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FULL PAPER

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Mycobiota of the Takamatsuzuka and Kitora Tumuli in Japan, focusing on the molecular phylogenetic diversity of *Fusarium* and *Trichoderma*

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Abstract In an effort to clarify the cause of the deterioration of the colorfully painted murals that adorn the inner walls of the small stone chambers in the Takamatsuzuka and Kitora Tumuli in Japan, we enumerated the fungi that were isolated from moldy spots on the plaster walls collected between May 2004 and April 2005. The 262 fungal isolates from 79 samples of both tumuli were identified as approximately 100 species based on their phenotypic characters. *Fusarium*, *Trichoderma*, and *Penicillium* species were the predominant colonizers in the stone chamber interior and adjacent areas of both tumuli. In addition to the 28S phylogeny, neighbor-joining and Bayesian phylogenies of partial *EF-1*-alpha gene sequences revealed 24 genetically diverse fusaria in the Takamatsuzuka and Kitora Tumuli. Most of the fusaria were nested in clade 3 of the *Fusarium solani* species complex (FSSC); however, a few isolates were members of the *F. oxysporum* species complex (FOSC) clade or the *F. avenaceum*/*F. tricinctum* species complex clade. The FSSC isolates were compared with those detected in the Lascaux cave in France. In addition, a partial *EF-1α* gene phylogeny indicated that 13 *Trichoderma* isolates clustered in the Harzianum-Virens clade and 5 isolates in the Viride clade or *Trichoderma* sect. *Longibrachiatum*. Our analyses suggest that most of the fungi recovered from both tumuli are typically soil dwellers.

Key words Archaeology and biodeterioration · Hypocreales · Molecular phylogenetic analysis · Mycobiota · Takamatsuzuka and Kitora Tumuli

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Introduction

The Takamatsuzuka Tumulus (hereafter, abbreviated to TT) and the Kitora Tumulus (hereafter, abbreviated to KT), which are thought to have been built toward the end of the later period of the Tumulus age (i.e., late 7th century), are well known to the general population of Japan as invaluable cultural heritage sites because of their beautiful mural (wall) paintings, which were drawn directly onto thin plaster in the small stone chamber interior (Fig. 1). The TT was discovered in 1972 and excavated in the same year, whereas the KT was discovered in 1983 and excavated only in early 2004. The Japanese government designated the TT and KT as Special Historic Sites in 1973 and 2000, respectively. These mural paintings have deteriorated since the excavation (Fig. 2).

The TT, a circular burial mound measuring only 20 m across, is located in the village of Asuka, Nara Prefecture, Japan (Fig. 2a). The stone chamber was constructed of cut slabs of tuff stone within the tumulus (tomb); the interior area is approximately 2.7 m deep, 1.0 m wide, and 1.1 m tall (see Fig. 1). The colorful paintings adorn the inner walls of the stone chamber with various colors, including red, green, yellow, blue, and gold. The figures comprise star constellations (*Seishuku*) on the ceiling, and the sun and the moon (*Nichi-Getsu*), the four heavenly guardian gods (*Shishin*), and groups of men and women on the four walls. The group of women on the west wall is called the "Asuka beauties" (Fig. 2d). All the paintings were designated as National Treasures by the government in 1974. In accordance with specialists' advice, the Japanese Agency for Cultural Affairs decided in 1973 to preserve the murals in the interior of the small stone chamber, which was never opened to the public. It was thought that the beauty and stability of the plaster paintings could be preserved if conditions of high humidity

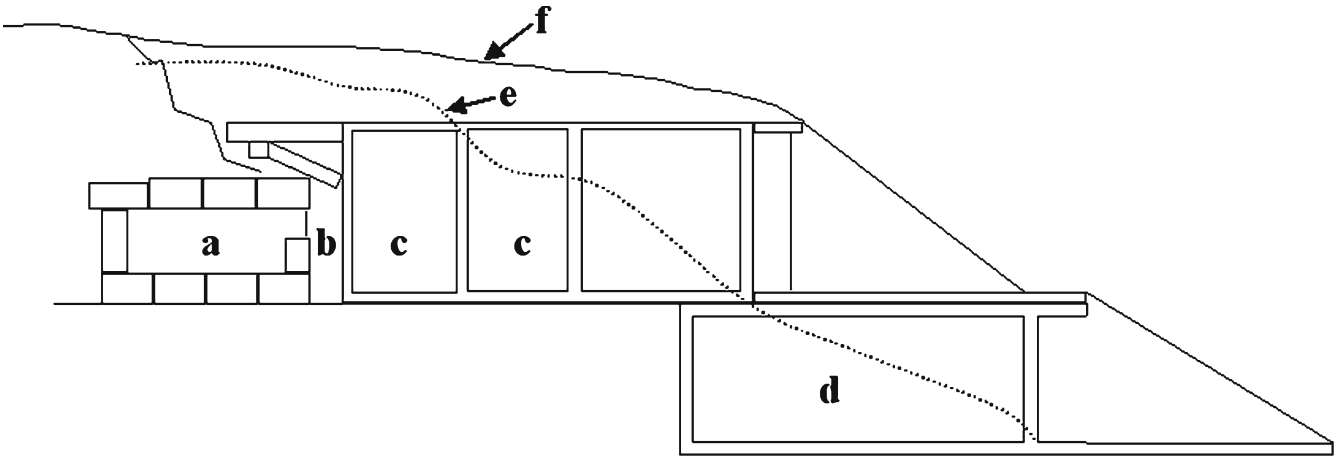

Fig. 1. Schematic diagram of a side view of the Takamatsuzuka Tumulus. *a*, stone chamber; *b*, adjacent space; *c*, adjacent rooms; *d*, mechanical room; *e*, line of the old mound; *f*, line of the recent mound

(100% RH) and cool temperature (14°–20°C) could be established. In addition, the stone chamber was kept in darkness except for regular inspections by the staff involved in its conservation. Because of the lack of light and because of conditions that were initially thought to be low in nutrients for growth of microorganisms and micro-animals, it was thought that the paintings would be protected against deterioration brought on by these organisms.

According to the official records (see the Agency homepage concerning the TT <http://www.bunka.go.jp/takamatsu_kitora/hekiga_hozonkanri.html>), *Alternaria* sp., *Cladosporium* sp., *Nigrospora* sp., and *Trichoderma viride* Pers.: Fr. were found in the stone chamber interior soon after the excavation in March 1972. In 1975, 3 years after excavation, fungi including a few species of *Doratomyces*, *Fusarium*, *Cladosporium*, and *Mucor* were detected as the main contaminants in the interior of the stone chamber (Arai 1984, 1987, 1990b; Agency for Cultural Affairs website, cited above).

Furthermore, during the periods of intense restoration between 1976 and the early 1980s, severe outbreaks of microbial contaminants such as *Doratomyces* sp. and *Streptomyces* sp. occurred on the walls inside the stone chamber (Arai 1984, 1987, 1990b; Agency for Cultural Affairs website, cited above). Fumigation with paraformaldehyde was performed at the end of the period of the outbreaks, after which microbial outbreaks became gradually less evident, in accordance with fewer entrances into the stone chamber (Kigawa et al. 2006b; Agency for Cultural Affairs website, cited above).

Species of *Aspergillus*, *Trichoderma*, and *Fusarium* along with actinomycetes and other molds were isolated in 1986–1987 (Arai 1984, 1987, 1990b); species of *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and other genera were found in the period 1994–2000 (Kigawa et al. 2006b). These fungi were constantly present in the stone chamber interior, but they did not cause serious damage to the murals. The mycobiota of the stone chamber interior was comparatively stable, and little damage occurred in these periods. However, particularly after the remediation work at the adjacent space of the stone chamber in spring 2001, the fungi again grew on the 1300-year-old murals, causing serious deterioration (Fig. 2d–g). In addition, viscous gels (i.e., biofilms that are mixtures of molds, yeasts, and bacteria), samples of which frequently contained mites (Fig. 2h) that were probably feeding on the fungi, were also visible on the wall plaster after 2004. Because of the state of deterioration, a plan to relocate the stone chamber began in April 2007, and the stone chamber was dismantled. By the end of June 2007, all the colorfully painted stone walls were relocated at an outside facility in the village of Asuka to save them from further deterioration and for necessary restoration. For more information on the situation relating to the murals, the stone chamber interior, and the adjacent space from the discovery to the end of 2000, see Kigawa et al. (2004, 2006b); for the details of the Agency's restoration project, see its homepage, cited above.

The KT is located 1.2 km south of the TT in the same village of Asuka. This circular tomb is approximately 14 m in diameter; the stone chamber interior is a little more than 2.4 m deep and 1 m wide and tall. A diagram of the stone chamber and related facilities is not illustrated here, but the KT adjacent small room corresponds to the adjacent space of the TT. Similar colorful murals are drawn on the thin plaster wall within the stone chamber, and a star chart on the ceiling is in the same style as in the TT. The interior environmental conditions are quite similar to those of the TT, and no conspicuous fungal colonies were seen for some time after the excavation. By the end of 2004, in the course of the excavation, species of *Acremonium*, *Fusarium*, *Penicillium*, and *Trichoderma* appeared as the main contaminants (Kigawa et al. 2005, 2006a, 2007). In August 2004, a species of *Phialocephala* was recovered from the stone wall in the adjacent small room of the stone chamber. In the case of the KT, the paintings on the plaster wall had become partly detached from their support. Considering their poor condition, the Agency decided to relocate these paintings to a controlled environment in July 2004, and by February

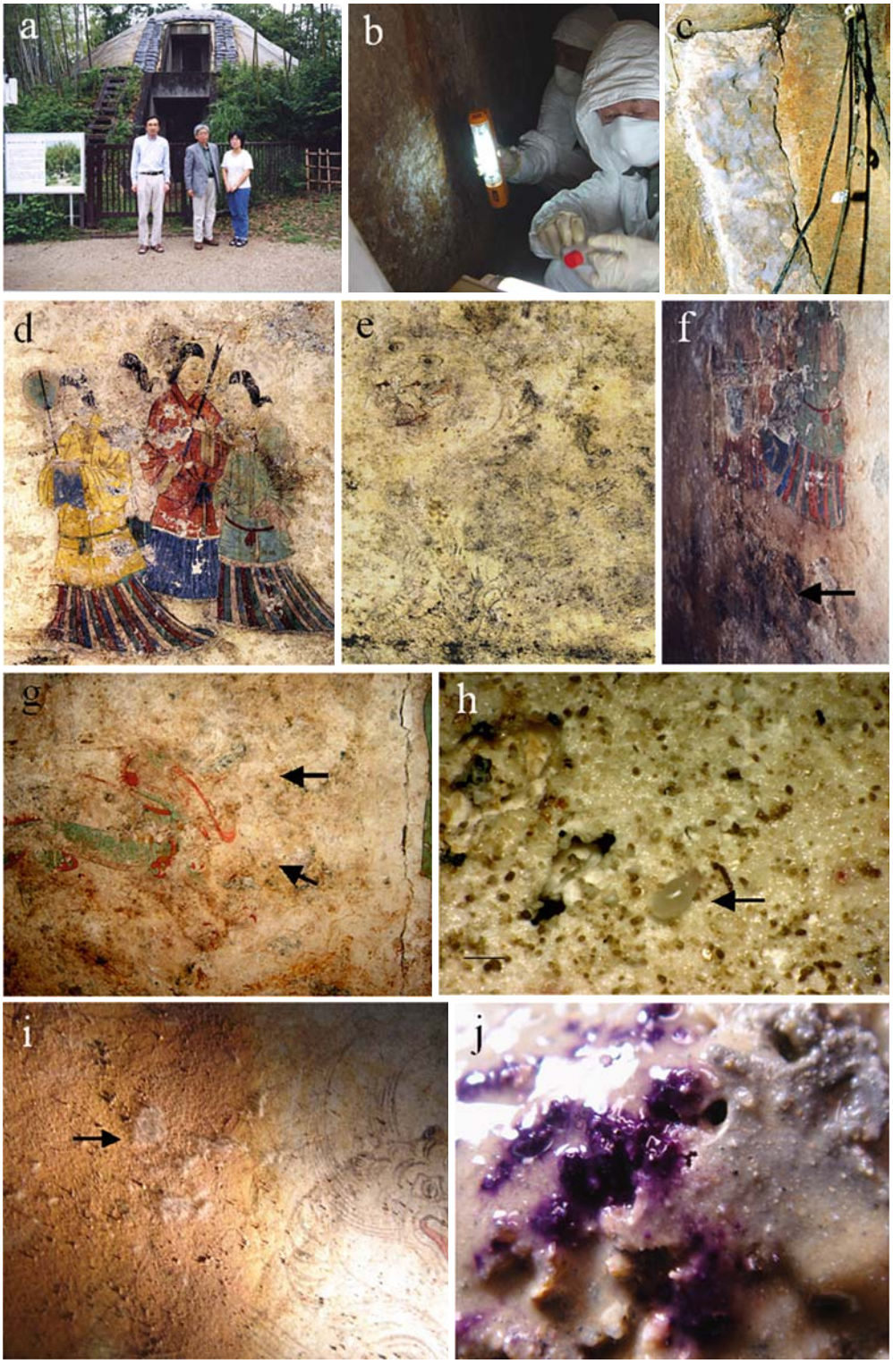

Fig. 2. Mural paintings with serious discolorations and blackening in the Takamatsuzuka Tumulus (**a**–**h**) and the Kitora Tumulus (**i**–**j**). **a** The Takamatsuzuka Tumulus in south view (photograph taken on May 19, 2004). **b** Sampling in the stone chamber interior and white mold on the west wall (photograph taken on Sept. 6, 2004). **c** White mold grew in the adjacent space after the remediation work (photograph taken on March 2001). **d** A group of women, the "Asuka beauties," on the west wall plaster with serious discoloration (photograph from the Agency for Cultural Affairs, 2004). **e** White tiger on the west wall with serious discoloration and blackening (photograph from the Agency for Cultural Affairs, 2004). **f** Black-stained region (*arrow*) under a group of women on the east wall plaster (photograph taken by J. Sugiyama on May 19, 2004). **g** Fungal colonies (*arrows*) on right part of a blue dragon on the east wall (photograph taken by R. Kigawa in May 2004). **h** A living mite (*arrow*) on the wall surface (photograph taken on Aug. 13, 2004). **i** White fungal colonies (*arrow*) on the left part of a white tiger on the west wall. **j** Purple-colored fungal colonies on the surface soil of the mound in the adjacent small room (photograph taken on May/April 2005)

2007 all except the star chart on the ceiling had been relocated.

In the present report, we identify the fungi isolated from both tumuli during the two survey periods: May to September 2004 for TT and June 2004 to April 2005 for KT. The fungi were isolated mainly using moist chamber and smear isolation methods. This study is the first to report species of *Fusarium* and *Trichoderma* as the predominant colonizers in the stone chamber interior and adjacent space or small room of both tumuli using molecular phylogenetic analyses. Cultural and morphological observations are presented in brief. The identity of the *Penicillium* isolates, which comprised the third dominant group found in the tumuli, will be published elsewhere.

Materials and methods

Sampling, isolating, and culturing

A total of 79 samples were collected from TT and KT; i.e., 37 samples from the former and 42 from the latter. Of the TT samples, 22 were taken from the stone chamber interior and 15 from the adjacent space between May and September 2004. Of the KT samples, 19 were taken from the stone chamber interior and 23 from the adjacent small room during June 2004 to April 2005. In addition to these, 11 strains (i.e., 6 from TT and 5 from KT) that were isolated by one of the authors (R.K.), using the smear method at the National Research Institute for Cultural Properties, were also examined in this study. These isolates are shown in Table 1 as TBT or TBK, respectively.

Sixty-five samples from both tumuli were collected using sterile moist cotton swabs, carefully avoiding damage to the paintings. We adopted the following three isolation methods. (1) A sterile moist cotton swab with the mold sample was rubbed immediately on moist filter paper in Petri dishes and incubated in a moist chamber at 25°C; these were observed at regular intervals for a period of about 2 months, and newly appearing colonies were isolated using a sterile needle under a stereo microscope (Gams et al. 1987; Krug 2004). (2) Swab samples were aseptically transported to the laboratory, where they were smeared directly on Petri dishes containing potato dextrose agar (PDA; Nihon Pharmaceutical, Tokyo, Japan) and then incubated at 25°C. (3) Opened Petri dishes were exposed to the air for 10 min and then incubated at 25°C to isolate air-borne fungi; 14 samples were recovered by this method. All fungal isolates were cultured on PDA in the dark at 25°C, and the first grouping was made at the generic level based on their cultural and morphological characters. All the isolates listed in Table 1 have been deposited in the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Wako, Saitama, Japan.

DNA extraction and PCR amplification

The isolates used for DNA sequencing are listed in Table 1, with the respective strain data. Additionally, we used 11 strains (6 isolates from TT during 2001 to 2004, and 5 recovered from KT from 2003 to 2005) isolated by one of the authors (R.K.); i.e., 3 *Cylindrocarpon* isolates, 3 *Fusarium* isolates, and 5 *Trichoderma* isolates were included for this study (cf. Table 1). However, the Kigawa's isolates were not included in Table 2. Their genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The two gene regions sequenced were 28S rRNA (rDNA) D1/D2 region (28 S) and part of the *EF-1*-alpha gene (= *tef1*; protein-coding gene translation-elongation factor 1-alpha; hereafter abbreviated *EF-1α*). Primers used included NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993) for 28S and EF-1F (5′-GTAAAACGACGGCCAGTATGGGTAAGGARGACAAGAC-3′) and EF-1 R (5′-GGAAACAGCTATGACCGGARGTACCAGTSATCATGTT-3′) for *EF-1α* of *Fusarium* and EF1-983F (5′-GTAAAACGACGGCCAGTGCYCCYGGHCAYGGTGAYTTYAT-3′) and EF1-2218R (5′-GGAAACAGCTATGACCATACRTGRGCRACRGTYTG-3′) for *EF-1α* of *Trichoderma*. Polymerase chain reactions (PCR) were performed using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA). Thermal cycling was performed using a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA) and the following cycling parameters: step 1, 5 min at 95°C; step 2, 40 cycles of 30 s at 94°C, followed by 30 s at 52°C for 28S or 50°C for *EF-1α* of *Fusarium* or 59°C for *EF-1α* of *Trichoderma*, followed by 1 min at 72°C; and step 3, 10 min at 72°C. The amplified DNA fragments were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

Sequencing

Sequencing reactions were performed using a BigDye Terminator Ready Reaction Mix contained in a BigDye Terminator Kit v3.1 (Applied Biosystems) and were performed in a GeneAmp PCR System 9600 (Applied Biosystems). All sequencing reactions were cleaned using a DyeExTM 2.0 Spin Kit (Qiagen). Sequences were determined using an ABI3100 DNA sequencer (Applied Biosystems). The sequences determined in this study were deposited in GenBank/EMBL/DDBJ; their accession numbers are given in Table 1. Other sequences downloaded from GenBank and the *Fusarium* database (http://fusarium.cbio.psu.edu/index.html; Geiser et al. 2004) are also shown in the respective molecular phylogenetic trees (Figs. 3–5).

Molecular phylogenetic analysis

The sequences were assembled using AutoAssembler (Applied Biosystems). Multiple alignments were performed using CLUSTAL W version 1.83 (Thompson et al. 1994); the final alignments were manually adjusted. Ambiguous positions and alignment gaps were excluded from the analysis. The neighbor-joining (NJ) tree (Saitou and Nei 1987) based on 28S and *EF-1α* sequences was constructed using the multiple alignment in MEGA ver3.1 (Kumar et al. 2004), with 1000 bootstrap replicates (Felsenstein 1985). Phylogenetic analyses of *EF-1α* sequences of *Fusarium* and *Trichoderma* were performed by NJ and Bayesian methods. The Bayesian approach to phylogenetic reconstruction (Rannala and Yang 1996) was implemented using MrBayes v3.1.1-p1 (Huelsenbeck and Ronquist 2001). The model of DNA substitution was calculated using Modeltest 3.0 (Posada and Crandall 1998), and the results were used in the General Time Reversible (GTR) model for *Fusarium* and *Trichoderma*. Bayesian Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses (Mau et al. 1999) were performed with MrBayes for phylogenetic estimation inferred from *EF-1α* sequences of *Fusarium* and *Tricho-*

Table 1. Strain data, with the GenBank accession numbers, for 28S and *EF-1α* gene sequences determined in this study

Taxon	Strain no.	JCM no.	Source	Tumulus[a]	Sampling date	GenBank accession no.	
						28S rDNA	EF-1α
Acremonium cf. *strictum*	K5217-2-2	15326	Black viscous gels behind the west wall in the stone chamber	K	17 Feb. 2005	AB294795	AB294844
Acremonium cf. *strictum*	K5217-2-3	15327	Black viscous gels behind the west wall in the stone chamber	K	17 Feb. 2005	AB294796	AB294845
Acremonium cf. *strictum*	K5225-6-1	15328	Yeast-like white colonies under the tortoise and the snake on the north wall in the stone chamber	K	25 Feb. 2005	AB294802	AB294851
Acremonium cf. *strictum*	K5225-13-3	15329	Air from northeast area in the stone chamber	K	25 Feb. 2005	AB294797	AB294846
Acremonium cf. *strictum*	K5225-14-3	15330	Air from northwest area in the stone chamber	K	25 Feb. 2005	AB294798	AB294847
Acremonium cf. *strictum*	K5225-15-1	15331	Air from southwest area in the stone chamber	K	25 Feb. 2005	AB294799	AB294848
Acremonium cf. *strictum*	K5225-19-1	15332	A piece of the fallen plaster in the west area in the stone chamber	K	25 Feb. 2005	AB294800	AB294849
Acremonium cf. *strictum*	K5225-19-2	15333	A piece of the fallen plaster in the west area in the stone chamber	K	25 Feb. 2005	AB294801	AB294850
Acremonium-like hyphomycete	K5225-10-5	15384	Air from west area in the adjacent small room	K	25 Feb. 2005	AB294803	–
Acremonium-like hyphomycete	K5225-14-1	15385	Air from northwest area in the stone chamber	K	25 Feb. 2005	AB294804	–
Acremonium-like hyphomycete	K5225-14-2	15386	Air from northwest area in the stone chamber	K	25 Feb. 2005	AB294805	–
Clonostachys sp.	T4519-4-1	15334	White cottony colonies on the lower center part of the south wall in the adjacent space	T	19 May 2004	AB294806	AB294852
Clonostachys sp.	T4519-4-2	15335	White cottony colonies on the lower center part of the south wall in the adjacent space	T	19 May 2004	AB294807	AB294853
Fusarium sp.	T4519-1-1	15348	White colonies on the left part of the south wall in the adjacent space	T	19 May 2004	AB294808	AB294854
Fusarium sp.	T4519-1-2	15349	White colonies on the left part of the south wall in the adjacent space	T	19 May 2004	–	AB294855
Fusarium sp.	T4519-3-1	15350	Green colonies on the plastic cover under the part of the hole dug by grave robbers in the adjacent space	T	19 May 2004	AB294817	AB294864
Fusarium sp.	T4519-5-2	15351	White colonies on the floor in the stone chamber	T	19 May 2004	AB294809	AB294856
Fusarium sp.	T4519-6-1	15352	White colonies on gauze on the floor in the stone chamber	T	19 May 2004	AB294810	AB294857
Fusarium sp.	T4519-7-1	15353	Green colonies on the floor in the stone chamber	T	19 May 2004	AB294811	AB294858
Fusarium sp.	T4519-9-1	15354	On the east wall in the stone chamber	T	19 May 2004	AB294812	AB294859
Fusarium sp.	T4519-9-2	15355	On the east wall in the stone chamber	T	19 May 2004	AB294813	AB294860
Fusarium sp.	T4519-9-3	15356	On the east wall in the stone chamber	T	19 May 2004	AB294814	AB294861
Fusarium sp.	T4519-10-2	15357	On the plaster near the west wall in the stone chamber	T	19 May 2004	AB294821	–
Fusarium sp.	T4906-10-1	15358	Black colonies near the blue dragon on the east wall in the stone chamber	T	6 Sept. 2004	AB294815	AB294862
Fusarium sp.	T4906-11-4	15359	Body surface of an Isopoda on the wall in the stone chamber	T	6 Sept. 2004	AB294818	AB294865
Fusarium sp.	T4906-11-5	15360	Body surface of an Isopoda on the wall in the stone chamber	T	6 Sept. 2004	AB294820	AB294867
Fusarium sp.	T4906-11-6	15361	Body surface of an Isopoda on the wall in the stone chamber	T	6 Sept. 2004	AB294819	AB294866
Fusarium sp.	T4922-8-2	15362	Viscous gels in the back part of the blue dragon on the east wall in the stone chamber	T	22 Sept. 2004	AB294816	AB294863

Fusarium sp.	K5225-2-1	15340	Pink colonies on soil in the adjacent small room	K	25 Feb. 2005	AB294829	AB294874
Fusarium sp.	K5225-19-3	15341	A piece of the fallen plaster in the west area in the stone chamber	K	25 Feb. 2005	AB294823	AB294869
Fusarium sp.	K5427-1-1	15342	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294822	AB294868
Fusarium sp.	K5427-1-2	15343	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294827	AB294872
Fusarium sp.	K5427-1-3	15344	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294828	AB294873
Fusarium sp.	K5427-1-5	15345	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294824	AB294870
Fusarium sp.	K5427-2-1	15346	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294825	–
Fusarium sp.	K5427-2-2	15347	Purple colonies in the adjacent small room	K	27 Apr. 2005	AB294826	AB294871
Trichoderma sp.	T4519-2-6	15373	Green colonies on the lower part of the south wall in the adjacent space	T	19 May 2004	AB294832	AB294877
Trichoderma sp.	T4519-2-7	15374	Green colonies on the lower part of the south wall in the adjacent space	T	19 May 2004	AB294833	AB294878
Trichoderma sp.	T4519-2-8	15375	Green colonies on the lower part of the south wall in the adjacent space	T	19 May 2004	AB294834	AB294879
Trichoderma sp.	T4519-5-7	15376	White colonies on the floor in the stone chamber	T	19 May 2004	AB294831	AB294876
Trichoderma sp.	T4519-7-5	15377	Green colonies on the floor in the stone chamber	T	19 May 2004	AB294836	AB294881
Trichoderma sp.	T4519-10-5	15378	On the plaster near the west wall in the stone chamber	T	19 May 2004	AB294830	AB294875
Trichoderma sp.	T4519-10-6	15379	On the plaster near the west wall in the stone chamber	T	19 May 2004	AB294835	AB294880
Trichoderma sp.	K5225-1-1	15367	Pink colonies on soil in the adjacent small room	K	25 Feb. 2005	AB294837	AB294882
Trichoderma sp.	K5225-3-1	15368	White colonies on soil in the adjacent small room	K	25 Feb. 2005	AB294841	AB294886
Trichoderma sp.	K5225-3-2	15369	White colonies on soil in the adjacent small room	K	25 Feb. 2005	AB294842	AB294887
Trichoderma sp.	K5225-6-6	15370	Yeast-like white colonies under the lower part of the tortoise and the snake on the north wall in the stone chamber	K	25 Feb. 2005	AB294838	AB294883
Trichoderma sp.	K5225-17-3	15371	On decayed root in the adjacent small room	K	25 Feb. 2005	AB294840	AB294885
Trichoderma sp.	K5225-18-3	15372	On decayed root in the west area in the adjacent small room	K	25 Feb. 2005	AB294839	AB294884
Cylindrocarpon sp.	TBT-1	15337	Under the tail of the tortoise and the snake on the north wall in the stone chamber	T	18 Dec. 2001	AB373716	AB373728
Cylindrocarpon sp.	TBT-2	15338	On the part of the south wall in the stone chamber	T	18 Dec. 2001	AB373717	AB373729
Fusarium sp.	TBT-3	15365	Near the tortoise and the snake on the north wall in the stone chamber	T	18 Dec. 2001	AB373718	AB373730
Fusarium sp.	TBT-4	15339	Black stain near the blue dragon on the east wall in the stone chamber	T	30 Oct. 2002	AB373719	AB373731
Trichoderma sp.	TBT-5	15382	Molds on soil mound in the adjacent space	T	16 Sept. 2003	AB373720	AB373732
Trichoderma sp.	TBT-7	15383	The upper part of the hole dug by grave robbers in the adjacent space	T	5 Apr. 2004	AB373721	AB373733
Trichoderma sp.	TBK-1	15380	Green molds on the west wall in the stone chamber	K	17 May 2004	AB373722	AB373734
Trichoderma sp.	TBK-3	15381	Green molds on soil that flowed into the stone chamber	K	17 May 2004	AB373723	AB373735
Trichoderma sp.	TBK-6	15366	A white floc of mold on soil of the central part in the adjacent small room	K	16 Sept. 2003	AB373724	AB373736
Fusarium sp.	TBK-8	15363	A white moldy area on soil where resin was applied in the adjacent small room	K	16 Sept. 2003	AB373725	AB373737
Cylindrocarpon sp.	TBK-22	15336	Brown spots in the lower part of the forefoot of the white tiger on the west wall in the stone chamber	K	17 Dec. 2005	AB373727	AB373738

[a]T and K indicate the Takamatsuzuka Tumulus and the Kitora Tumulus, respectively

Table 2. Comparison of fungal taxa isolated from the stone chamber interior and adjacent space or small room in the Takamatsuzuka and Kitora Tumuli

Taxon	Takamatsuzuka Tumulus		Kitora Tumulus	
	Stone chamber	Adjacent space	Stone chamber	Adjacent small room
Acremonium cf. *strictum*			8 (11.1)	
Acremonium (sect. *Gliomastix)* spp.	1 (1.1)		1 (1.4)	2 (3.3)
Acremonium-like hyphomycete			2 (3.0)	1 (1.7)
Arthrobotrys sp.	1 (1.1)			
Aspergillus spp.		1 (3.0)	1 (1.4)	2 (3.3)
Clonostachys sp. (*Clonostachys* cf. *rosea*)		3 (8.0)		
Cladosporium spp.	2 (2.2)	1 (2.5)	1 (1.4)	
Fusarium spp.	24 (27.1)	8 (20.0)	8 (11.1)	11 (18.3)
Gliocladium spp.	4 (5.0)	2 (5.0)	4 (6.0)	
Nigrospora sp.		1 (3.0)		
Paecilomyces spp.	2 (2.2)	2 (5.0)		
Penicillium sp. 1	11 (12.4)	8 (20.0)	8 (11.1)	2 (3.3)
Penicillium spp.	13 (14.6)	6 (15.0)	27 (37.1)	11 (18.3)
Phialocephala cf. *phycomyces*				2 (3.3)
Sporothrix sp.				1 (1.7)
Trichoderma spp.	22 (25.1)	8 (20.0)	1 (1.4)	18 (30.0)
Verticillium spp.	2 (2.2)		1 (1.4)	
Unidentified yeast spp.	6 (7.1)		3 (4.1)	6 (10.0)
Unidentified hyphomycete spp.	1 (1.1)		8 (11.1)	4 (6.7)
Total no. of isolates	89	40	73	60
Total no. of examined samples	22	15	19	23

Number of isolates and occurrence frequency: % = (no. of isolates/total isolates) × 100

derma. MrBayes was run for 6 000 000 generations for *EF-1α* of *Fusarium* and 21 000 000 generations for that of *Trichoderma*. Searches were conducted with four chains (three cold, one hot) with trees sampled every 100 generations. The average standard deviation of split frequencies was 0.009 at the end of the run. The support of nodes was measured by posterior probabilities obtained from the majority rule consensus after deleting the trees during burn-in.

Results and discussion

From a total of 79 samples (37 from TT, 42 from KT), about 262 fungal strains (including unidentified groups) were isolated, as listed in Table 2. They were identified to approximately 100 species based on their cultural and morphological characters, of which 39 species were recovered from TT and 61 from KT. A total of 129 strains were isolated from TT, i.e., 89 from the stone chamber interior and 40 from the adjacent space, respectively. By way of contrast, a total of 133 strains were isolated from KT, 73 from the stone chamber interior and 60 from the adjacent small room, respectively. One isolate, T4922-1-1 (see Table 2), proved to be an undescribed species of *Candida* that is described in Nagatsuka et al. (2008); the identity of the predominant *Penicillium* isolates will be published elsewhere.

Species of *Fusarium*, *Trichoderma*, and *Penicillium* were the dominant colonizers in both tumuli (see Table 2). From TT, 32 *Fusarium*, 30 *Trichoderma*, and 38 *Penicillium* isolates were recovered, whereas from KT, 19 *Fusarium*, 19 *Trichoderma*, and 48 *Penicillium* isolates were obtained. *Arthrobotrys* sp., *Clonostachys* sp., *Nigrospora* sp., and *Paecilomyces* spp. were only isolated from the TT. By way of contrast, *Acremonium* cf. *strictum* W. Gams, *Acremonium*-like hyphomycetes, *Fusarium* spp., *Phialocephala* cf. *phycomyces* W.B. Kendr., and *Sporothrix* sp. were only found in the KT. Arai (1984, 1987, 1990b) reported that *Doratomyces* sp., *Fusarium* sp., *Cladosporium* sp., and *Mucor* sp. were isolated as the predominant colonizers from the TT in March 1975, while a few *Trichoderma* sp. and *Penicillium* sp. were also isolated. Among these fungi, the dematiaceous genus *Doratomyces* and the zygomycetous genus *Mucor* have not been encountered in our survey from either tumulus during this period. Fungi recorded from other selected tumuli in Japan are summarized in Table 3 from literature published previously (Emoto and Emoto 1974; Emoto et al. 1983; Arai 1984, 1990a). Most representatives of these fungal genera are commonly found in soil (Ciferri 1999; Domsch et al. 2007).

Very recently, serious problems caused by members of the *Fusarium solani* (Mart.) Sacc. species complex (FSSC) in the prehistoric (15 000-year-old) painted cave of Lascaux in France were reported (Dupont et al. 2007), where the mold appeared to spread from the floor to the cave paintings (Holden 2003; Graff 2006; Orial and Mertz 2006). Members of the FSSC have been reported to deteriorate wall paintings (Dhawan et al. 1993; Garg et al. 1995). Dupont et al. (2007) also isolated species of *Chrysosporium*, *Gliocladium*, *Gliomastix*, *Paecilomyces*, *Trichoderma*, and *Verticillium* in addition to members of the FSSC in the cave.

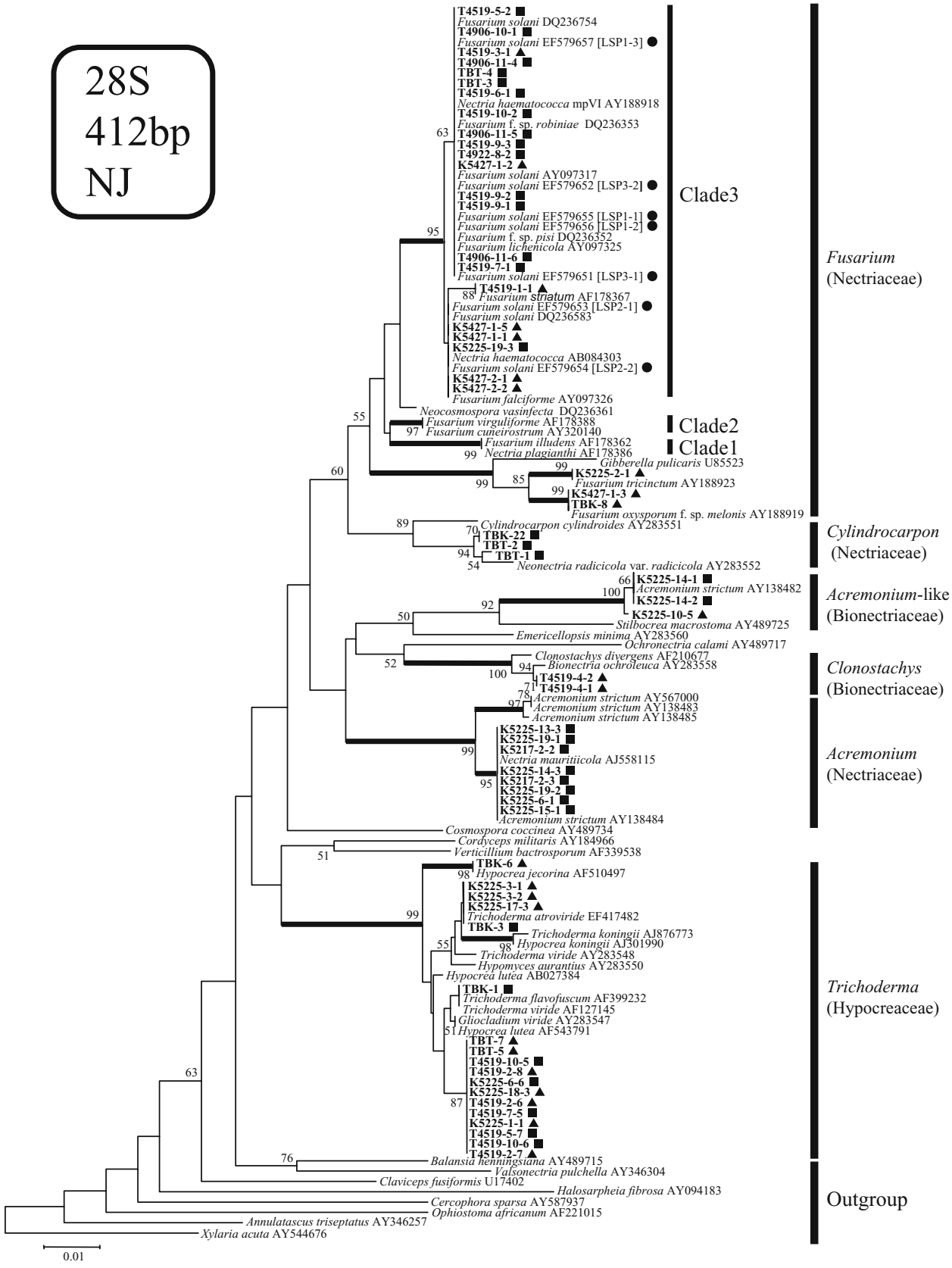

Fig. 3. Phylogenetic relationships among 59 Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT) isolates and 60 known Hypocrealean fungi with the accession numbers downloaded from GenBank, based on neighbor-joining (*NJ*) analysis of 28S rDNA-D1/D2 region sequence data of 412 aligned nucleotide sites using MEGA ver. 3.1. *Numbers* on the branch nodes represent bootstrap support values (%) based on 1000 replications; bootstrap values greater than 50% are indicated. *T* and *TBT* indicate isolates from the TT; *K* and *TBK* indicate isolates from the KT; ■ indicates the isolates from the respective stone chamber interiors; ▲ indicates those from the adjacent space or small room of both tumuli. Clades 1–3 refer to O'Donnell (2000) and Aoki et al. (2003); strain labels, ●, LSP1–LSP3 refer to Dupont et al. (2007). (For details, see text)

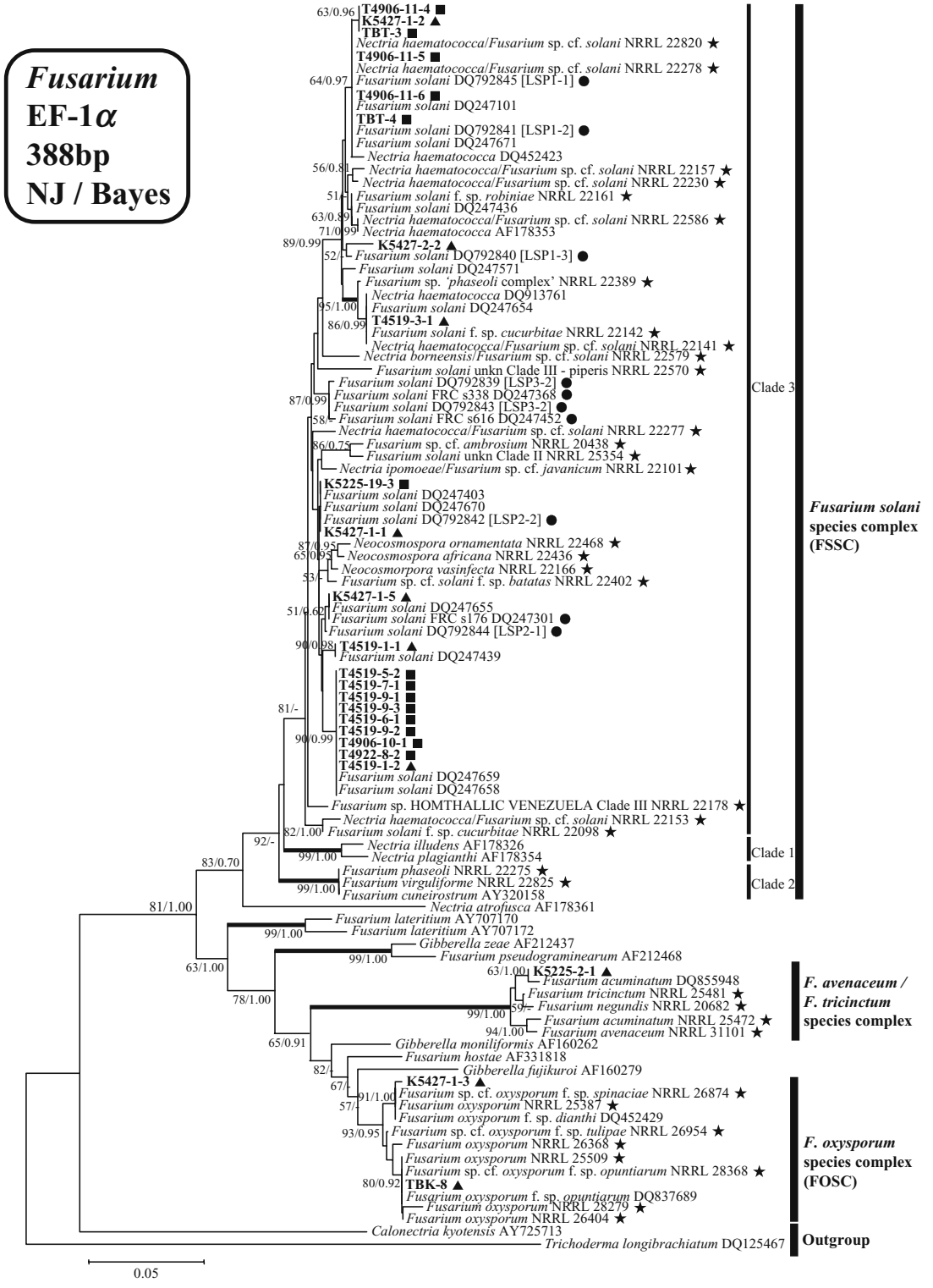

Fig. 4. Phylogenetic relationships among 24 *Fusarium* isolates from the Takamatsuzuka Tumulus (TT) and the Kitora Tumulus (KT), and known *Fusarium* species and relatives with the accession numbers downloaded from GenBank and the *Fusarium*-ID Database (http://fusarium.cbio.psu.edu/index.html; Geiser et al. 2004) based on *NJ* and Bayesian (*Bayes*) analyses of *EF-1α* gene sequences data of 388 aligned nucleotide sites, using MEGA ver. 3.1 and MrBayes. *Numbers* on branches indicate Bayesian posterior probability and bootstrap support values (%) based on 1000 replications; bootstrap values greater than 50% are indicated. *Right vertical bars* indicate the clades defined by O'Donnell (2000), O'Donnell et al. (2007), Yli-Mattila et al. (2002), and Aoki et al. (2003). ★ indicates the data from *Fusarium*-ID Database (Geiser et al. 2004; http://fusarium.cbio.psu.edu/index.html). (For other abbreviations, see Fig. 3)

Fig. 5. Phylogenetic relationships of 18 Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT) isolates, and known *Trichoderma* species and relatives with the accession numbers downloaded from GenBank based on NJ and Bayesian analyses of 675 aligned *EF-1α* nucleotide sites, using MEGA ver. 3.1 and MrBayes. *Numbers* on branches indicate Bayesian posterior probability and bootstrap support values (%) based on 1000 replications; bootstrap values greater than 50% are indicated. *Right vertical bars* indicate the sections and clades of *Trichoderma* defined by Samuels (2006). The *Hypocrea* clades in *parentheses* were first described by Chaverri and Samuels (2003). (For other abbreviations, see Fig. 3)

Table 3. Fungi recorded from selected tumuli in Japan

Tumulus	Locality (Prefecture)	Taxon[a]	Reference
Takamatsuzuka	Nara	***Doratomyces* sp., *Fusarium* sp., *Cladosporium* sp., *Mucor* sp.,** *Trichoderma* sp., *Pencillium* sp., *Fusarium* sp.[b], *Trichoderma* sp.[b], *Penicillium* sp.[b], *Verticillium* sp.[b], *Cladosporium* sp. (*Hormodendron* type)[b]	Arai 1984
Torazuka	Ibaraki	**Basidiomycete 1, Basidiomycete 2, *Penicillium* sp.,** *Alternaria* sp., *Trichoderma* sp., *Aspergillus* sp., *Cladosporium* sp., *Pestalotia* sp.	Arai 1984, 1990a; Emoto et al. 1983
Ozuka	Fukuoka	*Gliocladium virens*, *Gliomastix* sp., *Mucor* sp., *Penicillium purpurogenum* var. *rubri-sclerotium*, *Penicillium* sp., *Trichoderma viride*, *Verticillium* sp., *Alternaria alternata*[c], *Cladosporium herbarum*[c], *Epicoccum purpurascens*[c], *Fusarium oxysporum*[c], *Penicillium citrinum*[c]	Emoto and Emoto 1974
Chibusan	Kumamoto	*Gliocladium roseum*, *Idriella* sp., *Monodictys* sp., *Mortierella* sp., *Mucor* sp., *Penicillium citreoviride*, *Penicillium janthinellum*, *Penicillium oxalicum*	Emoto and Emoto 1974
Kiyotosako-oketsu	Fukushima	A whitish fungus	Arai 1984
Hayama-oketsu	Fukushima	*Trichoderma* sp. (a whitish fungus)	Arai 1984
Nakata-oketsu	Fukushima	*Phialophora* sp. (a pale white fungus)	Arai 1984

[a]The taxon names shown in bold font indicate the predominant group of the respective tumuli
[b]Species isolated from the adjacent space
[c]Species isolated as air-borne fungi

The fungal cultures isolated between 1975 and spring 2001, noted in the Introduction and Table 3, are no longer viable. Thus, detailed comparisons with the previously isolated strains from selected tumuli in Japan are impossible. However, comparisons based on the gene sequence data and molecular phylogenetic assignment for the Lascaux FSSC isolates (Dupont et al. 2007) follow.

Molecular phylogenetic placement of isolates of *Fusarium*, *Trichoderma*, and relatives (Fig. 3)

Using *Balansia henningsiana* (Möller) Diehl and seven other sordariomycetous species as outgroup taxa, the NJ phylogenetic tree (Fig. 3) was inferred from 412 bp of 28S sequences of 99 isolates of *Fusarium* and relatives, and an additional 11 strains (not included in Table 2) isolated by one of the authors (R.K.) between December 2001 and December 2005, from both tumuli. From Fig. 3, it can be seen that all are members of the Hypocreales and include species of *Acremonium* (Nectriaceae), an *Acremonium*-like hyphomycete (Bionectriaceae), *Clonostachys* (Bionectriaceae), *Cylindrocarpon* (Nectriaceae), *Fusarium* (Nectriaceae), and *Trichoderma* (Hypocreaceae). A good correlation was observed between the molecular phylogenetic placement and phenotypic characters for most of the isolates. The latter characterization will be published in the forthcoming paper.

In the 28S phylogeny (see Fig. 3), the biggest assemblage was made up of representatives of FSSC clade 3, which comprised 16 TT and 6 KT isolates. The remaining 3 KT isolates (i.e., these from the adjacent small room), with *Fusarium tricinctum* (Corda) Sacc. AY188923 and *Gibberella pulicaris* (Fr.: Fr.) Sacc. U85523, grouped outside the FSSC (O'Donnell 2000; Aoki et al. 2003). Further analyses and discussion of the TT and KT isolates, FSSC clades 1–3, and the Lascaux cave FSSC isolates (LSP1–LSP3 in Dupont et al. 2007) are given the following section.

Of the 16 isolates recovered, 13 were derived from the stone chamber interior, the remaining 3 from the adjacent space. Eight KT isolates (i.e., K5217-2-2, K5217-2-3, K5225-6-1, K5225-13-3, K5225-14-3, K5225-15-1, K5225-19-1, and K5229-19-2) were placed in the cluster represented by *Acremonium strictum* (Nectriaceae), including the ex-type strain CBS 346.70 (isolated from *Triticum aestivum* leaf) of *A. strictum* (AY138483; see Table 3 in Novicki et al. 2003) and *A. strictum* AY 138484 and AY 138485 (isolated from human blood; Novicki et al. 2003). Although cultural and morphological characteristics of these KT isolates agreed with those of *A. strictum* (Gams 1971; Domsch et al. 2007), additional molecular phylogenetic data are needed to identify KT isolates to species. However, no isolate assigned to *A. strictum* was obtained from the TT samples.

Three *Acremonium*-like isolates (K5225-10-5, K5225-14-1, and K5225-14-2), which were only recovered from the KT, grouped together in another cluster with a human isolate of *A.* cf. *strictum* AY138482 (as *A. strictum* genogroup II in Novicki et al. 2003). *Stilbocrea macrostoma* (Berk. & M. A. Curtis) Höhn. AY489725 (strain G.J.S. 73-26, = CBS 114375), having a synnematous anamorph *Stilbella aleuriata* (Berk. & M. A. Curtis) Seifert (Seifert 1985; Rossman et al. 1999), is related to these three KT isolates in this phylogenetic analysis. Only monophialidic conidiophores were observed on the PDA plates of these KT isolates.

Two *Clonostachys* isolates (T4519-4-1 and T4519-4-2) were only isolated from the adjacent space of the TT stone chamber; these grouped with *Clonostachys divergens* Schroers and *Bionectria ochroleuca* (Schwein.) Schroers & Samuels (with *Clonostachys rosea* (Link) Schroers & al. anamorph). Cultural and morphological characterization for the TT isolates fell in the generic definition of *Clonostachys* defined by Schroers (2001). *C. rosea* is known as a common soil fungus and a mycoparasite (Schroers 2001). Therefore, these data may suggest that the two *Clonostachys* isolates originated in the soil.

Three *Cylindrocarpon* strains (TBT-1, TBT-2, and TBK-22) clustered in the *Neonectria radicicola*/*Cylindrocarpon* clade (Nectriaceae) (clade III in Mantiri et al. 2001). This clade corresponds to *Cylindrocarpon* group 3 of Booth (1966). The *N. radicicola* clade (clade III) is characterized by production of microconidia, macroconidia, and abundant chlamydospores (Mantiri et al. 2001). Phenotypic characterization for our three isolates, producing microconidia, macroconidia, and chlamydospores, agreed well with the description of *Cylindrocarpon* group 3 (Booth 1966). Old PDA cultures of strain TBK-22, isolated from the brown spots near the front legs of the white tiger (Byakko) on the west wall in the KT stone chamber, became brown after 1 month. The cultural and morphological characteristics suggest that this species was involved in contamination of the wall plaster.

Molecular phylogenetic placement of *Fusarium* (Fig. 4)

The 28S gene sequences are too highly conserved to discriminate species of the commonly occurring genera *Fusarium* and *Trichoderma*. For putative identification of species in these genera, NJ and Bayesian analyses of partial *EF-1α* sequences were utilized (O'Donnell et al. 1998; Geiser et al. 2004; Samuels 2006).

Calonectria kyotensis Terash. ATCC 18834 and *Trichoderma longibrachiatum* Rifai DAOM 234103 were used as outgroup taxa for all phylogenetic-based identifications of *Fusarium* isolates. The NJ and Bayesian tree was inferred from 388 bp of *EF-1α* gene sequences, including data from GenBank and the *Fusarium*-ID Database (Geiser et al. 2004; http://fusarium.cbio.psu.edu/index.html).

Twenty-four TT and KT *Fusarium* isolates and known species of *Fusarium* retrieved from GenBank and the *Fusarium* Database, including their teleomorphs, were divided into five groups (see Fig. 4): these included FSSC clade 3 (O'Donnell 2000; Aoki et al. 2003), which is the biggest assemblage (O'Donnell 2000), the smaller FSSC clades 1 and 2 (O'Donnell 2000; Aoki et al. 2003), representatives of the *F. avenaceum*/*F. tricinctum* species complex (Yli-Mattila et al. 2002) clade, and the *F. oxysporum* species complex (FOSC) (O'Donnell et al. 1998, 2007) clade. Seven strains assigned to three distinct phylogenetic species (abbreviations: LSP1–LSP3) of the FSSC from the Lascaux cave in France reported by Dupont et al. (2007) were included in both trees (Figs. 3, 4).

The majority of the TT and KT *Fusarium* isolates were nested within the FSSC clade 3 along with one phylogenetic species (isolates LSP 1-1 and -2) from the Lascaux cave from Dupont et al. (2007) (see Figs. 3, 4). One comprised 5 TT (TBT-3, T4906-11-4, T4906-11-5, T4906-11-6, and TBT-4) isolates that were only recovered from inside the stone chambers, and 2 KT (K5427-1-2 and K5427-2-2) isolates only recovered from the adjacent small room. These isolates were characterized by cream to yellowish-white colonies on PDA with violet to reddish-brown pigments in the agar and relatively slender, straight to slightly curved macroconidia and chlamydospores. Another major group was represented by "*F. solani*" DQ247659 and included 2 Lascaux cave isolates of LSP 2, 10 TT cultures (T4519-1-1, T4906-10-1, T4519-6-1, T4922-8-2, T4519-9-3, T4519-1-2, T4519-5-2, T4519-7-1, T4519-9-1, and T4519-9-2), and 3 KT cultures (K5225-19-3, K5427-1-5, and K5427-1-1). The TT and KT isolates represented the major *Fusarium* contaminants and were characterized by white, cream to yellowish-white colonies on PDA, relatively wide, straight to slightly curved macroconidia, and chlamydospores. Two isolates from the Lascaux cave (LSP3 in Fig. 4) were closely related to two FRC (Fusarium Research Center Culture Collection) strains (s338 and s616) of *F. solani* from air sample (USA) and from soil (New Caledonia), respectively (cf. Dupont et al. 2007) in the FSSC. No TT and KT isolates were contained in this cluster. Members of FSSC clade 3 are well known as colonizers of a variety of plants, soils, mycetomas, and other substrates (Zhang et al. 2006; Domsch et al. 2007).

One of the minor colonizers, KT isolate K5225-2-1, was characterized by rose- to burgundy-colored colonies on PDA with dark red pigments in the agar and needle-like to sickle-shaped, slender, straight to slightly curved macroconidia, and chlamydospores. It was nested in the *F. avenaceum*/*F. tricinctum* species complex, with *F. avenaceum* (Fr.) Sacc. and *F. tricinctum* (Corda) Sacc. Members of this clade are known as plant pathogens and soil colonizers (Leslie and Summerell 2006).

Other minor colonizers, KT isolates TBK-8 and K5427-1-3, were characterized by pale violet- to burgundy-colored colonies on PDA with dark reddish-brown pigments in the agar and relatively slender, straight to slightly curved macroconidia, and chlamydospores. These were placed in the FOSC clade. The KT isolates agreed well with the description of *F. oxysporum* Schltdl.: Fr., which has a worldwide distribution mostly as a saprotroph in soil, as a parasite on a variety of host plants, and as human pathogens (O'Donnell et al. 2004; Domsch et al. 2007).

None of the fusaria from the Japanese tumuli or from the Lascaux caves of France clustered in FSSC clade 1 (a New Zealand clade) or 2 (a South American clade) (O'Donnell 2000; Aoki et al. 2003).

Most of the Japanese and French fusaria clustered in clade 3 of the FSSC. Taxonomy of the FSSC is unresolved (O'Donnell 2000). Further studies are required to fully elucidate the identity of TT and KT fusaria assigned to clade 3 (see Figs. 3, 4).

Molecular phylogenetic placement of *Trichoderma* isolates (Fig. 5)

The *Trichoderma* isolates were analyzed phylogenetically (Fig. 5) with *Hypomyces stephanomatis* Rogerson & Samuels G.J.S. 88-50 and *Nectria cinnabarina* (Tode) Fr. G.J.S. 91-111 (CBS 713.97) as outgroup taxa. The bootstrapped NJ and Bayesian tree was inferred from 675 bp of *EF-1α* gene sequences of 18 TT and KT isolates and other sequences of *Trichoderma*/*Hypocrea* retrieved from GenBank.

The TT and KT isolates were divided into three clades (Fig. 5); these included the Harzianum-Virens clade (Catoptron/Lixii clade), Viride clade (Rufa clade), and *Trichoderma* sect. *Longibrachiatum* (Chaverri and Samuels 2003; Samuels 2006).

Most of the isolates clustered in the Harzianum-Virens clade (Catoptron/Lixii clade), including nine TT isolates (T4519-2-6, T4519-2-7, T4519-2-8, T4519-5-7, T4519-7-5, T4519-10-5, T4519-10-6, TBT-5, and TBT-7) and four KT isolates (K5225-1-1, K5225-6-6, K5225-18-3, and TBK-1). The *EF-1α* phylogeny indicates the existence of haplotypes, particularly in *Hypocrea lixii* Pat., and the associated *Trichoderma harzianum* Rifai anamorph, as seen in the FSSC clade 3 (see Fig. 4). These TT and KT strains were isolated from the stone chamber interior and the adjacent space or small room (see Table 2). Members of this clade may be related to the cause of most of the deterioration of the wall plaster and murals, particularly in the interior of the TT stone chamber. The morphological species *T. harzianum* was the most commonly encountered *Trichoderma* from direct isolations, but it likely represents multiple phylogenetically distinct species (Samuels, personal communication). It is commonly isolated from soils (Domsch et al. 2007) and occurs as a colonizer of diverse plant substrates (Chaverii and Samuels 2002).

Four KT *Trichoderma* isolates (K5225-17-3, K5225-3-1, K5225-3-2, and TBK-3) formed a sister lineage of *Hypocrea rufa* (Pers.: Fr.) Fr. in the Viride clade (Rufa clade). *Hypocrea rufa* and its anamorph *T. viride* Pers., both of which were redefined and epitypified by Jaklitsch et al. (2006), show a north temperate distribution in soils and on decayed woods. The *EF-1α* phylogeny (see Fig. 5) suggests that these KT isolates are *H. rufa*. However, greater taxon sampling is needed to determine the taxonomic position of the KT isolates. These four strains were only isolated from soil that had been introduced inadvertently into the KT stone chamber interior and from soil/decayed roots in the adjacent small room. This *Trichoderma* sp. had been involved in contamination of the KT wall plaster because they were recovered from inside of the stone chamber.

One strain (TBK-6) isolated from the KT adjacent small room clustered with *Hypocrea jecorina* Berk. & Broome/*T. reesei* E.G. Simmons and *Trichoderma* cf. *citrinoviride* GJS 01-364 (= *Hypocrea* sp. GSJ 01-364 in Chaverri et al. 2003) (sect. *Longibrachiatum* clade; Samuels 2006) with strong bootstrap support. Members of section *Longibrachiatum*, which are colonizers of various plant substrates (Chaverri et al. 2003), are known for cellulase production (e.g., Kubicek et al. 1996).

In conclusion, our analysis of phenotypic characters of 262 fungal isolates from the TT and KT represented approximately 100 species. Among these, *Fusarium*, *Trichoderma*, and *Penicillium* species were the predominant colonizers in both tumuli. Our molecular phylogenies inferred from the 28S and partial *EF-1α* gene sequences indicate that these represent at least six *Fusarium* and four *Trichoderma* species from both tumuli. Most of these fungi were most likely carried into the stone chamber by water or other vectors (e.g., mites, other micro-animals, and air; Guglielminetti et al. 1994; Gorbushina and Petersen 2000) from the surrounding soils. The association of *Fusarium* and *Trichoderma* TT and KT isolates with human activities related to the conservation efforts (e.g., regular inspections, restoration work) will be discussed in forthcoming papers. Comparative studies of the genealogical relationships of *Fusarium* species/haplotypes, all with full strain data from both tumuli in Japan, the Lascaux cave in France, and the diverse environments are needed before their definite species identity can be determined.

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