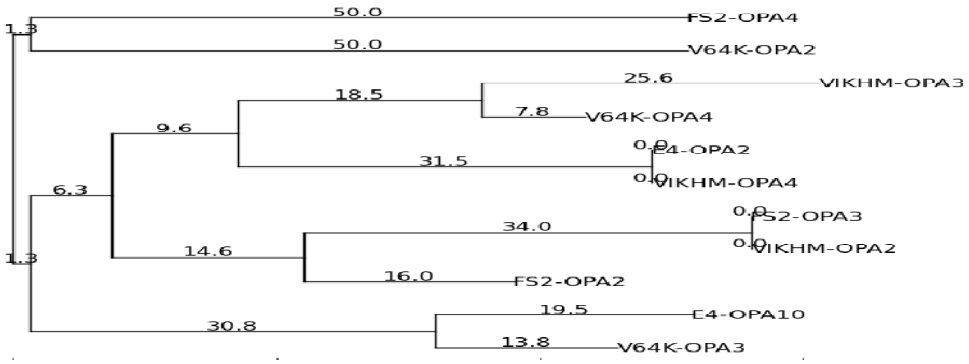

Fig-10

The taxonomy and diversity of *Aspergillus* fungi have been primarily studied using traditional morphological methods and rRNA sequences. These studies identified that the type of fungi that deteriorate sandstone monuments a highly reliable indicator about the evolutionary relationships among these species. Currently, available genomic sequences of aspergillus have made it possible for the evolutionary study at genomic level. The combined use of aspergillus sp. specific primers and culture-based techniques may benefit future studies of fungi .

Randomly Amplified Polymorphic DNA (RAPD) is a relatively easy technique and has been frequently used for genetic characterization and identification of individual isolates of fungi. The objective of the present study was to molecular identification and myco-ecological study of fungi, collected from different site. For this purpose one isolate of *Aspergillus sp.* was analyzed using 5 RAPD primers. All the tested genotypes in their RAPD assay generated variety of amplification products. Level of genetic polymorphism among the genotypes detected during present study varied from primer to primer. The quantification of PCR products by conventional PCR is limited, because during exponential amplification of the template, small variations in amplification efficiency can drastically change the yield of the PCR product, resulting in a non-quantitative assay. Co-amplification of the molecule of interest with a known amount of competitor molecules bearing primer sites identical to those of the target allows reproducible quantification of templates. The difference in size of the original PCR.

CONCLUSION

Fungal species were isolated from different sandstone monuments. The isolated species first identified morphologically and then tested. *Aspergillus* and *Rhizopus* species were identified genetically by sequencing-550 base pair and using RAPD Technique.

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