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Fungal diversity in the temple with an Internal Transcribed Spacer (ITS) molecular and morphological approach

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Abstract. *Wijayanti C, Kuswytasari ND, Shovitri M, Danilyan E, Zulaika E. 2024. Fungal diversity in the temple with an Internal Transcribed Spacer (ITS) molecular and morphological approach. Biodiversitas 25: 3151-3161.* Red bricks and andesite stones comprise most of Indonesia's temple architecture. Red brick is a highly porous building material that weathers rapidly in temples. Physical and biological factors are the main causes of temple weathering. Rain and other physical elements can raise the temple's humidity, encouraging the growth of lichen and fungus. Many studies have been conducted to investigate the fungal variety in temples made of andesite stones. Still, the diversity found in these studies has only been identified at the genus level. This research aimed to determine the variety of fungi in the temples by employing morphological and molecular approaches like Internal Transcribed Spacer (ITS) biomarkers. Macroscopic characterization involves visual inspection of a colony's color, edges, texture, diameter, shape, and surface for morphological identification. Microscopic characterization uses the slide culture method to observe reproductive and hyphal structures. Molecular identification includes DNA extraction with the Genomic ex Promega Corp Wizard kit, ITS marker-based DNA amplification, and visualization with a Gel Documenter, DNA sequencing, BLAST searches for species identification, and phylogenetic tree reconstruction using MEGA 11. Class *Eurotiomycetes*, which includes *Talaromyces funiculosus* (CB L1B isolate), *Talaromyces purpureogenus* (CB L3A isolate), *Penicillium oxalicum* (CB L4A isolate), *Penicillium cataractarum* (CT TL3 isolate), and *Aspergillus aflatoxiformans* (CT TL4 isolate), was the most prevalent among the identified species from the classes *Sordariomycetes* and *Eurotiomycetes*. *Purpureocillium lilacinum* (CBL1C isolate), *Trichoderma anaharzianum* (CB L3B isolate), and *Fusarium equiseti* (CT L41 isolate) were the discovered species from the Sordariomycetes class.

Keywords: Diversity, fungi, ITS, morphology, phylogenetic, relics

INTRODUCTION

Many historical and ancient relics are found in Indonesia, including the temple. The main materials used to make temple buildings are red bricks, andesite, and limestone; red bricks are building materials derived from clay (Siregar et al. 2023). Bricks have mechanical properties of absorption, which is the maximum ability of bricks to store or absorb water. Bricks are easily weathered compared to other materials because they have relatively high porosity, between 11-40% in contemporary buildings and 30-38% in historical buildings (Elert et al. 2003; Martinez et al. 2016; Fan et al. 2019). In addition to the material that makes up the rock, the location of the temple building in the open area also contributes to weathering, where the temple building's open location allows the surface of the temple stone to interact directly with the environment, making it vulnerable to damage. The most common damage or weathering of temple rocks is due to physical and biological weathering. Physical factors such as rainwater can increase the humidity in the temple rocks, leading to fungi and lichen flourish (Pinna 2021).

The fungi's role in rock weathering has been a subject of interest in various studies. Fungi can colonize the surface of rocks by penetrating hyphae on rock substrates, secreting enzymes, acids, and organic and inorganic pigments that can cause biological weathering, resulting in the rocks' discoloration (Warscheid and Braams 2000). Rock-surface fungi can act as significant biochemical weathering agents, producing lichen acids and chelating iron. TunTschu and Ching (1998) tested ectomycorrhizal fungi and associated microorganisms for their ability to weather limestone, marble, and calcium phosphate, and most fungi are ectomycorrhizal. van Schöll et al. (2008) discussed the role of "rock-eating" mycorrhizas in plant nutrition, biogeochemical cycles, and pedogenesis, highlighting that surface weathering by ectomycorrhizal fungi is more important than mineral tunneling. Fomina et al. (2010) demonstrated the ability of fungi to weather rocks through the excretion of organic acids and other metabolites, visualizing fungal tunnels and secondary mineral precipitation. Gerrits (2019) studied fungal olivine weathering, highlighting the role of fungi as mineral weathering agents and partners of phototrophs. Gerrits et al. (2020) investigated how the rock-inhabiting fungus *K. petricola* A95 enhances olivine dissolution through attachment, emphasizing the importance of fungal traits in weathering processes. The deposition of secondary minerals (carbonates and oxalates) in rocks formed during fungal colonization to form crusts on the rock surface can increase cracks and damage (Fomina and Gadd 2003).

Several studies have explored the diversity of fungal species that cause weathering in temple rocks (Gupta and Sharma 2011; Fan et al. 2019). Pangesti et al. (2022) obtained 7 genera of fungi on the rocks of Mendut Temple, where this temple is composed of andesite stone material. Fungal genera found include *Penicillium*, *Aspergillus*, *Acremonium*, *Scopulariopsis*, *Chrysonilia*, *Mucor*, and *Arthrinium*. The diversity identification in the study is still limited to the genus level; therefore, this study identifies the diversity of fungi in temple rocks with red brick constituent materials at the species level.

The phylogenetics approach is often used in systematics biology to understand the diversity of living things by reconstructing their genetic relationships (Ziemert and Jensen 2012). Therefore, fungi diversity can be studied with morphological and molecular approaches. Internal Transcribed Spacer (ITS) is a molecular approach which commonly used and according to Odorico and Miller (1997), this method has been utilized to determine molecular systematics at the species level. Mycologists use the ITS region as a standard DNA barcoding for fungi. Compared to other rDNA regions, the ITS region exhibits a higher rate of sequence variation, making it an excellent choice for genus-to-species level identification (Bradshaw et al. 2023). The study aimed to determine the diversity of fungi in temple rocks through morphological and molecular approaches using the ITS. The study's findings can be utilized to learn more about the variety of fungi that can erode the temple rocks' surface and help find substitute treatments to preserve the structure's integrity.

MATERIALS AND METHODS

Sampling and isolation

The selection of Brahu Temple and Tikus Temple for this study was based on their historical significance. Purposive sampling approach was used to target areas most likely to yield significant fungal diversity and potential biodeterioration. The criteria for identifying swabbing locations included visible signs of biodeterioration, such as discoloration, staining, or visible fungal growth, and surfaces frequently exposed to environmental elements like rain, sunlight, and humidity.

Sampling of fungi was carried out on the rock surface of Brahu Temple and Tikus Temple by swab method using sterile cotton buds moistened with sterile aquades (El Jaddaoui et al. 2023). Next, the cotton bud was inserted into the zip-lock plastic bag. Isolation was carried out on Potato Dextrose Agar (PDA) medium by streak method on the surface of the medium aseptically, incubated at room temperature for 24-72 hours. Fungi that grow and have different morphologies (color, mycelium type, colony texture, and colony dominance) were purified by the point method aseptically on a PDA medium and incubated at room temperature for 7 days.

Morphological identification

The fungi identification is done by observing macroscopic characters based on the reference book "Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species." (Watanabe 2010). Microscopic characterization was carried out using slide culture techniques, including hyphal structures and reproductive structures.

Species identification using Internal Transcribed Spacer (ITS) markers

Molecular identification using ITS marker was carried out through Griya Sain's "Center for Research and Publication Services." Those identification includes DNA extraction and DNA amplification with ITS primer 1 (5' TCCGTAGGTGAACCTGCGG 3') as forward primer and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') as a reverse primer, PCR product electrophoresis, and DNA sequencing. The PCR product was then electrophoresis using 2% agarose. In the amplification results, the band's position shows the length of the DNA fragment (bp), which refers to the size of the marker (Lee et al. 2012).

Species determination and phylogenetic tree reconstruction

Sequencing results were analyzed and processed using MEGA 11 software to identify up to the species level using the online Basic Local Alignment Search Tool (BLAST) with DNA sequence matching available on the National Center for Biotechnology Information (NCBI) database. The list of identified species is selected from the top with the highest percent similarity above 97% to cross-check with literature related to habitat or species origin. If the species name has habitat similarity, the species name was determined as the identified isolate (Kwaśna et al. 2008). The isolate sequences analyzed for kinship are reconstructed phylogenetic trees using MEGA 11 software, the Neighbor-Joining Tree method, and Bootstrap 1,000 replication.

RESULTS AND DISCUSSION

Isolates purification

Out of 19 distinct fungal isolates, eight pure fungal isolates were obtained: seven from Brahu Temple and twelve from Tikus Temple. Figure 1 shows the results of the purification process, with up to three isolates originating fromTikus Temple (CT) and up to five isolates from Brahu Temple (CB).

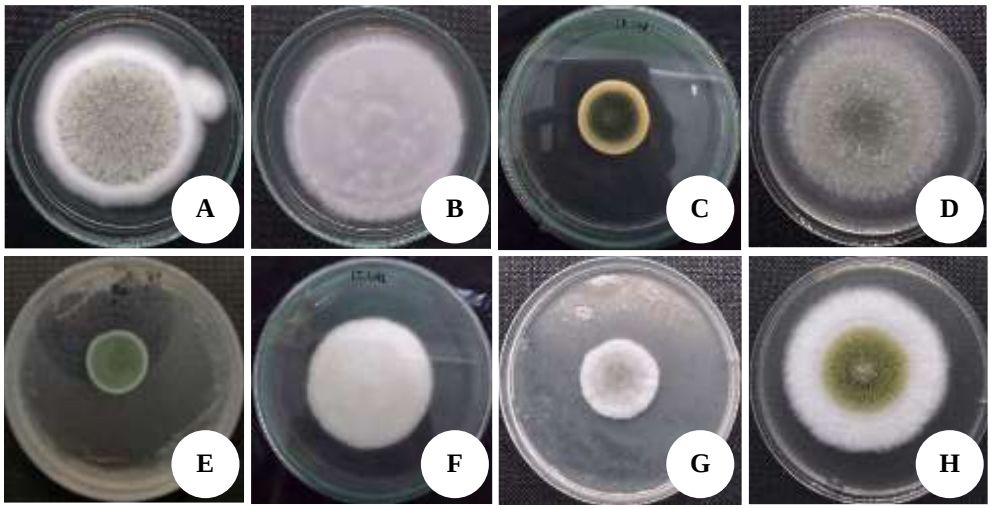


Figure 1. Purified fungal isolate: A. CB L1B Isolate; B. CB L1C Isolate; C. CB L3A Isolate; D. CB L3B Isolate; E. CB L4A Isolate; F. CT L41 Isolate; G. CT TL3 Isolate; H. CT TL4 isolate

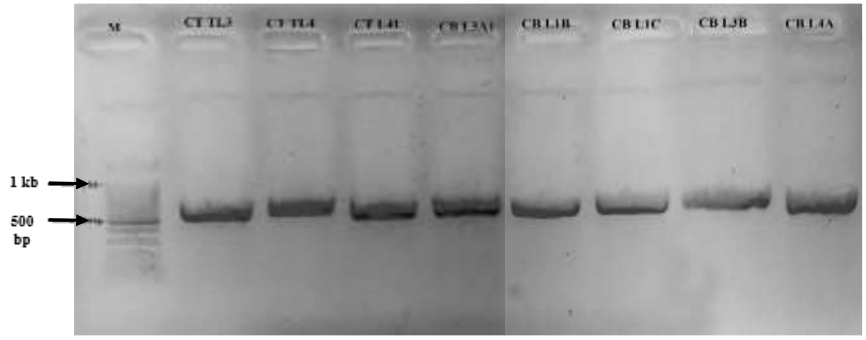


Figure 2. Visualization results of amplified DNA using Gel Doc; M: DNA Marker 1 kb

Table 1. Fungal isolate sequence similarity analysis with NCBI using BLAST

Isolate code	Nearest BLAST sequence	%Query cover	E-value	% identity	Accession number	Gap	Consensus sequence (bp)
CB L1B	*Talaromyces funiculosus* CBS 272.86	100%	0.0	98,19%	NR_103678.2	9/497	497
	Talaromyces malicola NRRL 3724	98%	0.0	98,16%	NR_165531.1	9/488	
	Talaromyces calidicanius CBS 112002	99%	0.0	97,17%	NR_103665.2	14/495	
CB L1C	*Purpureocillium lilacinum* NRRL 895	99%	0.0	99,80%	NR_165946.1	1/498	499
CB L3A	*Talaromyces purpureogenus* CBS 286.36	100%	0.0	97,90%	NR_121529.1	9/428	711
CB L3B	*Trichoderma anaharzianum* YMF 1.00383	100%	0.0	99,28%	NR_174890.1	4/554	553
	Trichoderma vermifimicola HMAS 248255	100%	0.0	99,10%	NR_171951.1	5/554	
	Trichoderma rifaii CBS 130746	100%	0.0	98,92%	NR_137305.1	6/554	
CB L4A	*Penicillium oxalicum* NRRL 787	98%	0.0	100,00%	NR_121232.1	0/497	504
	Penicillium soosanum CCF 3778	98%	0.0	98,60%	NR_173354.1	7/499	
	Penicillium araracuarense CBS 113149	97%	0.0	97,36%	NR_119814.1	13/493	
CT L41	*Fusarium equiseti* NRRL 26419	99%	0.0	99,57%	NR_121457.1	2/469	470
	Fusarium hainanense CGMCC 3.19478	92%	0.0	99,54%	NR_164597.1	2/437	
	Fusarium ipomoeae CGMCC 3.19496	92%	0.0	99,31%	NR_164596.1	3/436	
CT TL3	*Penicillium cataractarum* CBS 140974	100%	0.0	99,80%	NR_158822.1	1/501	501
	Penicillium laevigatum CGMCC 3.18801	98%	0.0	99,19%	NR_173366.1	4/495	
	Penicillium panissanguineum CBS 140989	100%	0.0	99,00%	NR_158825.1	5/501	
CT TL4	*Aspergillus flavus* ATCC 16883	100%	0.0	99,62%	NR_135329.1	2/527	716
	Aspergillus krugeri PPRI 8986	100%	0.0	99,05%	NR_135326.1	5/528	
	Aspergillus subflavus CBS 143683	99%	0.0	98,86%	NR_160622.1	6/526	

Molecular characterization

PCR product visualization with electrophoresis

PCR products were visualized using electrophoresis after amplification. The DNA on the gel was stained and viewed under UV light, revealing that all samples showed clear, single DNA bands at 500 bp, indicating successful amplification of the ITS region (ITS 1 and ITS 2) as expected (400-800 bp). Some smearing, caused by contaminants or residual extraction solutions, was observed. Sequencing was done using the reverse primer (ITS 4), and the resulting electropherograms were edited with MEGA11 to obtain complete consensus sequences. Good electropherograms had high, distinct peaks for the bases (T, C, A, G), while poor ones had low or overlapping peaks. After editing, the consensus sequences varied in length: CB L1B (497 bp), CB L1C (499 bp), CB L3A (711 bp), CB L3B (553 bp), CB L4A (504 bp), CT L41 (470 bp), CT TL3 (501 bp), and CT TL4 (716 bp) (Figure 2).

Similarity analysis using BLAST (Basic Local Alignment Search Tool)

The species identified through BLAST analysis are presented in Table 1, demonstrating a high level of homology >97% when compared to sequences in the NCBI. Additionally, species selection was guided by similarities in habitat or geographical origin.

Reconstruction of phylogenetic tree

Figure 3 presents the phylogenetic tree result of eight pure fungi isolates. The phylogenetic tree analysis used the Neighbour Joining method with Kimura's 2-parameter model and bootstrapping 1,000 replications. Six clades were formed from the Phylum Ascomycota: three from Eurotiomycetes (clades 1, 2, 3) and three from Sordariomycetes (clades 4, 5, 6). Clade 1 has two sub-clades: IA (CB L4A isolates with *Penicillium oxalicum*, bootstrap 100) and IB (CT TL3 isolates with *Penicillium cataractarum*, bootstrap 100), all in the genus *Penicillium*. Clade 2 (CT TL4 isolates with *Aspergillus flavus*, bootstrap 100). Clade 3 has two sub-clades: 3A (CB L3A isolates with *Talaromyces purpureogenus*, bootstrap 66) and 3B (CB L1B isolates with *Talaromyces funiculosus*, bootstrap 90), in the genus *Talaromyces*. Clade 4 (CB L1C isolates with *Purpureocillium lilacinum*, bootstrap 100). Clade 5 (CT L41 isolates with *Fusarium equiseti*, bootstrap 86). Clade 6 (CB L3B isolates with *Trichoderma anaharzianum*, bootstrap 100). The phylogenetic tree is reliable with bootstrap values over 70, indicating stable small clades. A value below 70 suggests possible changes in larger clades, while 25% indicates an unreliable clade.

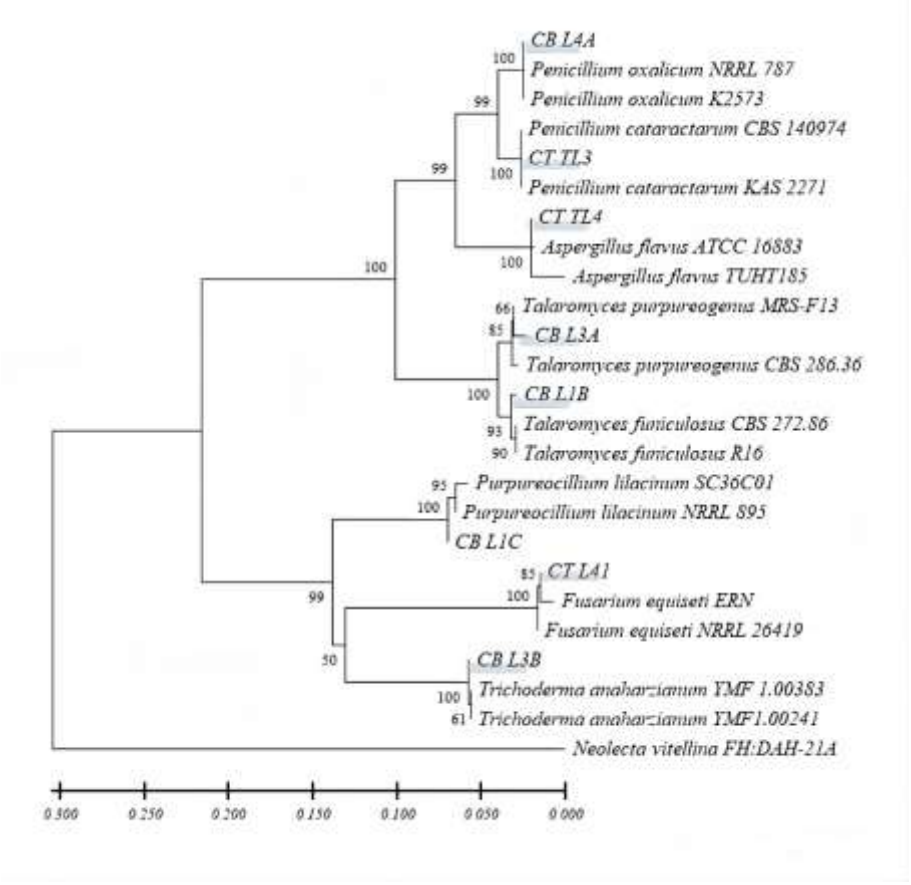
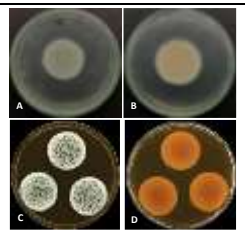
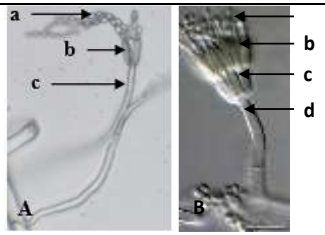
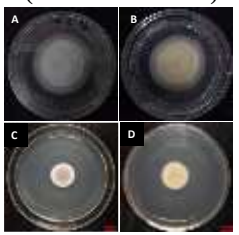
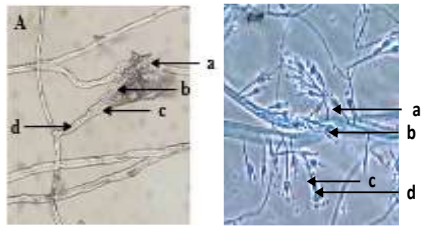
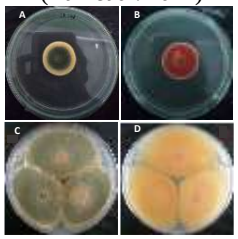
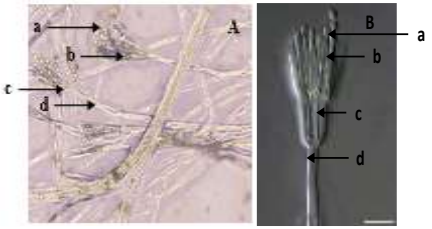
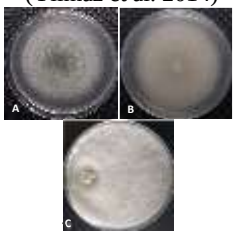
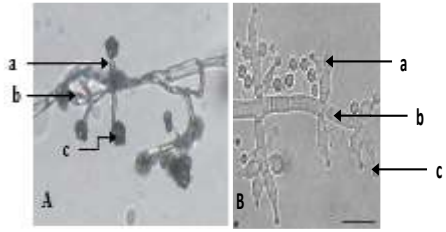
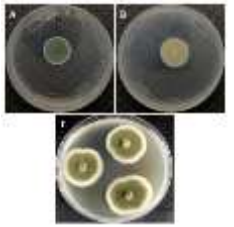
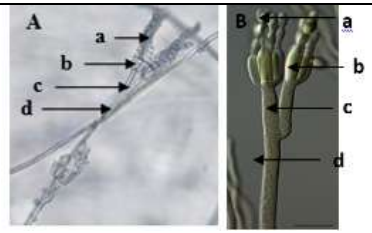
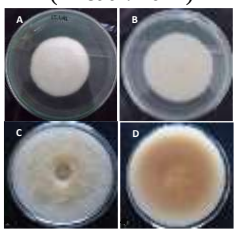
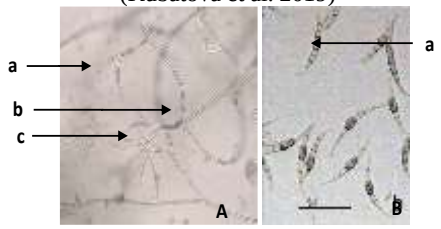
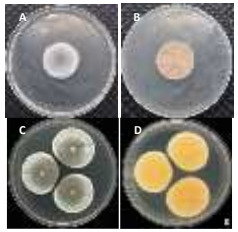
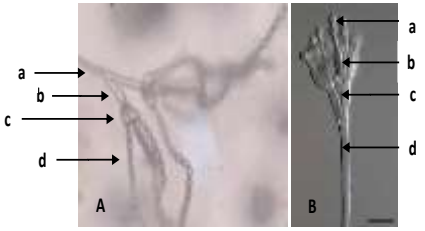
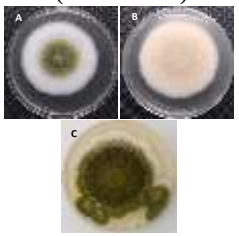
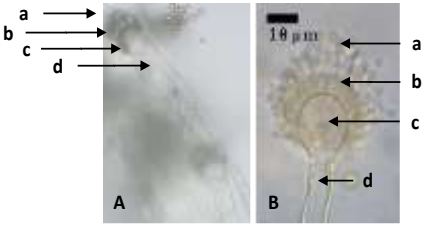


Figure 3. Phylogenetic tree reconstructed using Neighbor-Joining with Kimura 2-parameter model and bootstrapping 1,000 replications on MEGA11

Table 2. Macroscopic and microscopic species identification of eight pure fungi isolates

Isolate	Colony form	Colony texture	Diameter (mm)	Colony surface	Colony surface color	Reverse position color of the colony	Edge of the colony	Macroscopic view	Microscopic view
CB L1B	Circular	Velvety	32	Low, slightly raised in the central part of the colony	Grayish green with white edging	Yellowish white	Smooth	 (Yilmaz et al. 2014)	 (Yilmaz et al. 2014)
CB L1C	Circular	Cottony	22,6	Raised	Early colonies of white color then gradually become pink	Yellowish white	Smooth	 (Bali et al. 2022)	 (Bali et al. 2022)
CB L3A	Circular	Powder-like	45	Flat	Young colonies are yellow with white edging, then the middle of the colony gradually becomes dark green.	Red	Smooth, entire	 (Yilmaz et al. 2014)	 (Yilmaz et al. 2014)
CB L3B	Circular	Cottony	75	Flat	The growth begins in white; the colonies are light green to dark green over time.	Yellowish white	Filamentous	 (Zheng et al. 2021)	 (Zheng et al. 2021)

CB L4A	Circular	Powder-like	21	Flat	Grayish-olive green	Yellowish white	Smooth, entire	 (Li et al. 2021)	 (Kubátová et al. 2019)
CT L41	Circular	Cottony	55	Raised	White	White	Smooth, entire	 (Li et al. 2021)	 (Kubátová et al. 2019)
CT TL3	Circular	Clumped, radial furrow	18	Raised	Gray with white edging	Yellowish orange	Forming bars	 (Bonde 2014)	 (Pavlovic et al. 2016)
CT TL4	Circular	Powder (middle of colony), cottony (edge)	83.5	Low but at the center of the colony, slightly raised	The growth begins in white; over time, the colony is olive-green in the middle of the colony with a white edging	Yellowish white	Filamentous	 (Visagie et al. 2016)	 (Visagie et al. 2016)

Note: The image in the macroscopic view compares the: A. Surface morphology; and B. Reverse morphology of the eight pure isolates with the C. Surface morphology and D. Reverse morphology of the same species as documented in other literature. Additionally, microscopic scale comparisons are provided in the corresponding "Microscopic view" Column. Figure A comprises eight pure isolates, while Figure B consists of isolates from other literature

Identified species description

Following the purification process of the 19 fungal isolates, eight pure fungal isolates were obtained (Figure 1). Subsequently, the pure fungal isolates were characterized by molecular characterization. The DNA PCR results were visualized using electrophoresis (Figure 2), and the target gene amplification of the entire sample showed a DNA fragment measuring 500 bp. This shows that the ITS area (ITS 1 and ITS 2) is well amplified using the primary pair of ITS 1 and ITS 4 due to the size of the amplification product obtained following the statement by Badotti et al. (2017) and the ITS area has a size between 400-800 bp (Reich and Labes 2017). All samples showed a single, clear band of DNA, indicating high sample purities. Therefore, data visualization derived from electrophoresis is presented as an electropherogram. Subsequently, the resulting electropherogram was converted into sequence data using MEGA11 software. The CB L1B sequence has a length of 497 bp, CB L1C of 499 bp, CB L3A of 711 bp, CB L3B of 553 bp, CB L4A of 504 bp, CT L41 of 470 bp, CT TL3 of 501 bp, and CT TL4 of 716 bp.

The analyses using BLAST resulting in three species were selected based on the highest % identity value above 97% and the smallest gap in the alignment results in NCBI (Sukmawati et al. 2018). A value of >97% indicates that the sequence has a high homology rate and is the same species as the species sequence in NCBI (Kwaśna et al. 2008). Based on the results of BLAST analysis in Table 1, one identical identified species has been obtained. CB L1B isolate has the highest homology with *Talaromyces funiculosus* CBS 272.86, CB L1C isolate with *Purpureocillium lilacinum* NRRL 895, CB L3A isolate with *Talaromyces purpureogenus* CBS 286.36, CB L3B isolate with *Trichoderma anaharzianum* YMF 1.00383, CB L4A isolate with *Penicillium oxalicum* NRRL 787, CT L41 isolate with *Fusarium equiseti* NRRL 26419, CT TL3 isolate with *Penicillium cataractarum* CBS 140974, and CT TL4 isolates with *Aspergillus flavus* ATCC 16883. The reported fungal species generally come from natural environments such as soil, plants, and air (Zucconi et al. 2022). All the samples have an e-value of 0.0, so the sample sequence matches the database on Genbank (Newell et al. 2013).The species molecular characterization identification was utilized for phylogenetic tree reconstruction. In Figure 3, six clades are shown, all originating from the phylum Ascomycetes; three clades belong to the class Eurotiomycetes (clades 1, 2, 3), while the other three are from the class Sordariomycetes (clades 4, 5, 6). Clade 1 is subdivided into subclades 1.A and 1.B. Species in this clade belong to the genus *Penicillium*, where the genus *Penicillium* is characterized by branching in the conidiophore (penicillus). The branching pattern can be of a simple type to complex patterns with varying degrees, such as monoverticillate (one-branched stage), bi-verticillate (two-branched stages), terverticillate (three-branched stages), or more (Van Leeuwen 2009). While clade 3 is divided into subclades 3.A and 3.B, species in this clade belong to the genus *Talaromyces*; this genus has the characteristic of producing yellow ascomata, sometimes white, cream, pink, or reddish, and has yellow ascospores. Conidiophores are usually symmetrical bi-verticillate and acerose-shaped phialide (Vardasbi et al. 2021). In the phylogenetic tree, all listed clades or subclades exhibit branch length values ranging from 0.000 to 0.009, while bootstrap values range from 66 to 100. According to Anafarida and Badruzsaufari (2020), a bootstrap value with a range of 70-100 indicates a chance of change in a small clade, while a bootstrap value of less than 70 indicates a chance of changing the order of a large clade; if the bootstrap value is 25%, a clade cannot be trusted. Branch length represents the number of sequence changes occurring in the branch and describes how far/close the kinship is between species. The longer the branches of the phylogenetic tree, the greater the evolutionary changes that occur, so the more distant the relationship (Subari et al. 2021).

The species of each isolate has been determined based on the molecular characterization results. The isolates were further identified by comparing their macroscopic and microscopic characteristics with those described in existing literature shown in Table 2 for the respective species. The results of identifying the macroscopic and microscopic characteristics of the isolate are shown in Table 2.

The macroscopic appearance of CB L1B has a circular colony shape with a diameter of 32 mm and grayish green with white edging mycelium. Then, on microscopic examination with 1000X magnification, the CB L1B isolate has conidiophores, phialide, and conidia (C, B, and A in the figure). The CB L1B conidiophore is a monoverticillate type (has one branching stage), erect, and fine-walled conidiophore stalk. In 1 conidiophore, there are 4 phialides; the phialides are lanceolate in shape, and at the end of the phialide, there are smooth, subglobose (almost round) conidia. In comparison with the literature, the macroscopic appearance of CB L1B compared with *T. funiculosus*, according to Yilmaz et al. (2014), *T. funiculosus* has a circular colony shape, growth is directed upwards, especially in the middle part of the colony, the diameter ranges between 30-45 mm (incubation period 7 days), the mycelium is grayish green to dull green, and the lower surface of the colony is brownish yellow. *Talaromyces funiculosus* is an airborne fungus; its spores are dispersed in the air (Nageen et al. 2023). Microscopically, *T. funiculosus* has conidiophores with two branching stages (bi-verticillate), fine-walled stipes (conidiophore stalk), at the end of the conidiophore there are three to six medullas, the phialide is lanceolate (with a narrow body part tapering at the end), and the conidia are smooth, subglobose to ellipsoidal (Yilmaz et al. 2014). Based on the result comparison, CB L1B isolate was determined as *Talaromyces funiculosus* with classification, domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Trichocomaceae, genus *Talaromyces*.

Subsequently, based on macroscopic observations, CB L1C isolate has a circular colony shape with a cottony texture, the diameter of 22.6 cm, and early colonies of white color then gradually become pink. Based on microscopic observations with 400X magnification, CB L1C isolates have partitioned hyphae, conidiophores, medullas, phialide, and conidia (D, C, B, and A in Table 2). The conidiophore is upright and branched; the conidiophore has three branching stages (terverticillate). Conidiophore stalks are transparent and coarse-textured, consisting of vertical branches with two medulles. On the metula, there are 2-4 phialides that are cylindrical. Some conidia are oval (ellipsoidal) at the end of the phialide. Compared with *Purpureocillium lilacinum* (Luangsa-Ard et al. 2011), The CB L1C isolate shows similarities to *Purpureocillium lilacinum* based on both macroscopic and microscopic characteristics. Macroscopically, *P. lilacinum* forms circular colonies with a cotton-like texture that grows upwards and develops a hollow in the center. The

colony diameter ranges from 25 to 35 mm after a 7-day incubation period. Initially, the colonies are white, but they gradually turn purple as they grow. Microscopically, *P. lilacinum* features upright conidiophores with smooth or leathery stalks and vertical branches bearing two to four phialides. The conidiophores may be simple or branched and can range from mono-verticillate to tertiary or quaternary verticils. The metulae are typically one to two in number, straight to curved, with a slender neck. The phialides are cylindrical or ellipsoidal at the base, narrowing abruptly into a short neck. Conidia form in short chains, and are ellipsoid to fusiform, hyaline, and smooth-walled. Based on these characteristics, it can be concluded that the CB L1C isolate is a species of *Purpureocillium lilacinum*, classified within the following taxonomy: domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Ophiocordycipitaceae, genus Purpureocillium. The Order Hypocreales is the second most abundant order, accounting for 18% of the total fungi diversity reported at historical sites, including temples (Zucconi et al. 2022). *Purpureocillium lilacinum* was reported in Angkor Wat, Cambodia (Liu et al. 2018). *Purpureocillium lilacinum*, commonly found in soil, decaying vegetation, insects, nematodes, humans, animals, and air, demonstrates adaptability to harsh environments characterized by low nutrient concentrations and oxygen levels. This species can form biofilms in conjunction with other species like *Aspergillus*, *Fusarium*, and *Acremonium* (Luangsa-Ard et al. 2011).

The results of macroscopic and microscopic observations of the CB L3A isolate were compared with the literature. Macroscopically, the CB L3A isolate forms circular colonies with a powdery texture, a diameter of 45 mm, and a surface color that is yellow with white edges in young colonies. As the colony matures, the center gradually becomes dark green. Microscopic observations at 400x magnification reveal that CB L3A has conidiophores, metulae, phialides, and conidia, as shown in Table 2 (D, C, B, and A respectively). The conidiophores are of the bi-verticillate type (having two branching stages), upright, and hyaline. The ends of the conidiophores connect with 3-5 metulae. The phialides are lanceolate, and at their ends, smooth globose (round) conidia are present. A species with similar macroscopic and microscopic characteristics to the CB L3A isolate is *Talaromyces purpureogenus*. *T. purpureogenus* forms circular colonies with low, flat, and intact margins. The mycelia are dark green with bright orange edges, and the lower surface of the colonies ranges from brownish yellow to brownish orange. At 7 days of incubation, the diameter of the colony ranges from 30-45 mm (Yilmaz et al. 2014). Microscopically, according to the literature, *Talaromyces purpureogenus* has conidiophores with two stages of branching (biverticillate). The conidiophore stalks (stipes) are smooth walled. At the end of each conidiophore, there are three to five metulae. The phialides are lanceolate, with a narrow body tapering at the end. Each metula has three to six phialides. The conidia are smooth, ellipsoidal in shape, sometimes subspherical, and apiculate (Yilmaz et al. 2014). It can be concluded that the CB L3A isolate is *Talaromyces purpureogenus* with classifications, namely Eukaryota domain, Fungi kingdom, phylum Ascomycota, Eurotiomycetes Class, Order Eurotiales, Trichocomaceae Family, *Talaromyces* Genus. Order Eurotiales is the most widely reported order and is represented by more species. *T. purpureogenus* was found in the frescoes "St. Augustine" by Botticelli and "St. Jerome" by Ghirlandaio Italy in 1989. *Talaromyces purpureogenus* can cause biodeterioration due to its ability to utilize materials used in restoration as a source of nutrients (Zucconi et al. 2022). This species can be isolated from cornland farmland (Sun et al. 2023).

The macroscopic and microscopic observations of the CB L3B isolate were analyzed in comparison with existing literature. Macroscopically, the CB L3B isolate forms circular colonies with a cottony texture, a diameter of 75 mm, and a surface color that changes from light green to dark green over time. Microscopic observations at 1000x magnification reveal that the CB L3B isolate has conidiophores, phialides, and conidia. The conidiophores are branched, erect, and partitioned. The ends of the conidiophores connect to short and thick phialides, which are arranged in groups of 2-3. The phialides have subglobose conidia at their ends. According to Zheng et al. (2021), *Trichoderma anaharzianum* macroscopically exhibits a cotton-like texture with a white colony surface at the beginning of growth, which then turns dark green after four days. The colony growth is relatively fast, covering petri dishes in four days, and it produces abundant aerial mycelium. Microscopically, *T. anaharzianum* has hyaline (transparent) conidiophores that are erect and branched. These conidiophores typically contain paired branches that are perpendicular to the main axis and usually bear three to seven phialides. The phialides are characteristically elongated and lageniform, sometimes appearing singly directly from the main axis. At the ends of the phialides, the conidia are round to subglobose. According to Zin and Badaluddin (2020), a distinguishing feature of Trichoderma species is their branching conidiophores that resemble a pyramid, with repeated lateral branches at the bottom and shorter branches at the top. Based on these characteristics, it can be inferred that the CB L3B isolate is *Trichoderma anaharzianum*. Its classification is as follows: domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Hypocreaceae, genus Trichoderma. This species can be isolated from the soil and rhizosphere of tobacco (Zheng et al. 2021). According to Farooq (2015), several genera of fungi, such as *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, cause discoloration and structural damage to rock material. This genus of molds excretes organic acids (oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric acids) that play an important role in the chemical attack, leading to acidification of the substrate. These acids can cause the dissolution of cations and chelation of metal ions, which leads to the formation of stable metal complexes whose crystallization increases internal pressure, resulting in cracking and peeling.

Upon microscopic examination at 1000x magnification, the CB L4A isolate was identified to have conidiophores, metulae, phialides, and conidia, as shown in Table 2 (D, C, B, and A respectively). The conidiophores of CB L4A are long, branched, erect, smooth-walled, and have two stages of branching (biverticillate). The phialides are lanceolate, with a narrow base and tapered ends. The conidia are produced in short, fine-textured chains and are ellipsoidal in shape. The macroscopic appearance of CB L4A, as compared with the literature in Table 2, reveals that it has a powder-like texture and dense sporulation. The conidia are slightly yellowish green to grayish-olive green, the colony's lower surface is pale or medium yellow, and the colony surface is low. After seven days of incubation, the colony diameter ranges from 18-21 mm. According to BLAST and characterization results, CB L4A was identified as *Penicillium oxalicum*. Kubátová et al. (2019) describe P. *oxalicum* as having branched conidiophores that are usually bi-verticillate with 2-3 metulae or monoverticillate, with smooth stipes. The phialides are cylindrical and vertical, and the conidia are smooth and ellipsoidal. Therefore, the CB L4A isolate was determined to be *Penicillium oxalicum*. Its classification is as follows: domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Aspergillaceae, genus Penicillium. The order Eurotiales is the most widely reported and represented by a greater number of species, particularly in the genera Penicillium and Aspergillus. (Zucconi et al. 2022). *Penicillium oxalicum* was found in a mural painting of Chibusan Tumulus (a mound of earth and stone built over a tomb) in Kumamoto Prefecture, Japan, in 1974 (Zucconi et al. 2022). This species is an airborne fungus that can be isolated from soil and colonize various organic matter (e.g., corn), especially in the tropics (Kubátová et al. 2019). According to Farooq (2015), this genus of molds excretes organic acids (oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric) that play an important role in the chemical attack, leading to acidification of the substrate. These acids can cause the dissolution of cations and chelation of metal ions, which leads to the formation of stable metal complexes whose crystallization increases internal pressure, resulting in cracking and peeling.

Subsequently, the characteristics of CT L41 are detailed in Table 2. The isolate forms cottony circular colonies with a diameter of 55 mm and a white colony surface. Microscopically, CT L41 has conidiophores, microconidia, and macroconidia. The microconidia are small, elongated, oval-shaped, slightly pointed, and lack septa, forming on short conidiophores. The macroconidia are larger, elongated, pointed at both ends, shaped like curved crescents, and contain 3-5 septa. Comparing CT L41 with *Fusarium equiseti* according to Hami et al. (2021), both have similarities. Macroscopically, *F. equiseti* forms colonies with a cotton-like texture, abundant white aerial mycelium, and clotted (buff) appearance. The colony growth is relatively fast, covering almost the entire petri dish in six days. Microscopically, *F. equiseti* has hyaline conidiophores that are simple, short, and insulated. It produces two types of conidia: microconidia and macroconidia. The macroconidia are boat-shaped or crescent-like, with slightly tapered apical cells and twisted basal cells, containing 3-5 septa. The microconidia are ellipsoidal, hyaline, without septa, and are single or in chains. Additionally, *F. equiseti* has almost spherical hyaline chlamydospores. Based on the BLAST results and morphological characterization, it can be concluded that CT L41 is a species of *Fusarium equiseti*. Its classification is as follows: domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Nectriaceae, genus Fusarium. *Fusarium equiseti* was notably found in the 2017 mural painting "The Ariadne House" of Italian Pompeii (Veneranda et al. 2017). This species can be isolated from the soil and is a cosmopolitic soil-dwelling fungus (Wang et al. 2019).

Based on macroscopic observations, CT TL3 isolates have a circular shape with a clumped surface texture and form radial furrows. The mycelium is solid, and the growth of the isolates is relatively slow, reaching a diameter of 18 mm by the 7th day of incubation. The colony's surface is raised above the substrate. Initially, the mycelium appears gray with white edges, forming concentric circles, and does not change color as the colony grows. The lower surface of the isolate is yellow-orange. Microscopic observations at 1000x magnification reveal that CT TL3 isolates have conidiophores, metulae, phialides, and conidia. The conidiophores are long and hyaline with two stages of branching (biverticillate). Each conidiophore has two metulae. The phialides are lanceolate, with a narrow base and tapered ends. The conidia are produced in long, finely textured chains and are rounded in shape. Based on BLAST results in Table 1 and the morphological characterization described above, CT TL3 isolates are identified as *Penicillium cataractarum*. The classification is as follows: domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Aspergillaceae, genus Penicillium. This species has conidiophores that are biverticillate, terverticillate, and divaricate, very long in shape, and contain two to five regular metulae. The phialides narrow suddenly into long, ellipsoidal conidia, which are sometimes spherical. After 7 days of incubation, the colony diameter ranges from 34-42 mm. Microscopically, *P. cataractarum* exhibits a clumpy surface texture with low to deep margins and dense or sometimes buff mycelium. The colony surface is gray and turns pale yellow. However, some isolates may appear grayish-yellow (khaki) or grayish-orange (Visagie et al. 2016).

The last isolate identified was CT TL4. The results of macroscopic and microscopic observations of CT TL4 were compared to the literature. CT TL4 has circular-shaped colonies with a powdery center and cottony edges. The colonies have a diameter of 83.5 mm, starting white and turning olive-green in the middle, with a yellowish-orange reverse side. Microscopic observations at 1000x magnification reveal that CT TL4 isolates have conidiophores, vesicles, phialides, and conidia, as shown in Table 2. The conidiophores of CT TL4 are rough, thick-walled, and colorless, containing vesicles. The cells are

uniseriate, with the phialides growing directly on the vesicles. The conidia are spherical, with thin walls and a smooth texture. These characteristics match those of Aspergillus flavus. According to the literature, *Aspergillus flavus* has colorless, thick-walled, leathery conidiophores. The vesicles are subglobose or globose, and the cells can be uniseriate or biseriate. In the uniseriate form, the phialides grow on the vesicles, while in the biseriate form, the phialides grow on metulae. The conidia are rounded with thin walls and a rough texture. Based on these observations and comparisons, CT TL4 is identified as *Aspergillus flavus* (Thathana et al. 2017). Microscopically, *A. flavus* begins sporulation after 5 days from the inoculation point and develops radially, covering the colony's surface. As the colony grows progressively, it rises slightly, and the center becomes rough like powder. The formed conidia are olive-green. As sporulation spreads outward, a white mycelium forms (Thathana et al. 2017). It can be concluded that CT TL4 isolate is *Aspergillus flavus* with classifications, domain Eukaryota, kingdom Fungi, phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Aspergillaceae, genus *Aspergillus*. *Aspergillus flavus* is often found in monasteries, churches, temples, caves, and tombs, as this species is an airborne fungus. In 2011, *A. flavus* was found in India's Sita Devi Temple (Gupta and Sharma 2011).

In conclusion, analysis of ITS sequences identified eight fungal isolates from the temple rocks of the Trowulan Site, belonging to the classes Sordariomycetes and Eurotiomycetes. The Sordariomycetes class includes *Purpureocillium lilacinum* (CB L1C), *Trichoderma anaharzianum* (CB L3B), and *Fusarium equiseti* (CT L41). Species from the Eurotiomycetes class consist of *Talaromyces funiculosus* (CB L1B), *Talaromyces purpureogenus* (CB L3A), *Penicillium oxalicum* (CB L4A), *Penicillium cataractarum* (CT TL3 isolate), and *Aspergillus flavus* (CT TL4 isolate). In further studies, biodeterioration potential tests can be carried out on identified species to confirm whether the isolate has the potential to weatherize temple rocks. Research on methods to overcome fungal growth with environmentally friendly active ingredients must be done.

The identification of these fungal species has several practical applications and significant implications. Understanding the fungal species that colonize temple rocks is crucial for developing targeted conservation strategies. Conservationists can monitor these fungi to prevent the biodeterioration of historical sites, thereby preserving cultural heritage. Additionally, by identifying fungi that have the potential to weatherize temple rocks, this research provides a foundation for developing preventative measures. This could involve the application of antifungal treatments or the development of environmental controls to inhibit fungal growth. Furthermore, knowing which fungi contribute to material degradation can guide the formulation of materials resistant to fungal colonization.

Further studies should conduct biodeterioration potential tests on the identified fungal species to confirm their impact on temple rocks. This will help determine the threats each species poses and guide the development of effective conservation strategies. Research should focus on discovering and testing environmentally friendly active ingredients to inhibit fungal growth. Long-term monitoring of fungal populations on temple rocks should be undertaken to understand seasonal and climatic influences on fungal colonization and activity. This will provide insights into fungal growth dynamics and its impact on historical structures over time.

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