〔報文〕 Analysis of Fungal Flora within a Traditional Japanese-Style Shake Roof

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1. Introduction

Shake roofing is a traditional form of roof covering that can be seen on many buildings such as Japanese shrines and temples. A Japanese style shake roof is made from split logs, such as sawara cypress, Japanese cedar and Japanese chestnut etc. The length, breadth, and thickness of each wooden shake are about 300 mm, 300 mm, and 3 mm, respectively. Superimposed shakes with approximately equal spacing of ca. 30 mm are pegged by bamboo nail. Damage to roof materials is caused by microbial biodeterioration and meteorological conditions such as solar heat and rainwater. For these reasons, re-roofing is required approximately every 25 years in most cases. Even though shakes may appear superficially healthy on the outside, sometimes their inside cores are rotted. This phenomenon is not well understood, but it is thought to be due to moistness and retention of water resulting from methods of roofing and pegging nails[1].

To make the roof last long, copper (Cu) plates are often inserted between the shakes at equal spaces. It is a common belief among builders and operational managers that Cu plates prevent wood from decaying. A high concentration of Cu ion inhibits the growth of microbes[2,3], and the surge of Cu flowing out from the copper plates when a shake roof is exposed to rain is thought to inhibit the growth of wood-rotting fungi. Recently, the intensity of Cu K-α on the surface of shakes has been investigated by energy-dispersive X-ray fluorescence spectrometry[4,5]. However, the effectiveness of anti-fungal activity has yet to be revealed; further, the value of inserting copper plates between the shakes to prevent anti-fungal activity also needs to be confirmed. To this end, it is important to monitor changes of wooden roofs over time, especially the relationship between the degree of decay and the species of rotting fungi.

In this study, cultivation and gene analysis of fungi were conducted using two types of shake samples. Fungi in both samples were identified using a cultivation method on malt extract agar medium and a next-generation sequencing method[6,7].

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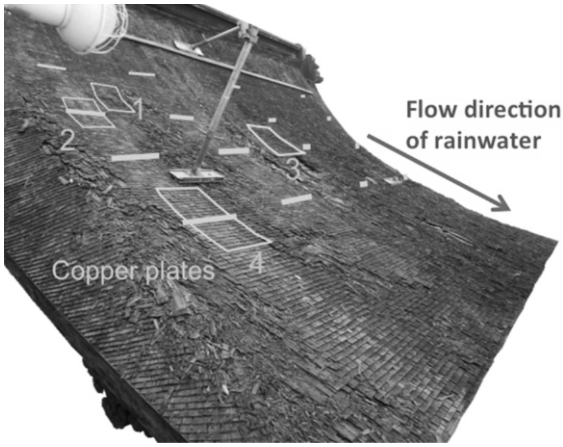
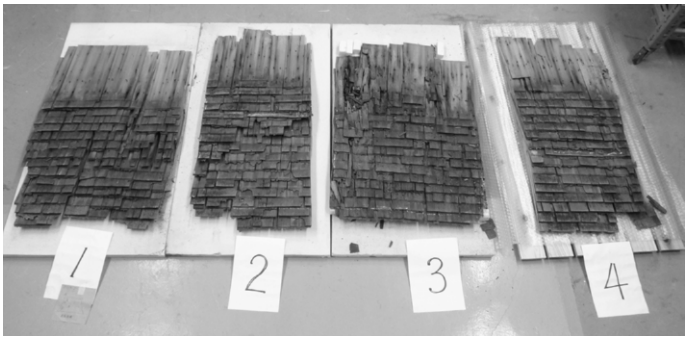


Figure 1 Locations of samples in a Japanese traditional shrine shake roof

Locations of all the samples, obtained from the main roof of the shrine, are surrounded with blue frames. Copper plates inserted between shakes are indicated by heavy yellow lines.

(A)



(B) Sample1 (Cu-) (C) Sample3-1 (Cu-)

(D) Sample2 (Cu+) (E) Sample4 (Cu+)

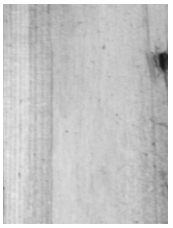


Figure 2 Samples obtained from the shake roof

All samples (Samples 1-4) were obtained from the shake roof. (A). Magnified pictures of representative portions were obtained from Sample 1 (B), 3 (C), 2 (D), and 4 (E). Cu- : distant from Cu plates, Cu+ : just below a Cu plate.

2. Materials and Methods

2－1. Samples

Two types of wood samples were obtained from a traditional Japanese shake roof which was re-roofed 25 years ago: Type 1, which consisted of samples situated distant from copper plates (Figure 1 and Figure 2, Samples 1 and 3), and Type 2, which consisted of samples just under copper plates (Figure 1 and Figure 2, Samples 2 and 4). Visually, some of the sections of wood that were situated distant from copper plates showed signs of decay, having falling and collapsed elements and exhibiting disarray and long, deep cracks; most of the wooden parts that were located under a copper plate were well preserved and had maintained their original form. These samples were tested within a month of sampling as described below.

2－2. Cultivation and identification of fungal strains from shake samples

To sterilize microbes on the surface of the samples, samples were cut into pieces of approximately 10×2×2 mm and soaked in 1 mL of 95% ethanol in individual 2-mL sterile tubes. The samples were vortexed twice for 1 min and then rinsed with sterile water. The samples were then set on malt extract agar (MA) plates and cultured at 25℃.

By testing the plates at approximately regularly spaced intervals, the fungal mycelia extending from the samples were isolated. Major strains were subjected to PCR and DNA sequencing to determine their taxonomic positions.

Then, DNA of fungal mycelia on MA plates was extracted using ISOPLANT (Nippon Gene) according to the total DNA isolation protocol supplied by the manufacturer. DNA samples were eluted with 100 μL of Tris-EDTA buffer (TE, pH 8.0). The eluted DNA solutions were used for PCR amplification of internal transcribed spacer (ITS) regions sequences between ribosomal DNA (rDNA) with ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [8] using a PCR thermal cycler (Dice® mini, TAKARA BIO). The PCR reaction mixture contained 1 μL of DNA solution, 0.8 μL of 10 μM forward primer, 0.8 μL of 10 μM reverse primer, 2.5 μL of 10x PCR buffer, 2.5 μL of 2 mM dNTPs, and 0.5 μL of DNA polymerase (KOD -Plus- Ver.2, TOYOBO). Thermal cycling conditions consisted of an initial denaturation at 94℃ for 2 m, followed by 30 cycles of 94℃ for 15 s, 60℃ for 30 s, and 68℃ for 20 s. The PCR products were directly sequenced using an ABI PRISM® 3130xl Genetic Analyzer (Life Technologies) following manufacture instructions.

2－3. Specific amplification of ITS regions in rDNA by PCR and Next Generation Sequencing

To obtain fungal DNA from wood, samples were shed finely by sterile scalpel. DNA was extracted from approximately 1 mg of the shed samples using ISOPLANT (Nippon Gene) as noted above in Section 2-2.

The ITS4 and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers[8] designed for fungi in general were used for PCR according to an amplicon library method manual

(http://454.com/downloads/my454/documentation/gs-junior/method-manuals/GSJunior_AmpliconLibrary Prep-RevJune2010.pdf). The purified PCR products were sequenced in a GS FLX sequencer (454 Life Sciences, Branford, Connecticut) according to a 454 sequencing system guidelines (http://my454.com/downloads/my454/applications-info/454Sequencing-System_GuidelinesforAmpliconExperimentalDesign_July2011.pdf).

2－4. Analysis of DNA sequence for identification of basidiomycete species

DNA sequences from Sample 1 and 2 obtained through next-generation sequencing were analyzed by SILVA comprehensive ribosomal RNA databases (http://www.arb-silva.de) to group according to the sequencing homology. BLAST searches based on the databases of GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/databases/) and Assembling the Fungal Tree of Life (http://aftol.org) were conducted using the sequences to determine refined taxonomic positions of fungi present in samples.

3. Results and Discussion

3－1. Identification of fungal strain obtained by culturation

Three basidiomycete and fifty-five ascomycete strains were detected through culturation (Table 1). The difference in detected fungal species between sterile and non-sterile (control) samples reflected the effect of surface sterilizing.

Notably, *Aspergillus* sp., *Exophiala* sp., *Paecilomycetes* sp., and *Penicillium* sp. (some of which were identified by microscopic observation) were mostly observed in non-sterile control samples. *Aspergillus* sp. and *Penicillium* sp. in particular are well known as

Table 1 Identification of fungal strains obtained by the cultivation method

Sample 1 (Cu -)[a]

Cu[a]	Sample	Treatment[b]	Isolate No.	Fungi[c]	Morphological classification	Identification by ITS DNA sequencing
−	1	EtOH	1-1-A	A	−	A type of Pezizomycotina
			1-1-B	A	−	A type of Pezizomycotina
			1-1-C	A	*Paecilomyces* sp.	−
		CTL	1-1-A-CTL	B	−	*Antrodia sordida*
			1-1-B-CTL	A	*Penicillium* sp.	−
			1-1-C-CTL	A	*Paecilomyces* sp.	−
			1-2-A-CTL	A	*Paecilomyces* sp.	−
			1-2-B-CTL	A	−	−
			1-2-C-CTL	A	*Exophiala* sp.	−
			1-2-D-CTL	A	−	−
			1-2-E-CTL	A	*Penicillium* sp.	−
			1-2-F-CTL	A	−	−
			1-3-A-CTL	A	*Paecilomyces* sp.	−

Sample 3 (Cu -)[a]

Cu[a]	Sample	Treatment[b]	Isolate No.	Fungi[c]	Morphological classification	Identification by ITS DNA sequencing
−	3	EtOH	3-1-A	A	*Paecilomyces* sp.	−
			3-1-B	A	−	*Penicillium polonicum*/*Penicillium* sp. related to *Penicillium viridicatum*
			3-2-A	A	−	*Hypoxylon* sp.
			3-3-A	A	−	A type of Dothideomycetes
		CTL	3-1-A-CTL	A	*Paecilomyces* sp.	*Paecilomyces* sp. related to *Paecilomyces variotii*
			3-2-A-CTL	A	*Aspergillus* sp.	−
			3-3-A-CTL	A	−	−
			3-3-B-CTL	A	*Paecilomyces* sp.	−
			3-3-C-CTL	A	−	−
			3-3-D-CTL	A	*Paecilomyces* sp.	−
			3-3-E-CTL	A	−	−
			3-3-F-CTL	A	*Paecilomyces* sp.	−

Sample 2 (Cu +)[a]

Cu[a]	Sample	Treatment[b]	Isolate No.	Fungi[c]	Morphological classification	Identification by ITS DNA sequencing
+	2	EtOH	2-1-A	B	−	*Bjerkandera adusta*
			2-1-B	A	−	A type of Herpotrichiellaceae/Chaetothyriales
			2-1-C	A	−	A type of Herpotrichiellaceae/Chaetothyriales
			2-2-A	B	−	*Dacryopinax* sp. related to *Dacryopinax spathularia*
			2-2-B	A	−	A type of Herpotrichiellaceae/Chaetothyriales
			2-2-C	A	−	A type of Pezizomycotina
			2-3-A	A	−	*Annulohypoxylon annulatum*
			2-3-B	A	−	*Exophiala* sp./ A type of Herpotrichiellaceae
		CTL	2-1-A-CTL	A	*Aspergillus* sp.	−
			2-1-B-CTL	A	−	−
			2-1-C-CTL	A	*Exophiala* sp.	−
			2-1-D-CTL	A	*Exophiala* sp.	−
			2-1-E-CTL	A	*Exophiala* sp.	−
			2-2-A-CTL	A	*Aspergillus* sp.	−
			2-3-A-CTL	A	*Aspergillus* sp.	−

Sample 4 (Cu +)[a]

Cu[a]	Sample	Treatment[b]	Isolate No.	Fungi[c]	Morphological classification	Identification by ITS DNA sequencing
+	4	EtOH	4-1-A	A	−	A type of Capnodiales
			4-1-B	A	−	A type of Herpotrichiellaceae/Chaetothyriales
			4-3-A	A	−	*Hypoxylon* sp.
			4-3-B	A	−	*Hypoxylon* sp.
			4-3-C	A	−	*Coniochaeta* sp. (*Lecythophora* sp.)
			4-3-D	A	−	*Hypoxylon* sp.
			4-3-E	A	−	*Annulohypoxylon bovei* var. *microspora*
		CTL	4-1-A-CTL	A	*Aspergillus* sp.	−
			4-1-B-CTL	A	*Penicillium* sp.	−
			4-2-A-CTL	A	*Cladosporium* sp.	−
			4-2-B-CTL	A	−	−
			4-2-C-CTL	A	−	−
			4-2-D-CTL	A	*Exophiala* sp.	−
			4-3-A-CTL	A	*Penicillium* sp.	−
			4-3-B-CTL	A	*Penicillium* sp.	−
			4-3-C-CTL	A	−	−
			4-3-D-CTL	A	−	−
			4-3-E-CTL	A	−	−

*a: Cu-, distant from Cu plates; Cu+, just below a Cu plate
*b: EtOH; sterilized by ethanol treatment; CTL, non-treatment (control)
*c: Classified by the absence of presence of clamp connections as ascomycetes (A) and basidiomycetes (B), respectively.

airborne fungi; further, these four genera fungi decompose polysaccharide components derived from wood[9].

Antrodia sordida in the non-sterile sample, *Bjerkandara adusta,* and *Dacryopinux* sp. in the sterile sample were detected as basidiomycetes. However, differences in the quantitative ratio of detected fungal species between the samples taken from the areas adjacent to Cu plates and the samples taken from the areas without Cu plates remains uncertain. Analyzing differences in biological properties of these fungal strains such as copper tolerance is needed for the future.

3－2. Identification of fungal strains using next-generation sequencing method

In total, 12,826 (Type 1, distant from Cu plates) and 7,086 sequences (Type 2, under a Cu plate) were yielded by next-generation sequencing (Figure 3).

Sequences from Type 1 samples fell into three groups. The main group (11,619 sequences) was highly homologous to *Dacryopinux* sp. or *Dacrymyces* sp. classified as basidiomycetes, sharing 85% similarity with a representative sequence. The other two, accounting for less than 8.37% of the total, were a type of Verrucariales (1,050 sequences, sharing 83% similarity) and a type of Chaetothyriales (10 sequences, sharing 83% similarity), which are classified as ascomycetes (Figure 3 (A)).

On the other hand, sequences from Type 2 samples were classified into many groups and the proportion of the sequences identified as ascomycetes was higher than for Type 1. The main group (4350 sequences) was highly homologous to *Dacryopinax* sp. related to *Dacryopinax spathularia,* sharing 95% similarity with a representative sequence. Other groups (*Exophiala* sp., 1494 sequences; a type of Pezizomycotina, 1,053 sequences; a type of Dothideomycetes, 43 sequences) were ascomycetes and accounted for more than 37.36% of the total (Figure 3 (B)).

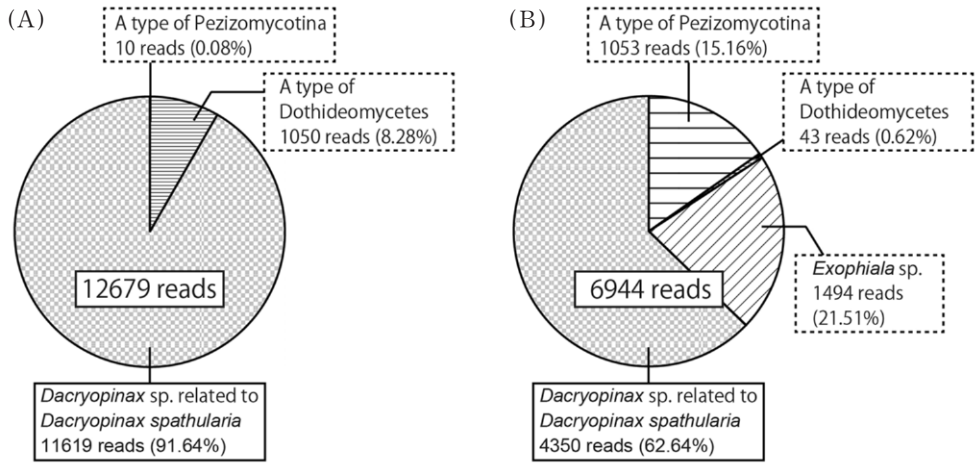


Figure 3 Proportions of identified fungal strains from Sample 1 (A) and 2 (B)

Values in parentheses are respective percentages of the totals. Solid line and dotted line boxes are basidiomycetes and ascomycetes, respectively.

There was a large and obvious difference between the kinds of species identified in samples of Type 1 and Type 2 in terms of the quantitative ratio of detected fungal species (Table 1, Figure 3). In addition, the intensity of Cu-Kα on surfaces of Samples 3 and 4 was also measured with an energy-dispersive X-ray fluorescence spectrometer[10]. The results were that surfaces of Type 2 (with a Cu plate) showed high intensities of Cu-Kα (0.14–0.40 g/m^2) while Type 1 (without a Cu plate) showed low intensities (lower than 0.13 g/m^2). This is perhaps due to differences in the levels of decay, which presumably relate to the presence or absence of copper plates. Although the number of samples is low, these results nonetheless suggest that Cu from copper plates affect the distributions of fungal flora and ultimately prevents wood decay.

Recently developed next-generation sequencing technologies have made high-throughput sequence determination of the rDNA-ITS regions and simultaneous sequence number counting possible[11,12,13]. It has been pointed out that the elucidation of accurate component ratios of fungi is difficult because copy numbers of rDNA vary across species and because tandemly repeated rDNA may let PCR products act as templates[14,15]. However, the method is considered a convincing assessment tool to infer what trends are occurring between floral species.

4. Conclusions

In this study, visual evaluation found that the samples situated just under a copper plate were evidently better protected than the samples located distant from copper plates. The cultivation method did not yield complete information on the differences between the detected fungal species from the two sample types in terms of the quantitative ratios of their fungal flora. We also attempted to identify fungal species in these samples using next-generation sequencing technology. Subsequently, a majority of ITS DNA sequences from samples of Type 1 were classified as wood-rotting fungi. On the other hand, sequences from Type 2 samples were classified into many groups and the proportion of the sequences identified as ascomycetes was increased compared to Type 1. We believe that the differences in fungal species composition identified by next-generation sequencing manifest themselves in the different levels of decay observed in wood that was near to versus distant from copper plates. These results clearly support the long-held belief that copper mainly protects against the growth of basidiomycetes, thereby conserving wooden architecture.

However, as this study focuses on one case study only, further research is required in order to fully understand the effects of using copper plates as a building material and its properties.

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キーワード：柿（こけら）；葺き屋根 (shake roof)；銅板(copper plates)；菌叢 (fungal flora)；腐朽 (decay)；同定(identification)

柿（こけら）葺き屋根材中に存在する菌類の菌叢解析

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柿（こけら）葺きは，サワラ，スギ，クリ，ヒノキなどの木材から長さ１尺（約 30 cm），厚さ一分（約 3 mm）の大きさに薄く剝いだ板（こけら板）を，一寸（約 30 mm）ずつずらして重ね，竹釘で留めながら葺き上げていく日本の伝統的な屋根葺き工法の一つであり，社寺建築や数寄屋建築に用いられている。古来よりこけら板の腐朽を抑制し耐用年数をより長くする目的で，屋根を葺く際に薄い銅板が葺き込まれることが多々ある。銅の木材に対する防腐効果は一般的に知られており，こけら板についても同様に銅板から雨水によって銅イオンが溶脱し，残存する銅成分がこけら板の保存に役立っているとする検討もあるが，こけら屋根の劣化のメカニズムや銅板の防腐効果については不明な点が多い。

本研究では，銅板を使用する事で腐朽を抑制していると思われるこけら葺き屋根から，目視で健全であると思われるこけら板，および腐朽が深刻であるこけら板を銅板直下，銅板と接していない箇所から採取し，それらの試料中に存在する菌類のモニタリングを行った。培養法による同定の結果，多糖類を分解する空中浮遊菌として知られる *Aspergillus* 属菌，*Penicillium* 属菌などの子嚢菌が数多く検出された。非培養法である次世代シーケンシング技術を用いて DNA 分析を行った結果，銅板と接していない箇所の試料からは 12,828 塩基配列を得て，その多く（91.64%, 11,679 配列）は木材腐朽菌に分類される担子菌であった。その一方で銅板直下から採取した試料からは 7,086 塩基配列を得ており，検出される子嚢菌の種類や割合（37.36%，2,590配列）が高くなった。以上から，培養法と非培養法では同定される菌種やその割合が異なること，銅板の有無の違いによって菌叢が異なることなどが明らかとなった。このことから銅板の存在下では木材腐朽担子菌の生育が抑制されることでこけら材が健全な状態を保ち，こけら葺き屋根材の長寿命化が達成されていることが示唆された。

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