〔報告〕 Microbial Flora Analysis of Neolithic Tree Remains from the Yenikapi Excavation Site in Istanbul, Turkey, Using Denaturing Gradient Gel Electrophoresis

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1. Introduction

During construction of the Yenikapi metro station of the Marmaray railway line in Istanbul, Turkey (Fig. 1A), between 2004 and 2012, a number of archaeological remains were discovered. These included Byzantine and Ottoman period ruins and remains, for example an ancient Byzantine Port, Istanbul's oldest city wall, a Byzantine Church, and shipwrecks and their associated artifacts. In addition, many Neolithic tree stump remains in standing position were unearthed (Figs. 1B–D). The Neolithic tree remains were found in a small local swamp, 8.5 m below sea level, under a 4–5 m deep layer of marine sand and sea shells[1].

Dogu *et al.*[2] and Yilgor *et al.*[3] identified and characterized some of these Neolithic remains. They identified the trees as *Fraxinus* spp. (ash) and *Quercus* spp. (oak) that were severely degraded by soft-rot fungi and bacteria. The remains also had a high sulfur and iron content, which is typical for marine-archaeological wood found in anoxic conditions such as those found underwater or in swamps[3,4].

Submerged wooden remains are in general waterlogged, and are usually kept in water after excavation until conservation procedures can be carried out. These procedures, such as the polyethylene glycol method[5,6] and the sugar alcohol method[7], are used to prevent the shrinkage and cracking that is caused by dehydration. Once waterlogged wooden objects have shrunk, it is almost impossible to restore them to their original state. However, microscopic analysis has shown that while submerged for preservation, the wood tends to be degraded by bacteria and soft-rot fungi[4,8,9].

Many wooden cultural artifacts on land are degraded by wood-rotting fungi, and there are many studies on the wood biodegradation caused by such organisms. However, the degradation mechanism that operates in waterlogged and excavated wooden artifacts is completely different, and little attention has been given to the specific microorganisms that

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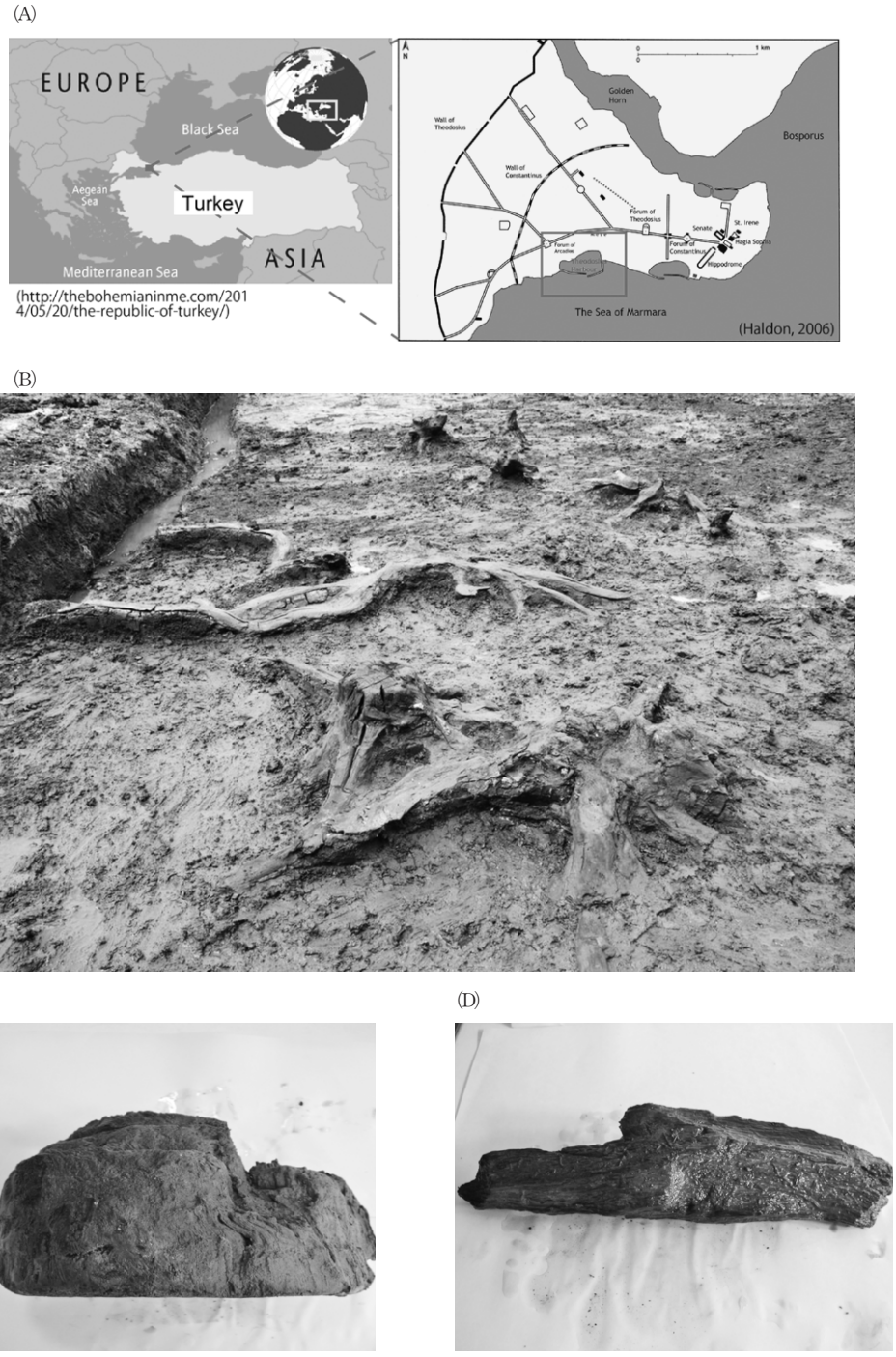

Figure 1. Neolithic tree remains excavated at the Yenikapi excavation site, Istanbul. Map showing the location of the study site (A), example of tree remains (*Fraxinus* spp.) *in situ* (B), and portions of the remains once excavated (C, *Fraxinus* spp. and D, *Quercus* spp.).

degrade waterlogged wood[9].

We used the denaturing gradient gel electrophoresis (DGGE) method to identify fungi and bacteria in waterlogged and excavated wooden remains from the Yenikapi excavation site. Based on the DGGE profiles and sequence types thus produced, we aimed to describe the molecular fungal and bacterial diversity associated with this waterlogged wood.

2. Materials and Methods

2－1. Samples

Archaeological excavations began in 2004 at the Yenikapi excavation site, which is located on the southern coast of the European side of Istanbul, Turkey (Fig. 1A). And excavations in Neolithic layer were begun in 2008 and a large number of the Neolithic tree remains were unearthed on May 2009 from 8.5–8.9 m below sea level. We used data concerning the archaeological sites and 12 samples, with sample reference numbers as follows: a) G-38 No.3; b) I-34; c) J-38; d) K-34; e) L-37; f) L-39; g) M-36; h) M-38; i) M-41; j) M-35; k) N-39; and l) F-38 (Table 1). Among them, 11 were identified as *Fraxinus* (Fig.1B and C) and 1 as *Quercus* (sample l, i.e. F-38, Fig.1D)[2]. The Neolithic tree remains used in this study were immediately preserved at 4℃ in de-mineralized water until the DNA extraction was performed in October 2013 and the water was renewed at two-three-week intervals.

2－2. PCR amplification for DGGE

To obtain DNA from microorganisms, all samples were collected by shedding using sterile scalpel as previously reported[10,11,12]. DNA was extracted from the shed samples, each of which weighed ca. 100 mg, using ISOIL (NIPPON GENE), according to the DNA isolation protocol supplied by the manufacturer. DNA samples were eluted with 50 μL of Tris-EDTA buffer (TE, pH 8.0). Then the eluted sample solutions and the fungal-specific primers ITS1-F-DGGE[13] (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCC CTTGGTCATTTAGAGGAAGTAA-3') and ITS4[14] (5'-TCCTCCGCTTATTGATATGC-

Table 1. List of samples of Neolithic tree remains from the Yenikapi excavation site, Istanbul, Turkey used in this study.

Sample name	Samples reference	Wood species
a	G-38 No.3	*Fraxinus* spp.
b	I-34	*Fraxinus* spp.
c	J-38	*Fraxinus* spp.
d	K-34	*Fraxinus* spp.
e	L-37	*Fraxinus* spp.
f	L-39	*Fraxinus* spp.
g	M-36	*Fraxinus* spp.
h	M-38	*Fraxinus* spp.
i	M-41	*Fraxinus* spp.
j	M-35	*Fraxinus* spp.
k	N-39	*Fraxinus* spp.
l	F-38	*Quercus* spp.

3') and the eubacterial-specific primers 8F-GC[15] (5'-CGCCCGCCGCGCCCCGCGCCCGTCC CGCCGCCCCCGCCCGAGAGTTTGATCCTGGCTCAG-3') and 519R[16] (5'-GTATTACCGC GGCTGCTGG-3') were used as a template for the PCR.

The PCR reaction mixture contained 4 μL of DNA solution, 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 12.5 μL of 2× Gflex PCR buffer, and 0.5 μL of Tks Gflex™ DNA polymerase (TAKARA BIO). The thermal cycling conditions for fungi consisted of an initial denaturation at 95℃ for 5 min, followed by 30 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s. The conditions for bacteria consisted of an initial denaturation at 95℃ for 5 min, followed by 30 cycles of 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s. PCR amplification was performed in triplicate and checked using gel electrophoresis with GelRed™ (Biotium), and the agarose gels were photographed on a UV light transilluminator (Figs. 2 and 3).

2－3. Separation of PCR products by DGGE and sequence analysis

DNA bands excised in appropriate size (fungi, 500-900 base; bacteria, 400-600 base) from the agarose gel were purified using Wizard® SV Gel and the PCR Clean-up System (Promega), and separated by DGGE on an 8% polyacrylamide gel containing a 20–70% urea-formamide denaturing gradient (fungi) and 30–60% gradient (bacteria). Wells were washed with TE buffer, and 5 μL of amplified PCR product and 5 μL of 2× DGGE loading buffer (NIPPON GENE) were mixed and loaded per lane and run at 80 V for 15 h at 60℃.

The resulting gel was stained using SYBR Green I (TAKARA BIO) and photographed (Figs. 4 and 5). Detected bands were excised from the gel and extracted DNA using Wizard® SV Gel and the PCR Clean-up System (Promega). Purified DNA was reamplified

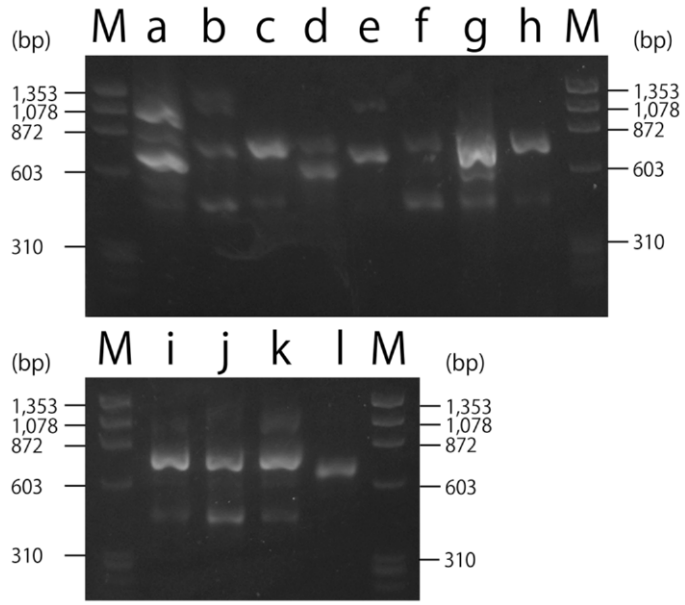

Figure 2. PCR of template DNA extracted from Neolithic tree samples, using a fungal-specific primer pair. Lane labels denote sample names. M: Molecular weight marker (ϕ×174Hae III digest).

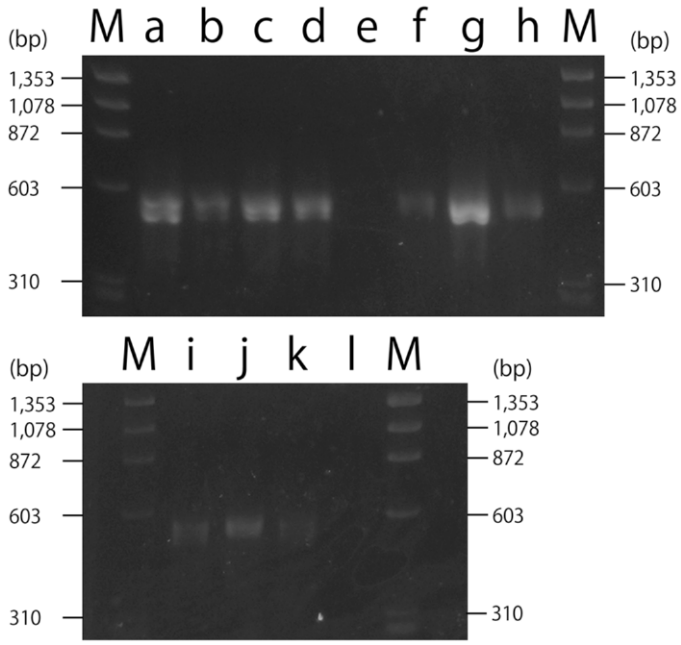

Figure 3. PCR of template DNA extracted from Neolithic tree samples, using a fungal-specific primer pair. Lane labels denote sample names. M: Molecular weight marker ($\phi \times 174$Hae III digest).

using the primer pairs described above. The reamplified PCR products were purified using Wizard® SV Gel and the PCR Clean-up System (Promega) and sequenced using the ABI PRISM® 3130xl Genetic Analyzer (Life Technologies). The BLAST algorithm, based on GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), was used to analyze the sequences to determine the refined taxonomic positions of the fungi and bacteria present in the samples.

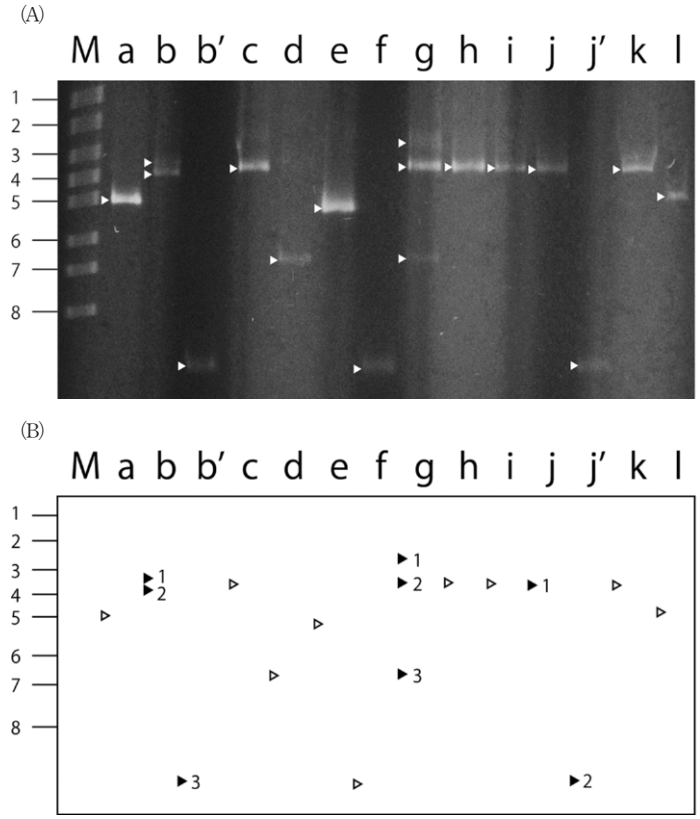

Figure 4. Denaturant gradient gel electrophoresis (DGGE) profiles (20–70% gradient) of amplified fungal ribosomal RNA gene fragments (A), and labeling pattern of bands excised for sequence analysis (B). Lane labels denote sample name. Because of the amplicon size, bands from the PCR of samples b and j were divided into two as follows: sample b: c. 900 bp (lane b) and c. 700 bp (lane b'); sample j: c. 700 bp (lane j) and c. 500 bp (lane j'). M: DGGE Marker I (NIPPON GENE).

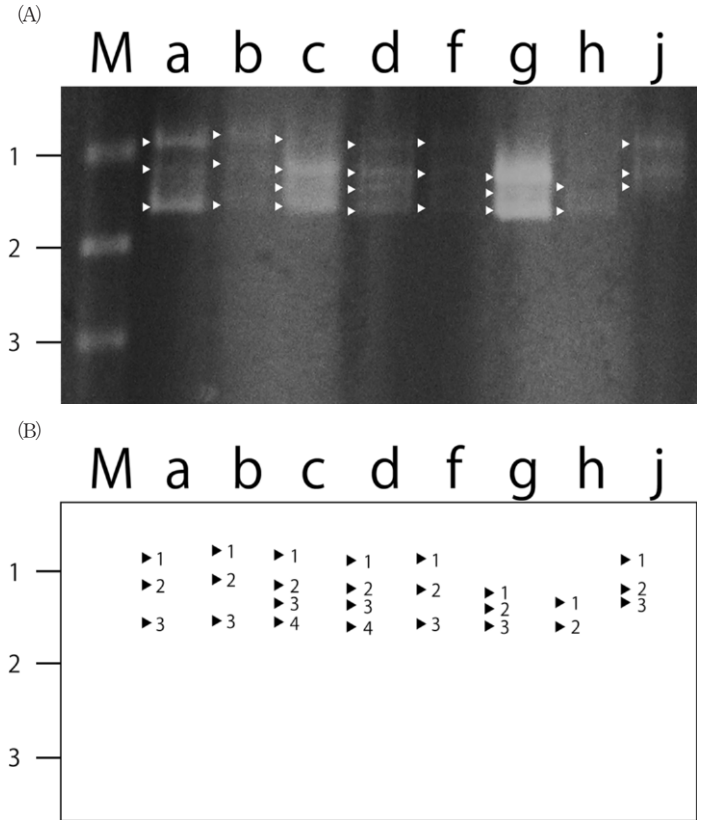

Figure 5. Figure 5. DGGE (30–60% gradient) profiles of amplified bacterial ribosomal RNA gene fragments (A), and labeling pattern of bands excised for sequence analysis (B). Lane labels denote sample name. M: DGGE Marker IV (NIPPON GENE).

3. Results and Discussion

3－1. DGGE and sequence analysis of fungi

Using fungal-specific primers, DNA bands were detected (Fig. 2) and separated by DGGE in all samples (Fig. 4). In total, 17 DGGE bands were obtained and subjected to DNA sequence analysis. Altogether, 9 ascomycete species were identified (Table 2). The majority of the identified fungi obtained from 5 samples was highly homologous with *Aspergillus penicillioides* (length 440 base, coverage 97%, identity 85%). Another species were highly homologous with *Cadophora fastigiata* (3 samples, length 330 base, coverage 100%, identity 99%), *Pichia pastoris* (2 samples, length 303 base, coverage 100%, identity 99%), *Aspergillus clavatus* (1 sample, length 624 base, coverage 70%, identity 84%), *Penicillium megasporum* (1 sample, length 559 base, coverage 41%, identity 91%) and *Protoventuria alpina* (1 sample, length 593 base, coverage 99%, identity 100%). And the other 3 fungal species were highly homologous with uncultured fungus clones (2 sample (H-Tj-143), length 276 base, coverage 100%, identity 100%; 1 sample (WZF-14), length 404 base, coverage 62%, identity 88%; 1 sample (WME35), length 330 base, coverage 100%,

identity 99%).

In particular, *Aspergillus* and *Penicillium* species are well known globally as airborne fungi, and as soft-rot fungi that decompose the polysaccharide components of wood[19]. The *Aspergillus* and *Penicillium* species detected in this study have been already recorded from various substrates/habitats in Turkey[20].

Protoventuria alpina was first described by Barr in 1993[21]; however, this species has not yet been studied in detail. Some *Cadophora* spp. such as *C. luteo-olivacea* and *C. malorum* are known as plant pathogen[21] and soft-rot fungi[22]. Especially *Cadophora fastigiata* causes stains in soft wood timber[21]. The presence of *Pichia pastoris*, which is a methylotrophic yeast, is thought to mean that the wood had hardly been degraded at all in the soil or during storage.

3－2. DGGE and sequence analysis of bacteria

The PCR amplification for bacteria was not effective in sample-e, l, i and k (Fig. 3), so it was not possible to conduct a DGGE analysis of these samples. The DGGE analysis was performed for the other samples and totally 25 DGGE bands were obtained and sequenced.

Although sample b- fragment 1 (Fig. 5) and sample d- fragment 3 (Fig. 5) were not analyzable because of mixed DNA sequences, other samples were highly homologous with bacteria genes and seven bacterial species were identified (Table 2). The species detected in the remaining 6 samples (a, c, d, f, g, and h) was highly homologous with *Methylovirgula ligni* (length 445 base, coverage 100%, identity 99%), and an additional species identified in sample b, c, d, f, and g was highly homologous with uncultured soil bacterium isolate 2313F152 (length 442 base, query coverage 100%, identity 95%). These 2 species constituted the majority of the bacteria we identified. Other species were highly homologous with *Acidithiobacillus ferrooxidans* strain CK (3 samples, length 429 base, coverage 100%, identity 98%), *Acidisphaera* sp. SBLE1E7 (1 sample, length 409 base, coverage 100%, identity 95%), uncultured Hydrogenophilaceae gene S133 (1 sample, length 328 base, coverage 99%, identity 90%), uncultured Acetobacteraceae bacterium Elev_16S_1845 (1 sample, length 394 base, coverage 95%, identity 92%), and *Micromonospora* sp. AV135 (1 sample, length 343 base, coverage 82%, identity 80%).

Methylovirgula ligni was isolated from beech wood blocks being decayed by the white-rot fungus, *Hypholoma fasciculare*[23]. It is reported that this bacterium can utilize not only methanol but also assimilate carbon via the ribulose-bisphosphate pathway[23]. The genus *Micromonospora* belongs to the Micromonosporaceae family, Actinomycetales order and it might be expected to degrade cellulose[24,25]. It is thought that *Methylovirgula ligni* and *Micromonospora* spp. might play an important role in the degradation of organic matter such as waterlogged wood.

Acidithiobacillus ferrooxidans is a chemolithoautotrophic, γ-proteobacterium that uses energy from the oxidation of iron- and sulfur-containing minerals for growth under anoxic conditions[26]. In a previous study, iron and sulfur were the most common elements after carbon and oxygen in an ancient ash wood sample[2,3]. This suggested that bacteria in waterlogged wood use iron and sulfur from the soil for their anaerobic metabolism. Even

Table 2. Fungal and bacterial species identified following DGGE analysis of Neolithic tree remains from the Yenikapi excavation site, Istanbul, Turkey.

Sample name	Fungi			Bacteria		
	Fragment No.	The highest homologued species	Accession No.	Fragment No.	The highest homologued species	Accession No.
a		Uncultured fungus clone H-Tj-143	EU486145.1	1	Uncultured *Hydrogenophilaceae* gene (S133)	JN217058.1
				2	*Methylovirgula ligni*	FM252035.1
				3	*Methylovirgula ligni*	FM252035.1
b	1	*Protoventuria alpina*	EU035444.1	1	NA*	
	2	*Gadophora fastigiata*	GU067761.1	2	Uncultured soil bacterium isolate 2313F152	FR732446.1
	3	*Aspergillus clavatus*	NR_121482.1	3	Uncultured Acetobacteraceae bacterium Elev_16S_1845	EF020298.1
c		*Cadophora fastigiata*	GU067761.1	1	*Micromonospora* sp. AV135	JN409364.1
				2	Uncultured soil bacterium isolate 2313F152	FR732446.1
				3	*Methylovirgula ligni*	FM252035.1
				4	*Methylovirgula ligni*	FM252035.1
d		*Aspergillus penicillioides*	GU017494.1	1	*Acidithiobacillus ferrooxidans* strain CK	KJ944318.1
				2	Uncultured soil bacterium isolate 2313F152	FR732446.1
				3	NA*	
				4	*Methylovirgula ligni*	FM252035.1
e		Uncultured fungus clone WFZ-14	JX374767.1		NA*	
f		*Pichia pastoris*	FN392325.1	1	*Acidithiobacillus ferrooxidans* strain CK	KJ944318.1
				2	Uncultured soil bacterium isolate 2313F152	FR732446.1
				3	*Methylovirgula ligni*	FM252035.1
g	1	Uncultured fungus clone H-Tj-143	EU486145.1	1	Uncultured soil bacterium isolate 2313F152	FR732446.1
	2	*Penicillium megasporum*	GU092967.1	2	*Methylovirgula ligni*	AB254794.1
	3	Uncultured fungus clone WME35	JN847480.1	3	*Methylovirgula ligni*	FM252035.1
h		*Aspergillus penicillioides*	GU017494.1	1	*Methylovirgula ligni*	FM252035.1
				2	*Methylovirgula ligni*	FM252035.1
i	1	*Aspergillus penicillioides*	GU017494.1		NA*	
j	1	*Aspergillus penicillioides*	GU017494.1	1	*Acidithiobacillus ferrooxidans* strain CK	KJ944318.1
	2	*Pichia pastoris*	FN392325.1	2	*Acidisphaera* sp. clone SBLE1E7	FJ228342.1
				3	*Acidisphaera* sp. clone SBLE1E7	FJ228342.1
k		*Aspergillus penicillioides*	GU017494.1		NA*	
l		*Cadophora fastigiata*	GU067761.1		NA*	

*NA: not analysable

though the relation between the presence of *A. ferrooxidans* and wood degradation is unclear, the species closely related to *A. ferrooxidans* were also found from the shipwreck, King Henry VIII's warship the *Mary Rose*[4].

The genus *Acidisphaera* contains a single species, *A. rubrifaciens,* which is aerobic, mesophilic, acidophilic and chemo-organotrophic[27]. The Hydrogenophilaceae are Betaproteobacteria; this family includes two genera – *Hydrogenophilus* and *Thiobacillus*. However, the role of these species in wood degradation remains unclear.

These analyses have shown that there are some fungal and bacterial species with the potential ability to degrade wood in these waterlogged Neolithic tree remains. However, this is the first time these groups have been surveyed in this environment. Further detailed analyses are therefore required to develop a better understanding of degradation mechanisms in waterlogged wood. For example, microscopic photographs, scanning and transmission electron microscope photographs would show degree of decay and presence of fungi and bacteria. In addition, a vast of DNA sequences obtained by using next generation sequencing technique would be used for microbial flora analysis. Combining these data, relation between wood degradation and microbial flora of waterlogged archeological

woods might be able to be revealed.

4. Conclusions

Using DGGE analysis, fungal and bacterial species were identified in degraded Neolithic tree remains. Related species of some fungi (*Aspergillus* sp. and *Penicillium* sp.) detected in this study are well known as soft-rot fungi. In addition, some bacteria (*Methylovirgula ligni* and *Micromonospora* sp.) are known as degraders of polysaccharide components derived from wood. The role of the other fungal and bacterial species that we detected remains unclear. Further research is in progress to clarify the mechanisms of degradation, for the purposes of remediation and restoration of waterlogged archeological woods.

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キーワード：denaturing gradient gel electrophoresis（DGGE, 変性剤濃度勾配ゲル電気泳動法）；microbial flora（微生物叢）；soft-rot fungi（軟腐朽菌）；bacteria（細菌類）；tree remains（出土樹木）

変性剤濃度勾配ゲル電気泳動法を用いた，トルコ，イェニカプ遺跡から発掘された新石器時代の出土樹木中に存在する微生物群の菌叢解析

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トルコ・イスタンブールにあるイェニカプで，マルマラ海峡地下トンネル工事中に，ビザンチン帝国時代，オスマン帝国時代の沈没船や教会などの遺跡，遺物の他，新石器時代に伐採された樹木が地下8.5m の深さから発掘された。Dogu らによって本樹種は *Fraxinus* spp. (トネリコ属)あるいは *Quercus* spp. (コナラ属)であると同定された。

このような超高含水率の出土樹木や木製品は，保存処理を行うまでの期間，乾燥防止を目的として通常水中で保管されるが，これによって軟腐朽菌や細菌類による腐朽が生じ易くなることが問題点として指摘されている。このような出土樹木や木製品はこれまで数多く発掘されているが，このような水中における樹木，木材などの腐朽機構についてはほとんど調査されてこなかった。そこで本研究では，変性剤濃度勾配ゲル電気泳動法を用いて新石器時代の出土樹木中に存在する菌類，細菌類の菌叢解析を行った。

その結果，9 種の菌類，7 種の細菌類が同定された。そのうち，*Aspergillus* sp.や *Penicillium* sp.といった軟腐朽菌，*Methylovirgula ligni* や *Micromonospora* sp.といった細菌は木材の主要構成成分であるセルロースや多糖を分解する能力を有することが知られており，木材分解において重要な役割を果たしていることが考えられた。その他の菌類，細菌類については，一部は分解残渣などを栄養源として生育していることが示唆されたが，残りの多くの菌類，細菌類の役割については依然不明である。

これまで類似の研究例はあまり報告されていないため，今後木材劣化に関わる微生物の塩基配列情報や顕微鏡写真といったより多くのデータを集積し，水中における樹木・木材等の腐朽機構の解明に役立てることは，出土木製品などの保存，修復のために重要であると考えられる。

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