Invited Review

Polyphasic insights into the microbiomes of the Takamatsuzuka Tumulus and Kitora Tumulus

(Received December 5, 2016; Accepted January 25, 2017; J-STAGE Advance publication date: March 24, 2017)

Junta Sugiyama,^{1,*} Tomohiko Kiyuna,² Miyuki Nishijima,² Kwang-Deuk An,^{2,#} Yuka Nagatsuka,^{2,†} Nozomi Tazato,² Yutaka Handa,^{2,‡} Junko Hata-Tomita,² Yoshinori Sato,³ Rika Kigawa,^{3,\$} and Chie Sano³

¹ TechnoSuruga Laboratory Co. Ltd., Chiba Branch Office, 3-1532-13 Hasama-cho, Funabashi, Chiba 274-0822, Japan

² TechnoSuruga Laboratory Co. Ltd., 330 Nagasaki, Shimizu-ku, Shizuoka-city, Shizuoka 424-0065, Japan

³ Tokyo National Research Institute for Cultural Properties, 13-43 Ueno Park, Taito-ku, Tokyo 110-8713, Japan

Microbial outbreaks and related biodeterioration problems have affected the 1300-year-old multicolor (polychrome) mural paintings of the special historic sites Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT). Those of TT are designated as a national treasure. The microbiomes of these tumuli, both located in Asuka village, Nara, Japan, are critically reviewed as the central subject of this report. Using culture-dependent methods (conventional isolation and cultivation), we conducted polyphasic studies of these microbial communities and identified the major microbial colonizers (*Fusarium* spp., *Trichoderma* spp., *Penicillium* spp., dark *Acremonium* spp., novel *Candida* yeast spp., *Bacillus* spp., *Ochrobactrum* spp., *Stenotrophomonas tumulicola*, and a few actinobacterial genera) and noteworthy microbial members (*Kendrickiella phycomyces*, *Cephalotrichum verrucisporum* ($\equiv$ *Doratomyces verrucisporus*), *Sagenomella striatispora*, *Sagenomella griseoviridis*, two novel *Cladophialophora* spp., *Burgoa anomala*, one novel species *Prototheca tumulicola*, five novel *Gluconacetobacter* spp., three novel *Bordetella* spp., and one novel genus and species *Krasilnikoviella muralis*) involved in the biodeterioration of mural paintings, plaster walls, and stone chamber interi-

ors. In addition, we generated microbial community data from TT and KT samples using culture-independent methods (molecular biological methods, including PCR-DGGE, clone libraries, and pyrosequence analysis). These data are comprehensively presented, in contrast to those derived from culture-dependent methods. Furthermore, the microbial communities detected using both methods are analytically compared, and, as a result, the complementary roles of these methods and approaches are highlighted. In related contexts, knowledge of similar biodeterioration problems affecting other prehistoric cave paintings, mainly at Lascaux in France and Altamira in Spain, are referred to and commented upon. Based on substrate preferences (or ecological grouping) and mapping (plotting detection sites of isolates), we speculate on the possible origins and invasion routes whereby the major microbial colonizers invaded the TT stone chamber interior. Finally, concluding remarks, lessons, and future perspectives based on our microbiological surveys of these ancient tumuli, and similar treasures outside of Japan, are briefly presented. A list of the microbial taxa that have been identified and fully or briefly described by us as known and novel taxa for TT and KT isolates since 2008 is presented in Supplementary Materials.

*Corresponding author: Junta Sugiyama, TechnoSuruga Laboratory Co. Ltd., Chiba Branch Office, 3-1532-13 Hasama-cho, Funabashi, Chiba 274-0822, Japan.

Tel: +81-47-404-5738 E-mail: jsugiyam@tecsrg.co.jp

[#]Present address: Macrogen Japan Corp., International Science Innovation Building 3F, Kyoto University, 36-1 Yoshida-Honmachi, Sakyo-ku, Kyoto 606-8501, Japan.

[†]Present address: Mukaishima-cho, Onomichi, Hiroshima 722-0073, Japan.

[‡]Present address: CAF Laboratories Inc., 1257-1 Michinoue-Kannabe-cho, Fukuyama, Hiroshima 720-2104, Japan.

^{\$}Present address: Kyushu National Museum, Museum Science Division, 4-7-2 Ishizaka, Dazaifu, Fukuoka 808-0118, Japan.

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Key Words: bacteria; biofilm; cultural properties; culture-dependent methods; culture-independent methods; fungi; microbial community analysis; yeasts

Abbreviations: Agency for Cultural Affairs, ACA; D1/D2 domains of nuclear large subunit ribosomal RNA, LSU; Investigation Committee for Elucidating the Cause of Deterioration of the Mural Paintings of the Takamatsuzuka Tumulus, ICECDMPPTT; = Heterotypic (taxonomic) synonym; ≡ Homotypic (nomenclatural) synonym; Kitora Tumulus, KT; Takamatsuzuka Tumulus, TT

1. Introduction

Microorganisms characteristically decompose or assimilate substrates, ranging from monosaccharides to the most complex polymers. Some bacteria and fungi, particularly the basidiomycetous fungi, can decompose polysaccharides, cellulose, and lignin. The undesirable effects of these activities, such as the destruction or decomposition and spoilage of useful materials and artifacts by living organisms, are categorized as biodeterioration: more general saprotrophic activities are categorized as biodegradation (Deacon, 1997; Pinna and Salvadori, 2008). Biodeterioration is simply the consequence of extensive biodegradation of a material (Carlile and Watkinson, 1994). A variety of objects of cultural significance, including mural and cave paintings, ruins, historic buildings, and monuments, are suitable habitats for destructive microorganisms (e.g., Allsopp et al., 2004; Caneva et al., 2008; Saiz-Jiménez, 2003, 2014, 2015; Sterflinger, 2010).

Herein, we focus mainly on the biodeterioration by microorganisms and related consequences to the mural paintings, plaster, and plaster walls at two ancient Japanese tumuli, both in Nara: the Takamatsuzuka Tumulus (TT; Fig. 1a) and the Kitora Tumulus (KT; Fig. 1o). Both of these 1300-year-old tumuli contained multicolor (polychrome) mural paintings that were directly drawn onto thin plaster inside the respective small stone chambers. The mural paintings and plaster walls of both tumuli were confined inside enclosed stone chambers under environmental conditions to be described in detail later. However, these mural paintings and plaster walls faced serious microbial outbreaks and disturbances after respective excavations in 1972 (TT) and 2004 (KT). Eventually, the TT and its stone chamber were dismantled, and the 16 stone blocks, i.e., comprising eight stone (side) walls and four ceiling stones, bearing mural paintings, and four floor stones (Fig. 2), were relocated to a restoration facility in Asuka village during April to August 2007. On the other hand, the mural paintings and plaster of the stone chamber interior of KT were detached from their stone walls by the end of 2009. Repair work of all the mural paintings, stone walls and ceilings has been recently completed. Protective measures against microbial damage have been undertaken for these treasures in Japan (e.g., Agency for Cultural Affairs,

Japan, 1987; Ishizaki and Kigawa, 2011), and, similarly, for the Paleolithic caves in southern Europe, such as the Lascaux Cave in France, and the Altamira and other caves in Spain (e.g., Coye, 2011; Saiz-Jiménez, 2014, 2015).

Our collaborative research team has conducted microbiological surveys to identify the microbiota (fungi and bacteria, in particular) involved in the biodeterioration of the mural paintings, plaster, stone walls and ceilings of both tumuli (Sugiyama et al., 2009). This work began at the Takamatsuzuka Tumulus in 2004 at the height of the serious contamination phase termed the “Explosive Appearance of Molds in Heisei (the Second Crisis),” which was named by the Investigation Committee for Elucidating the Cause of Deterioration of the Mural Paintings of Takamatsuzuka Tumulus (ICECDMPPTT, 2010). At that time, the attention of both the scientific community (e.g., ICECDMPPTT, 2010) and society in general (e.g., Mohri, 2007) was focused on identifying the molds and possible invasion routes involved in the biodeterioration of TT mural paintings, such as “Asuka Beauties (*Asuka Bijin*)” (Fig. 1j) and “White Tiger (*Byakko*)”, and how to conserve and restore them at the site.

The original published papers and accounts based on our research are referred to, and cited, within this review. A better understanding and elucidation of the causes and mechanisms involved in the biodeterioration are essential for the proper conservation and restoration of mural paintings and related cultural properties. The aims of this review are to identify and describe the microorganisms involved in the biodeterioration of the mural paintings and plaster walls of these national treasures. The artifacts described herein were maintained *in situ* in a subterranean environment specific to the interior of the tumulus stone chamber. This review also aims to elucidate the mechanisms involved in biodeterioration, using the insights gained from data collected at the Lascaux, Altamira, and other caves. Covering nearly all of the data and evidence regarding the microbiology of the Takamatsuzuka and Kitora Tumuli, this review is expected to provide a guide for future work with such artifacts so as to facilitate keeping them at the original site.

2. A Brief History of the Major Phases, Microbial Disturbances, and Related Biodeterioration Problems of the Paintings and Other Artifacts at Selected Ancient Tumuli and Caves

The selected ancient tumuli (tombs) and caves are divided into four groups: TT, KT, other tumuli in Japan, and caves outside Japan. The profiles, microbial outbreaks, environmental conditions, and related deterioration problems encountered at these various tumuli and caves are briefly reviewed.

2-1. The Takamatsuzuka Tumulus

The TT is located on the south slope of a small hill in Asuka-mura (Asuka village), Takaichi-gun (Takaichi county), Nara Prefecture, Japan. This tumulus was constructed by compacting layers of soil to form a two-tiered dome. The diameter of the lower dome is 23 m and that of the upper dome is 17.7 m (Ishizaki and Kigawa, 2011).

Fig. 1. Takamatsuzuka (a–n) and Kitora (o–x) Tumuli and their diagnostic biodeteriorated samples.

(a) The TT seen from the south (photo taken during the first sampling on May 19, 2004). (b) Sampling inside the TT stone chamber and white mold on the west wall (photo taken on Sep 6, 2004). (c) A blue dragon on the east wall with serious discoloration (photo taken on May 10, 2004). (d) Appearance of the stone chamber on Mar 28, 2007. (e) A living mite on the wall surface (photo taken on Aug 13, 2004). (f) A mite collected from the viscous gels (sample No. T7426-5). (g) Two springtails (Collembola) collected from the stone chamber interior (sample No. T61213-14). (h, i) Viscous gel (biofilm) occurred on the walls of the stone chamber interior of TT and its light micrograph (sample No. T5916-1). (j, k) Black stains/spots occurred on the mural painting of a group of women (“Asuka beauties”) on the west wall of TT, and conidiophores/conidia of dark *Acremonium* (sample No. T6517-11). (l–n) A piece of plaster collected from the space between the west stone walls 3 and 2 of TT, and SEM micrographs (sample No. T7510-1). (o) The Kitora Tumulus in the south view (photo taken in 1997, Asuka Village Board of Education). (p) Entrance to the KT stone chamber (photo taken on Mar 3, 2006). (q) Sampling inside the KT stone chamber (photo taken on Jun 17, 2008). (r) White fungal colonies (arrows) on the left part of a white tiger on the west wall. (s) Black small hole on the surface of the plaster wall (photo taken on Sep 29, 2005). (t) Viscous gel (biofilm) occurred on the walls of the stone chamber interior of KT (sample No. K9218). (u) A mite collected from the viscous gels (sample No. K9218). (v) Light micrograph of the viscous gel (sample No. K9703-1). (w) A part of the astronomical (star) chart on the ceiling stone wall in the KT (photo taken on Apr 28, 2006) with black particles (arrows). (x) Bristle-like structures on the stone wall in the adjacent small room of the KT (photo taken on Sep 10, 2004). a: reproduced from Kiyuna et al. (2008); h, i, l–n: reproduced from Sugiyama et al. (2009); k: reproduced from Kiyuna et al. (2011); o: reproduced with permission from the Asuka Village Board of Education; b–e, j, p–s, w, x: reproduced with permission from the Agency for Cultural Affairs, Japan.

Fig. 2. Schematic diagram of the Takamatsuzuka Tumulus and its stone chamber.

(a) The panoramic view of the tumulus from the south. (b) Cross-section diagram of the conservation facility and stone chamber viewed from the east (modified from Kiyuna et al., 2008). (c) Outward from the stone chamber. (d) Development diagram of the stone chamber; each stone wall was assigned by its direction and number.

Built around the late 7th or early 8th century during the Asuka Era, TT is well known to the Japanese people as an important cultural heritage site because of its multicolor murals. It was discovered and excavated in March 1972. The Japanese government designated TT as a special historic site in April 1973 and the mural paintings as a national treasure in April 1974. A conservation facility was built during the period 1974 to 1976 to protect the tumulus environment from desiccation while allowing access to the chamber (Fig. 2). This facility enabled the early treatment of the murals and periodic inspections of the interior conditions. The characteristics of TT, including the stone chamber and mural paintings, and related environmental data, have been fully described in the 2004–2005 excavation survey reports (Agency for Cultural Affairs, Japan, 1987; Nara National Research Institute of Cultural Properties, 2006).

The stone chamber was constructed within the tumulus using cut slabs of tuff stone. The interior is approximately 2.7 m deep, 1.0 m wide, and 1.1 m high, which makes it a difficult place in which to work (Fig. 2). There is a big hole (85 cm east-west, 60 cm top-bottom) on the south stone wall that was made by thieves in the 12th or 13th century (during the Kamakura period). Polychrome mural paintings were drawn on the relatively flat, thin plaster surface of the wall (2–7 mm thick; erroneously written as 2–7 cm thick in the Takamatsuzuka Tumulus Emergency Conservation Research Committee report, 1972; cf., Sano

et al., 2009) inside the stone chamber using many colors, including red, green, yellow, blue, black and gold. The paintings on the eight side walls comprise groups of men and women, the sun and the moon (*Nichi-Getsu*), the four heavenly guardian gods (*Shishin*), viz. “Blue Dragon” (*Seiryu*; Fig. 1c), “White Tiger” (*Byakko*), “Vermilion Bird” (*Suzaku*), and “Black Snake-Tortoise” (*Genbu*), in addition to star chart/constellations (*Seishuku*) on the ceiling. The group of women on the west wall is entitled “Asuka Beauties” (*Asuka Bijin*) (Fig. 1j). In accordance with specialists’ advice, the Agency for Cultural Affairs (ACA), Japan, decided in 1973 to preserve the mural paintings in the small stone chamber interior *in situ*, and it was never opened to the public. It was thought that the beauty and stability of the plaster paintings could be preserved if the conditions of high humidity (100% relative humidity [RH]) and cool temperature (14–20°C) could be established (Ishizaki and Kigawa, 2011; Kigawa et al., 2006b). In addition, the stone chamber was kept in darkness, except for regular inspections by the staff involved in the conservation. Because of the darkness, the high humidity and cool temperature were initially considered to be less likely to support microbial growth; also, it was thought that the paintings would be protected against microbial disturbances and biodeterioration.

A brief summary of the selected records and events of microbiological inspections and related activities in TT since its discovery in 1972 is presented in Table 1. Ac-

Table 1. Brief overview of selected records of microbiological inspections in the stone chamber of the TT, after its discovery.*

Date	Events / countermeasures	Dominant/problematic microorganisms**
1972	Discovery	<i>Alternaria</i> , <i>Cladosporium</i> , <i>Nigrospora</i> , <i>Trichoderma viride</i>
	Excavation and the first microbiological inspection	
1975	Microbiological survey	<i>Doratomyces</i> *** , <i>Fusarium</i> , <i>Cladosporium</i> , <i>Mucor</i>
1976	Construction of the restoration facility	
	Annual inspections started	
1976–Early 1980s	Periodic intense restoration	<i>Doratomyces</i> *** , <i>Streptomyces</i>
The First Crisis (1980–1984): “Explosive Appearance of Molds in the Showa Era”		
1980–1981	Fungal outbreak	
1982	Stability and balance were gradually recovered	
1986–1987		<i>Aspergillus</i> , <i>Fusarium</i> , <i>Trichoderma</i> , actinomycetes
1994–2000		<i>Penicillium</i> , <i>Aspergillus</i> , <i>Fusarium</i> , <i>Trichoderma</i>
The Second Crisis (2001–2005): “Explosive Appearance of Molds in the Heisei Era”		
Spring 2001	Remediation work at the adjacent space	<i>Penicillium</i> , <i>Aspergillus</i> , <i>Fusarium</i> , <i>Cladosporium</i> , <i>Cylindrocarpon</i> , <i>Gliomastix</i> [sic] , <i>Trichoderma</i>
	Fungal outbreak	<i>Penicillium</i> , <i>Fusarium</i> , <i>Trichoderma</i>
2002		<i>Penicillium</i> , <i>Fusarium</i> , <i>Trichoderma</i> , <i>Candida</i> , <i>Bacillus</i> , <i>Ochrobactrum</i> , <i>Stenotrophomonas</i>
May 2004	Viscous gels (biofilms) were visible on the wall plaster and floor	
Sep 2005	Cooling of the burial mound started	
Feb 2006	Outbreak of black stains/spots on the “Asuka Beauties”	
May–Dec 2006	Outbreak of black stains/spots on the murals	<i>Acremonium</i> (sect. <i>Gliomastix</i>) , <i>Penicillium</i> , <i>Bordetella</i>
Apr–Aug 2007	Dismantling and relocating the stone chamber	
2008–2009	Excavation and investigation of burial mound	
Oct 2009	Completion of maintenance work on the burial mound	

* For details, refer to Arai (1984, 1987, 1990b), Ishizaki and Kigawa (2012), Kigawa et al. (2004, 2006b, 2007a, 2008a, 2009a), and the ACA, Japan website (http://www.bunka.go.jp/seisaku/bunkazai/takamatsu_kitora/).

** Genera in bold show the major microbial colonizers.

*** Heterotypic synonym of *Cephalotrichum* (for details, see Subsection 7-3 of this review).

cording to the official records of the ACA, a Japanese website (<http://www.bunka.go.jp/takamatsu_kitora/hekiga_hozonkanri.html>), Kigawa et al. (2004, 2006b), and ICECDMPPT (2010), the deterioration of mural paintings of TT is attributed to several events that disrupted the static balance of microbial activity in the tumulus. Of these events, the following are noteworthy from the standpoint of biodeterioration or contamination: (1) thieves in the 12th or 13th centuries, (2) the 1972 discovery and excavation, (3) restoration work in 1976–1981, and (4) renovation work in the adjacent space in 2001. Subsequently, the “restoration work in 1976–1981” and the “renovation work in the adjacent space in 2001” were deemed responsible for the “Explosive Appearance of Molds in the Showa Era” (the First Crisis) and the “Explosive Appearance of Molds in the Heisei Era” (the Second Crisis), as defined by ICECDMPPT (2010).

Black spot/stain-forming fungi were identified as *Alternaria* sp., *Cladosporium* sp., and *Nigrospora* sp., and green spot-forming fungi found in the stone chamber interior soon after the excavation in March 1972 were identified as *Trichoderma viride*. In 1975, three years after the excavation, the fungi *Doratomyces* sp., *Fusarium* sp., *Cladosporium* sp., and *Mucor* sp. were identified as the predominant contaminants in the stone chamber interior (Arai, 1984, 1987, 1990b; ACA, Japan website <<http://www.bunka.go.jp/>>). 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., appeared on the walls of the stone chamber interior (Arai, 1984, 1987, 1990b; ACA, Japan website (<<http://www.bunka.go.jp/>>)). Paraformaldehyde fumigation was performed for a period

of five years starting from June 1981 to control the growth and appearance of microorganisms containing *Doratomyces*. Subsequently, microbial outbreaks gradually became less evident because fewer entries into the stone chamber were made (ACA, Japan website <loc. cit.>; Arai, 1987; Kigawa et al., 2006b). For the matter of *Doratomyces*, see Subsection 7-3. *Aspergillus* sp., *Trichoderma* sp., and *Fusarium* sp., as well as other molds and actinomycetes, were isolated during 1986–1987 (Arai, 1984, 1987, 1990b), whereas *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp., and others, were found during 1994–2000 (Kigawa et al., 2006b). These fungi continuously inhabited the stone chamber interior; however, they did not cause serious damage to the mural paintings. The mycobiota of the stone chamber interior was comparatively stable, and little damage occurred during these periods.

In February 2001, renovation was performed in the space adjacent to the TT stone chamber. Because the conservation facility was aging, leakage of soil and rainwater occurred (Kigawa et al., 2009a). In March 2001, following the remediation work in the space adjacent to the TT stone chamber, there were significant occurrences of *Aspergillus* sp., *Cladosporium* sp., *Fusarium* sp., and *Penicillium* sp., in the adjacent space. In September and December 2001, a fungal outbreak occurred on the stone wall of the grave hole and the wall paintings of the stone chamber interior; *Penicillium* sp., *Aspergillus* sp., and *Fusarium* sp. were identified. In December 2001, brown fungi (*Cylindrocarpon* sp.) and black fungi (dark *Acremonium*, viz. *Acremonium* sect. *Gliomastix*) were isolated for the first time near the paintings “Black Snake-Tortoise” (*Genbu*) on the north wall, “Blue Dragon” (*Seiryu*) on east

Table 2. Brief overview of selected records of microbiological inspections in the stone chamber of the KT, after its discovery.*

Date	Events/countermeasures	Dominant/problematic microorganisms detected **
1983	Discovery	
2003	Fungal appearances in the adjacent small room	<i>Aspergillus</i> , <i>Fusarium</i> , <i>Penicillium</i> , <i>Trichoderma</i> , <i>Cunninghamella</i>
Mar–Jul 2004	Excavation	<i>Acremonium</i> , <i>Penicillium</i> , <i>Trichoderma</i>
Aug 2004	Fungal colonies found on the murals	
Aug 2004	Outbreak of bristle-like fruiting bodies on the stone walls in the adjacent small room	<i>Phialocephala</i> (subsequently reidentified as <i>Kendrickiella</i>)
Sep 2004	The Agency decided to detach all of the murals	
Oct–Dec 2004		<i>Fusarium</i> , <i>Penicillium</i> , <i>Trichoderma</i> , <i>Cylindrocarpon</i> , <i>Aspergillus</i> , <i>Bacillus megaterium</i>
2005	Viscous gels (biofilms) were visible on the wall plaster and floor	<i>Penicillium</i> , <i>Fusarium</i> , <i>Trichoderma</i> , <i>Cylindrocarpon</i> , <i>Acremonium</i> , <i>Phialocephala</i> , <i>Candida</i> , <i>Stenotrophomonas</i> , <i>Serratia</i> , <i>Sphingomonas</i> , <i>Pseudomonas</i>
Sep 2005	Small holes were seen in the plaster walls	<i>Gluconoacetobacter</i>
Apr 2006	Outbreak of black particles (“ <i>Kuro-tsubu</i> ”) on the ceiling	<i>Burgoa</i> , <i>Penicillium</i>
Oct 2006	Outbreak of black spots/stains on the walls	<i>Acremonium</i> (sect. <i>Gliomastix</i>), <i>Penicillium</i>
Feb 2007	All paintings, except the star chart on the ceiling, were relocated	
May 2007	Further outbreak of black spots / stains on the walls	<i>Acremonium</i> (sect. <i>Gliomastix</i>), <i>Cladophialophora</i>
Nov 2008	All paintings were relocated	
Mar 2009	UV irradiation was started to reduce growth of microorganisms	
Nov 2010	All plaster relocation was finished	
Oct 2013	Stone chamber closed	

* For details, refer to Ishizaki and Kigawa (2011), Kigawa et al. (2005, 2006a, 2007b, 2008b, 2009a, 2009b, 2010c, 2011, 2012a), the ACA, Japan website (http://www.bunka.go.jp/seisaku/bunkazai/takamatsu_kitora/), and this text.

** Genera in bold show the major microbial colonizers.

wall 2, and above the painting “White Tiger” (*Byakko*) on west wall 2 (Kigawa et al., 2006b, 2009a; cf., Fig. 2). These black and brown fungi were of particular concern because they produced a soluble pigment that remained on the wall paintings. In October 2002, black stains appeared near the painting “Blue Dragon” on east wall 2 and near the painting of a group of women on east wall 3. It was not possible to remove such stains on site.

In May 2004 (Fig. 1a), we began a series of microbiological surveys, focusing primarily on the dynamics of microbiota (mycobiota and bacteriobiota) to elucidate the cause of the biodeterioration of TT mural paintings. After May 2004, viscous gels (biofilms composed of mixtures of molds, yeasts, bacteria, and microanimals such as mites) also appeared on the wall plaster (Kigawa et al., 2009a). Because of the continuing deterioration of fragile supports (plaster walls and cut slabs of tuff stone), in addition to the serious contamination and blackening of the mural paintings, the ACA of Japan decided in March 2005 to dismantle the stone chamber to save and restore the mural paintings. Cooling of its mound began in September 2005 to slow fungal growth before dismantling. In February 2006, despite cooling the interior, black spots (Figs. 1j and k) appeared on the painting of a group of four women, known as “Asuka Beauties” (*Asuka Bijin*), on west wall 3. In May 2006, the temperature of the stone chamber interior was kept stable at approximately 10°C; however, the black spots expanded on the plaster walls (Kigawa et al., 2007a, 2009a).

Changes in the microbiota were recognized during the period from May 2004 to December 2006, and, subsequently, the stone chamber interior was cooled. Between April and August 2007, the stone chamber was dismantled and relocated by the ACA of Japan (Kigawa et al., 2008a), and all of the stone walls and ceilings with mural paintings were moved to a restoration facility in Asuka village by the end of August for restoration and protection from further deterioration. Excavation and archaeo-

logical surveys of the mound were conducted from 2008 to 2009. While excavating the mound and dismantling the stone chamber, a variety of samples, such as blackish plaster pieces, plant roots, and soil, were collected, and fungi and bacteria were isolated from the selected samples (Sugiyama et al., 2009).

The mound has now been temporarily restored to its former state. The 16 stone blocks (Fig. 2), including the stone walls and ceiling stones with mural paintings, are being repaired at a restoration facility in Asuka village. The ACA of Japan is now polyphasically investigating the return of all of the mural paintings and stone blocks to their original positions after the restoration is completed.

2-2. The Kitora Tumulus (KT)

The KT is located 1.2 km south of the TT in the same Asuka village (Fig. 1o). This circular tomb, which is thought to have been built around the same time as the TT, was also made by compacting soil layer-by-layer to form a two-tier dome. The diameter of the lower dome is 13.8 m and that of the upper dome is 9.4 m. The stone chamber interior is slightly more than 2.4 m deep, 1.0 m wide, and 1.2 m high. Similarly multicolored murals are painted on the thin plaster walls within the stone chamber. The star chart on the ceiling is in a style similar to that in the TT. In November 1983, the mural painting called “Black Snake-Tortoise” (*Genbu*) on the north wall of the KT stone chamber interior was discovered using a fiberscope that was passed into the stone chamber interior through the grave-robber (thieving/thief) hole (on the south wall) cut at the same time as that in the TT. This was only the second discovery of a polychrome mural painting in Japan, the first being in the TT (Nara National Research Institute for Cultural Properties, 2008). The Japanese government designated the KT as a special historic site in 2000. Subsequently, “Blue Dragon” (*Seiryu*) on the east wall, “White Tiger” (*Byakko*) on the west wall (Fig. 1r), the star chart/constellations (*Seishuku*; Fig. 1w) (the old-

Fig. 3. Schematic diagram of a floor plan (second floor) of the conservation facility for the Kitora Tumulus (modified from Mabuchi and Sano, 2007).

est example of ceiling star constellations in East Asia), and “Vermilion Bird” (*Suzaku*) on the south wall were discovered in 2001 using a compact digital camera. The remaining mural painting depicts all four of the guardian gods (Nara National Research Institute for Cultural Properties, 2008). The profile of this tumulus, including the stone chambers interior and mural paintings, and related environmental data, have been fully described based on the excavation survey in 2002–2004 (Nara National Research Institute of Cultural Properties, 2008).

The environmental conditions inside the stone chamber of the KT were nearly the same as those of the TT. However, the mural paintings deteriorated after its excavation (Fig. 1r). Table 2 summarizes the events and records of microbiological inspections and related activities at the KT after its discovery.

In 2003, a conservation facility was constructed adjacent to the stone chamber (Fig. 3). In January 2004, before excavation, Sano et al. (2005; cf., 2004) conducted the first investigation of the atmospheric composition as an environmental factor and counted airborne fungi. The carbon dioxide (CO_2) concentration was approximately three times higher than that of the outside atmosphere. CO_2 was believed to have been introduced through the plaster walls, which consist primarily of calcium carbonate (CaCO_3) (Sano et al., 2005; cf., 2004). There were no poisonous gases, and the number of airborne fungal colonies was low. The latter was considered to be an indication of low fungal activity in the air of the stone chamber interior (Sano et al., 2005; cf., 2004). In early 2004, the KT was excavated. The interior environmental conditions were very similar to those of the TT, and no conspicuous fungal colonies were observed for some time after excavation (Kigawa et al., 2005). However, with the excavation and restoration work, the mural paintings encountered serious microbial disturbance (Figs. 1r and s). In addition, the paintings on the plaster walls became partially detached from the supporting stone wall. Considering the unstable condition of the paintings, the ACA of Japan decided to detach and relocate all of the mural paintings to a controlled environment in September 2004.

Over the course of the excavation, *Acremonium* sp., *Fusarium* sp., *Penicillium* sp., and *Trichoderma* sp. ap-

peared as major contaminants (Kigawa et al., 2005). In June 2004, we started microbiological surveys concurrent with those at the TT, focusing primarily on the dynamics of the microbiota (mycobiota and bacteriobiota) involved in the biodeterioration of the KT stone chamber interior. In August 2004, a “bristle-like” fungus (Fig. 1x), *Phialocephala* sp. (later re-identified as *Kendrickiella phycomyces* by Kiyuna et al., 2012, and mentioned later in Subsection 7-2 of this review), was detected on the KT tuff stone wall of the small room adjacent to the stone chamber (Kigawa et al., 2005, 2006a).

In the summer of 2005, viscous gels or biofilms that were mixtures of molds, yeasts, bacteria, and micro-animals such as mites (Fig. 1u) also appeared on the KT wall plaster, similar to that seen at the TT (Kigawa et al., 2006a; Sugiyama et al., 2009). In September 2005, small holes (approximately 10 mm in diameter; Fig. 1s) caused by acetic acid bacteria (initially identified as members of the genus *Gluconacetobacter* and subsequently proposed as two novel species, *Gluconacetobacter tumulicola* and *Gluconacetobacter asukensis*, by Tazato et al., 2012) became apparent on the plaster wall of the ceiling of KT (Kigawa et al., 2009b, 2012b; Tazato et al., 2012). In April 2006, the basidiomycetous anamorphic fungus *Burgoa* sp. (later re-identified as *Burgoa anomala* by Kiyuna et al., 2015, and mentioned later in Subsection 7-2 of this review), referred to as “black particles,” (Fig. 1w) appeared on the ceiling stones (Kigawa et al., 2007b). In May 2007, a further outbreak of black spots/stains caused by *Acremonium* (sect. *Gliomastix*) sp. occurred on the walls (Kigawa et al., 2008b). By the end of 2008, almost all of the paintings on the side walls and the star chart on the ceiling were relocated using the detachment method, except for those that may have been hidden by a thin layer of mud on the walls (Ishizaki and Kigawa, 2011). In 2010, almost all of the plaster on the stone walls was also detached and relocated.

In April 2009, intermittent UV irradiation in the stone chamber interior began to arrest the appearance and growth of various microorganisms (Kigawa et al., 2010c); and their appearance decreased thereafter (Kigawa et al., 2011, 2012b, 2013b, 2015). However, some microorganisms (fungi and bacteria) seemed to survive in places, such as inside the cracks of the stone walls, inside the mud layer, and in viscous gels (biofilms), where sufficient UV light did not penetrate (Kigawa et al., 2011, 2012b, 2013b, 2015). Incidentally, the detached paintings have been repaired at a restoration facility in Asuka village and moved to a new exhibition facility in the same village in September 2016. A comprehensive summary of the surveys of microorganisms in this tumulus from 2004 to 2013 was recently published by Kigawa et al. (2015).

2-3. Other ancient tumuli in Japan

According to the ICECDMPTT’s report (2010), approximately 600 tumuli with mural paintings (known as ‘decorated tombs’) have been found in Japan. Compared to the TT and the KT, the other decorated tombs differ somewhat with respect to mural decoration methods, areas of distribution, and construction dates. As mentioned above, both the TT and the KT are located in Nara Prefecture and

Table 3. Brief overview of selected records in the Lascaux Cave after its discovery.*

Date	Events/countermeasures	Dominant microorganisms**
Sep 1940	Discovery	
1948	Opened to the public	
First microbial crisis, 1955–1963		
1955–1962	Increasing number of visitors (30,000 per year) and increased carbon dioxide levels	
1960–1963	Development and spread of green spots (called “la maladie verte” or “green disease”) and spread of calcite deposits on the walls (called “white disease”)	Green algae (Chlorophyte algae), <i>Bracteacoccus minor</i>
Apr 1963	Decision to close the cave to the public	
1979	Designated by UNESCO as a World Heritage Site.	Stable condition until the end of 2001
1999–2000	Replacement of the decrepit air conditioning system	
Second microbial crisis, 2001–2007		
Sep 2001	Outbreak of white molds and white mucous colonies	<i>Fusarium solani</i> , with <i>Pseudomonas fluorescens</i>
Aug 2001	First biocidal treatments started	
Dec 2001	First observations of small black “spots” on the ceiling of the entrance airlock chamber	<i>Gliomastix murorum</i> [sic]
2001	Monitoring of air biocontamination began	
Third microbial crisis, 2006–Present		
2006–	Appearance of obvious black “spots”	<i>Ochroconis</i> (formerly identified as <i>Scolecobasidium</i>), <i>Ulocladium</i> , <i>Gliomastix</i> , <i>Gliocladium</i> , <i>Fusarium solani</i>
2008	Biocide treatments (quaternary ammonium derivatives) used again	

* For details, see Coye (2011), Oriol et al. (2011), and Martin-Sanchez et al. (2015).

** Generic/species names in bold show the major or noteworthy microbial colonizers.

date to around the late 7th or early 8th century, and their multicolor murals were painted directly onto thin plaster on the stone walls and ceiling stones. In contrast, the other decorated tombs were built in the 5th to 7th century. They are located in the northern Kyusu, Kanto, and Tohoku regions, and their murals were painted directly onto the stone walls without plaster. The exceptions are the Kadumayama and Marukoyama Tumuli in Nara Prefecture and the Houtouzuan Tumulus in Gunma Prefecture (ICECDMPTT, 2010).

Records of microorganisms, especially fungi, detected from selected Japanese ancient tumuli, excluding the TT and the KT, are summarized in Table S1. Several microbiological surveys, focusing primarily on fungi, have reported on biodeterioration problems in the following decorated tombs: Torazuka Tumulus in Ibaraki Prefecture (Arai, 1984, 1990a; Emoto et al., 1983), Chibusan Tumulus in Kumamoto Prefecture (Emoto and Emoto, 1974), Ozuka Tumulus in Fukuoka Prefecture (Emoto and Emoto, 1974), Hayama-oketsu Tumulus in Fukushima Prefecture (Arai, 1984), Kiyotosaku-oketsu Tumulus in Fukushima Prefecture (Arai, 1984), Nakata-oketsu Tumulus in Fukushima Prefecture (Arai, 1984), and Mezurashizuka Tumulus in Fukuoka (Sano, pers. comm.).

The Torazuka Tumulus, built around the 7th century, was completely sealed off by 2–3 m of surrounding soil, without cracks or signs of burglary in the surrounding soil. Therefore, the burial stone chamber is considered to have been without ventilation for more than 1000 years (Arai, 1984, 1990a; Emoto et al., 1983). A series of reports on the conservation of mural paintings have included microbiological investigations. After excavation, the number of *Cladosporium* sp. isolated from the air in the stone chamber increased to 500–600 cells/m³ from only 40–60 cells/m³ before excavation (Arai, 1984, 1990a; cf., Section 10 of this review; Emoto et al., 1983). In addition, some fungi were isolated from the air in the stone chamber interior (i.e., a basidiomycete and *Penicillium* sp.), followed by species of *Alternaria*, *Aspergillus*, *Pestalotia*, and *Trichoderma* (Arai, 1984, 1990a). These reports did not dis-

cuss the origins of the fungi or their roles in biodeterioration, although Arai (1984, 1990a, 1990b) has discussed the roles of amines (low-molecular-weight alkylamines) as antimicrobial factors detected inside the stone chamber of the Torazuka Tumulus.

Emoto and Emoto (1974) reported on microbiological investigations of the Chibusan and Ozuka Tumuli and their paintings. In the Chibusan Tumulus in Kumamoto Prefecture, moldy spots appearing on the stone walls were collected using sterile cotton wool and tweezers, and *Gliocladium roseum*, *Idriella* sp., *Monodictys* sp., *Mortierella* sp., *Mucor* sp., and three species of *Penicillium* (i.e., *P. citreoviride*, *P. janthinellum*, and *P. oxalicum*) were detected. In contrast, in the Ozuka Tumulus in Fukuoka Prefecture (discovered in 1934), moldy spots appearing on the stone walls, soil, and a wooden ladder were collected using the same sampling tools, and airborne fungi were collected using the open-plate sampling method (Emoto and Emoto, 1974). Fungi including *Gliocladium virens*, *Gliomastix* sp., *Mucor* sp., two *Penicillium* spp., *Trichoderma viride*, and *Verticillium* sp. were detected in the stone chamber interior, and *Alternaria alternata*, *Cladosporium herbarum*, *Epicoccum purpurascens*, *Fusarium oxysporum*, and *Penicillium citrinum* were isolated from the air. These authors reported that these organisms caused many stains on the surface of the wall paintings and suggested that many of these microorganisms may have been introduced into the stone chamber interior by humans contaminated with fungal-spore-containing dust. Furthermore, they noted that the ladders and wood used for roofs to conserve these ancient tombs became a nutritional source for fungi, which grew and spread because of insufficient disinfection (Emoto and Emoto, 1974).

In the Hayama-oketsu Tumulus in Fukushima Prefecture (discovered in 1973), *Trichoderma* sp. appeared on the ceiling. This fungus is thought to have spread from a wooden ladder used during the investigation conducted after the discovery of the tumulus (Arai, 1984). In the Kiyotosaku-oketsu Tumulus in Fukushima Prefecture (dis-

covered in 1967), a whitish fungus was found on the left side of the spiral design of straw mats covering the tumulus entrance (Arai, 1984). In the Nakata-oketsu Tumulus in Fukushima Prefecture (discovered in 1969), a pale white fungus, *Phialophora* sp., appeared near the ceiling of the main chamber. Arai (1984) assumed that this fungus originated from soil carried in by workers during the excavation or construction of the preservation facility.

These reports identified the microorganisms using culture-dependent methods (for isolation) and microscopic observations. However, accurate taxonomic identification at the species level was not performed; viz., the taxonomic rank for identification was usually genus. At that time, no molecular approaches were applied. In addition, none of the fungal isolates or specimens described in these reports were preserved in any public culture collections or herbaria. Therefore, precise comparisons between these reports and subsequent studies are not possible.

2-4. Representative decorated caves outside of Japan: Lascaux, Altamira, and others

Biodeterioration of cave paintings by microorganisms has been reported worldwide, particularly in Europe (Garg et al., 1995; Karbowska-Berent, 2003). The paleolithic decorated or painted caves of Lascaux (Dordogne, France) and Altamira (Cantabria, Spain), with paintings as old as 18,000–20,000 years, are especially famous worldwide. These cave paintings are also remarkable in terms of the serious microbial disturbance and contamination reported (Bastian and Alabouvette, 2009; Bastian et al., 2010; Geneste, 2011; Martin-Sanchez et al., 2015; Saiz-Jiménez et al., 2011). By measuring the decay of uranium atoms in the calcite coating of paintings, a team of UK and Spanish researchers recently determined that a large, claviform-like symbol in the famous Techo de los Policromos at the Altamira Cave is at least 35,600 years old (Pike et al., 2012). Here, the historical background and events at the Lascaux and Altamira caves are briefly reviewed, as these caves faced biodeterioration problems and crises similar to those of TT and KT (Coye, 2011; Martin-Sanchez et al., 2015; Sano, 2009).

Table 3 summarizes selected records of microbiological inspections and related activities in the Lascaux Cave since its discovery. The paintings of the Lascaux Cave are thought to have been kept for a very long time in a buried environment with a damp internal atmosphere having a stable temperature and RH, and the images are painted directly onto limy substances (Coye, 2011; cf., Sano, 2009). Lascaux is a descending-type cave, with the entrance situated in the upper part of the hill. It has a medium-sized cavity, approximately 60 m in length from the entrance to the end of the Axial Gallery and approximately 110 m from the entrance to the bottom of the gallery on the right. The accessible cave system has a volume of about 320 m³ in the Hall of the Bulls and 1,300 m³ in the area on the right (Oriol et al., 2011). The environmental conditions inside the cavity are approximately 12°C and 99% RH (Oriol et al., 2011).

The Lascaux Cave was discovered in September 1940, classified as a historic monument in December 1940, and designated by UNESCO as a World Heritage Site along

with other caves in 1979. In July 1948, the Lascaux Cave was opened to the public. In 1955, the first indications of cave wall deterioration were reported. This damage was caused by excessive CO₂ levels from a large number of visitors (30,000 per year) and lighting that favored microbial and algal growth (Oriol et al., 2011). Subsequently, an air recirculation system was installed under the entrance stairway. In September 1962, the first spots were detected and identified as a single unicellular green alga, *Bracteacoccus minor* (a chlorophyte). Green spots (“la maladie verte” or “green disease”) subsequently developed and spread on the walls (Lefevre, 1974; Martin-Sanchez et al., 2015; Oriol et al., 2011). “White disease”, caused by the spread of calcite on the walls, also occurred. These phenomena were called the “first bioclimatic crisis (1955–1962)” by the Commission for the Scientific Study and Preservation of Lascaux Cave (Coye, 2011). In 1963, after green algal contamination promoted by continuous lighting, the cave was closed to the public (Coye, 2011).

After renewal of the air conditioning system in 2001, the first evidence of a white mold outbreak caused by the *Fusarium solani* species complex (FSSC) appeared in the cave (Dupont et al., 2007; Oriol and Mertz, 2006; Oriol et al., 2011). Dupont et al. (2007) detected some haplotypes in the FSSC and suggested that the FSSC could have invaded the cave through percolating water from the upper soil, outside soil carried in by people, small animals or running water, or all of these routes. Dupont et al. (2007) reported six fungal genera, namely *Chrysosporium*, *Gliocladium*, *Gliomastix* (sic), *Paecilomyces*, *Trichoderma*, and *Verticillium*, in addition to the FSSC. However, species identification and the roles and origins of these fungi were not discussed. Furthermore, Dupont et al. (2007) indicated that human activity could be a probable cause of the alteration in the cave environment and introduction of fungi. Bastian et al. (2010) suggested that the data reported by Dupont et al. (2007) indicated a strong association between the cave fungi and arthropods because these fungal genera contain many entomopathogenic species. In addition to these fungi, localized white, mucous colonies associated with the gamma-proteobacterial species *Pseudomonas fluorescence* appeared as a contaminant in 2001. This bacterial contamination was treated using a specific anti-bacterial agent (streptomycin plus polymyxin) (Martin-Sanchez et al., 2015; Oriol et al., 2011).

The first biocidal treatments (benzalkonium chloride solutions plus streptomycin and polymyxin) were then initiated. After the outbreak of the FSSC, black spots/stains caused by *Gliomastix murorum* (sic) appeared on the cave walls and became problematic in December 2001 (Oriol et al., 2011). These phenomena are referred to as the “second bioclimatic crisis (2001–2007)” (Coye, 2011). The microbial crises in the Lascaux Cave have been described in several scientific reports (Bahn, 2008; Fox, 2008; Graff, 2006). The monitoring of biocontaminants in the air was initiated in 2001 and has continued since then.

A novel black spot/stain phenomenon has been observed since 2006 and is present in the ceiling and clay banks extending back into the cave. These black stains contain many fungal genera, including *Ulocaldium*, *Gliomastix*, *Scolecobasidium tshawytschae*, and *Verticillium* (Bastian

and Alabouvette, 2009; Bastian et al., 2010). The appearance of *S. tshawytschae* has been linked to the availability of carbon sources, such as, possibly, benzalkonium chloride and its degradation products (Bastian et al., 2010). Furthermore, relationships between the springtail *Folsomia candida* (Collembola, Isotomidae), found on and around black stains, and two fungi from the Lascaux outbreaks (*Fusarium solani* and *S. tshawytschae*) are discussed in Bastian et al. (2010). Two fungi have been suggested to be food for collembolans, which may disperse them within the cave environment (Bastian et al., 2010).

Fungal and bacterial community analyses using molecular biological methods (clone analysis) were conducted by Bastian et al. (2009a, b, c). Based on their findings, they extensively discussed the ecology of microbial communities (microbiomes) in the Lascaux Cave (Bastian et al., 2010). The fungal communities are primarily composed of entomopathogenic fungi, such as *Geosmithia namyslowskii* and *Isaria farinosa* (Bastian et al., 2009b, 2010). These fungi play an important role in the cave, and arthropods contribute to the dispersion of their conidia (Bastian et al., 2009b, 2010; Jurado et al., 2008). In contrast, the bacterial communities were shown to be composed of human pathogenic bacteria as a result of biocide selection (Bastian et al., 2009a, 2009c, 2010). Bastian et al. (2010) noted that a careful study of the fungal ecology is required to completely elucidate the cave food web and control the black stains that threaten the Paleolithic paintings.

Martin-Sanchez et al. (2012a, b) identified and proposed two new species of *Ochroconis* (including that formerly identified as *Scolecobasidium*), *Ochroconis lascauxensis* and *O. anomala*, as major contributors to the black stain. They suggested that the development of these fungi and other fungal outbreaks were likely linked to the presence of unusual organic carbon and nitrogen sources supplied by the intensive biocide treatments, and provided data on their physiology and cellular fatty acid profiles. In addition, Porca et al. (2011) reported that these melanized fungi were the predominant groups in the air.

The Altamira Cave in Spain, with its 15,000-year-old paintings, was discovered in 1879 and was opened to the public in 1917. It was designated a World Heritage Site by UNESCO along with the Lascaux Cave in 1940 (Lasheras et al., 2011). A large number of visitors (175,000) during the 1960s and 1970s severely altered the microclimatic conditions, resulting in the increasing deterioration of the paintings. The cave was closed to the public in 1977. In 1982, after a microclimatic survey, the cave was reopened to the public on a limited basis. In 2002, however, the cave was again closed to the public because of the appearance of phototrophic microorganisms on the paintings (Porca et al., 2011; Saiz-Jiménez et al., 2011). Reopening of the cave has been under consideration since 2010 (Saiz-Jiménez et al., 2011). In this cave, surveys of the microorganisms affecting the wall paintings have primarily focused on bacterial communities/colonizers (Cuezva et al., 2012; Gonzalez et al., 2006, 2008a, 2008b; Jurado et al., 2009; Porca et al., 2012; Portillo and Gonzalez, 2009a, 2011; Saiz-Jiménez et al., 2011;

Schabereiter-Gurtner et al., 2002a, b). The walls and ceiling were primarily colonized by actinobacteria (Groth and Saiz-Jiménez, 1999; Groth et al., 1999; Laiz et al., 1999), acidobacteria (Cuezva et al., 2012; Schabereiter-Gurtner et al., 2003; Zimmermann et al., 2005), and sulfate-reducing bacteria (Portillo and Gonzalez, 2009b). There have been only a few reports on contamination by fungi (Jurado et al., 2009; Porca et al., 2011; Saiz-Jiménez et al., 2011) and phototrophic cyanobacteria (Cañaveras et al., 2001; Saiz-Jiménez et al., 2011). Jurado et al. (2009) reported the detection of entomopathogenic (*Isaria farinosa* and *Trichothecium asperum*) and other fungi (i.e., *Acremonium strictum*, *Bionectria ochroleuca*, *Cladosporium cladosporioides*, *Lecanicillium psalliotae*, and *Paecilomyces marquandii*). They discussed the role of arthropods in the dispersal of these fungal propagules.

In addition to the Lascaux and the Altamira Caves, recent reports describe microbiological problems and conservation efforts in the Mogao Grottoes with ancient wall paintings in Dunhuang, China (Ma et al., 2015), a Pleistocene cave with rock art (at least 39,900 years old) in Sulawesi, Indonesia (Aubert et al., 2014; Roebroeks, 2014), Naracoorte Caves in southeastern South Australia (Adetutu et al., 2011), and the Cave of Bats with rock art in Zuheros, Spain (Urzi et al., 2010). Microbial diversity and biodeterioration problems in mural (wall) painting environments have also been mentioned and discussed (e.g., Caneva et al., 2008; Saiz-Jiménez, 2003; Vanderwolf et al., 2013).

2-5. Principal differences between the TT and KT and Lascaux Cave

Some of the important differences between the TT and KT and Lascaux are briefly summarized by Ishizaki and Kigawa (2011). One of the big differences between these sites is their geographical location and age. The TT and KT are located in Japan, a corner of East Asia, and both tumuli are dated to around the late 7th or early 8th century, during the Asuka period. In contrast, the Lascaux Cave is located in France, southern Europe, and the paintings and engravings are dated to be prehistoric, 18,600 ± 190 years BP (e.g., Geneste, 2011). The support layers of the painting in the TT and KT are made of plaster, whereas those of Lascaux are composed of limestone. The pigments differ between the TT/KT and Lascaux, and those of both tumuli are thought to be sensitive to certain chemical disinfectants and fungicides (Ishizaki and Kigawa, 2011). The stone chambers of both tumuli (Figs. 1d, 1p, and 2) are constructed of rectangular stones of volcanic tuff (tuff brecca) and their enclosed, interior spaces (Figs. 1b and q) are extremely narrow compared to those of the Lascaux Cave. The former spaces make many types of work difficult (e.g., inspection, treatments, and sampling; Figs. 1b and q). In addition to these differences, TT and KT have never been open to the public, whereas the Lascaux Cave was opened to tourists from 1948 to 1963 (60 visitors/h; 1000 visitors/d; 100,000 visitors/y). As a result, the Lascaux Cave was subjected to destabilization of the temperature and RH, and an increasing concentration of CO₂ in the air (Pallot-Frossard et al., 2009).

Fig. 4. A scheme of the microbiological methods adopted in our studies (modified from Sugiyama et al., 2009).

Abbreviations: SM, Stereo microscope; LM, Light microscope; SEM, Scanning electron microscope.

3. Microbiological Approaches Adopted in Determining the Cause of the Biodeterioration of the TT and KT Mural Paintings and Plaster Walls

It is important to fully understand the features of the tumulus, stone chamber, mural paintings, and surrounding environmental conditions when planning a microbiological survey. Both the TT and KT are special historic sites, and the mural paintings are national treasures or the equivalent. In Japan, the “Law of the Protection of Cultural Properties” (<<http://law.e-gov.go.jp/htmldata/S25/S25HO214.html>>) only permits the use of “non-destructive methods” for the handling of cultural properties. This regulation qualitatively and quantitatively limits our research strategies. As a general rule, only the use of “non-destructive methods” is applied. Considering these limitations, we adopted the following methodologies for determining the identity of microorganisms and the cause of biodeterioration. The approaches adopted in our series of surveys and studies of the tumuli, stone chambers, and murals are illustrated in Fig. 4.

3-1. Sampling and sampled materials

Samples inside the stone chamber were collected using sterile moist cotton swabs, carefully avoiding damage to the paintings and other substances. A total of 716 samples, including mold spots, viscous gels (biofilms), and mixtures of plaster fragments and soil, were collected from the TT stone chamber interior, spaces between the stone walls, and the stone chamber exterior, between May 2004 and February 2009 (Fig. S1). These TT samples were classified, according to the sampling area, into the following categories: stone chamber interior, adjacent space, joint space of the stone walls, stone chamber exterior, and

mound (Fig. 2, Fig. S1). The types of sampled materials and the respective numbers of samples are shown in Fig. S1. A total of 217 samples were collected from the KT stone chamber interior and exterior from June 2004 to February 2013 (Fig. 3, Fig. S2). Similarly, these KT samples were categorized according to the sampling area as follows: stone chamber interior, adjacent small room, adjacent rooms, and mound and external air (Fig. 3, Fig. S2). From these collected samples of the TT and KT, selected samples were adopted to isolate (or examine?) fungi and bacteria.

3-2. Direct observation of samples

Before isolation and cultivation, the samples from both tumuli were carefully observed macroscopically and with stereo and light microscopes (Fig. 4). These data helped to specify the major fungal colonizers using the cultivation method. A few microphotographs based on these observations are shown in Figs. 1f–i, k, t–v. To examine the microbial invasion of the substrates, scanning electron microscopy (SEM) was used to observe selected plaster pieces and viscous gels (biofilms) on plaster (Sugiyama et al., 2009; Figs. 1l–n). Our SEM observations clearly demonstrated fungal hyphae and microbial cells penetrating the plaster of the TT stone chamber interior, thereby damaging the texture of the plaster (Figs. 1m and n).

3-3. Culture-dependent methods: Isolation, cultivation, and identification

Various methods and techniques have been used to investigate the biodeterioration of cultural heritage/properties such as mural paintings. These methods have been summarized by many authors (e.g., Allsopp et al., 2004; Ciferri, 1999; Dakal and Arora, 2012; Heyrman and

Swings, 2003; Pinzari et al., 2010; Salvadori and Urzi, 2008). Traditionally, investigations of microbes/microbial communities related to the biodeterioration of cultural heritage/properties have been conducted using culture-dependent methods (e.g., Berner et al., 1997; Guglielminetti et al., 1994). We used the following three isolation methods (Fig. 4): (1) sterile moist cotton swabs bearing the mold samples were rubbed immediately onto moist filter papers in a Petri dish, incubated in a moist chamber at 25°C, and observed at regular intervals over a period of approximately two months. The newly-appearing colonies were isolated using a sterile needle under a stereomicroscope (Gams et al., 1987; Krug, 2004); (2) swab samples were aseptically transported to the laboratory, where they were smeared directly onto plates containing potato-dextrose agar (for fungi) (PDA; e.g., Kiyuna et al., 2008) and nutrient agar (CM3; e.g., Tazato et al., 2012) and then incubated at 20–25°C and 30°C, respectively, for a suitable period under aerobic conditions; and (3) uncovered plates were exposed to air for 10 min and then incubated at 25°C to isolate airborne microbes. All fungal isolates were cultured on PDA in the dark at 25°C. After purification of the fungal and bacterial colonies, the respective isolates were grouped by morphological characteristics. In addition to the full phenotypic characterization of the selected/representative isolates, gene sequencing was performed, mainly on the LSU and other genes for fungi and 16S rRNA gene for bacteria. Based on the sequence data, molecular phylogenetic analyses were made using the standard method. Representative studies that used our methods include Kiyuna et al. (2008, 2011, 2012, 2015, 2017) and An et al. (2009) for the major/noteworthy fungal colonizers, Nagatsuka et al. (2009, 2016, 2017) for yeast and yeast-like colonizers, and Tazato et al. (2012, 2015), Nishijima et al. (2013, 2017a, 2017b), and Handa et al. (2016, 2017), for the major/noteworthy bacterial colonizers. These original papers have revealed the identities of the TT and KT microbial isolates in question, proposing one novel genus, seventeen novel species, and six novel combinations in total, as listed in Supplementary Materials of this review.

3-4. Culture-independent (molecular) methods: PCR-DGGE, clone libraries, and pyrosequence analyses

Molecular community analyses (polymerase chain reaction-denaturing gradient gel electrophoresis [PCR-DGGE], clone library, and pyrosequence analyses) of the selected samples were used to clarify the microbial diversity of various biodeteriorated environments (Gurtner et al., 2000; Portillo et al., 2008, 2009; Saiz-Jiménez, 2003; Zengler, 2008). Details of our culture-independent methods (Fig. 4) and the data obtained have been described in Sato et al. (2013, 2015), Nishijima et al. (unpubl.), and Section 8 of this review.

3-5. Biological characterization (bioprofiling) of the selected microbial isolates

To identify the organisms in question, we used the usual biological (physiological) characterization (Fig. 4; e.g., growth range at different temperatures, responses on fermentation and assimilation tests in yeasts, growth range

at different temperatures and pH, and utilization of various carbon sources in bacteria). In addition, we used the following assessments and bioprofiling techniques: (1) utilization of ethanol and isopropanol as carbon sources (Kigawa et al., 2010b); (2) the effect of a biocide (Kathon GC) on isolates from viscous gels (biofilms) (Kigawa et al., 2007c); (3) carboxylic acid productivity (Sano et al., 2010); (4) acetic acid production by *Gluconacetobacter* isolates (Kigawa et al., 2013a); (5) growth tests on consolidants of mural paintings [paraloid B72, hydroxyl propyl cellulose, methyl cellulose, and others (Kigawa et al., 2010a, 2012a)]; (6) decomposition of calcium carbonate (CaCO₃) as a major component of the plaster by *Gluconacetobacter* isolates (Kigawa et al., 2013a); and (7) investigating the effects of intermittent UV irradiation (applied inside the KT stone chamber from 2009 to 2013 after relocation of the mural painting in 2008) on selected fungal (e.g., dark *Acremonium* spp., *Penicillium paneum*, and *Burgoa anomala*) and bacterial (e.g., *Bacillus* and *Stenotrophomonas* spp.) isolates (Kigawa et al., 2010c, 2013b). These studies were conducted to reveal the biological characteristics of the major/selected TT/KT microbial isolates and their involvement in the biodeterioration of mural paintings and wall plaster and to design proper measures for controlling microbial contaminants.

4. Dynamics of Microbiota of the TT Stone Chamber Interior and Neighboring Environments from 2004 to 2009 Using Culture-Dependent Methods

4-1. Mycobiota of the TT

In this section, the dynamics and changes of mycobiota, especially molds, are described, followed by that of the yeast biota.

A total of 236 selected samples, including mold spots, viscous gels (biofilms), and mixtures of plaster fragments and soil (Fig. S1) were collected from the stone chamber interior, spaces between the stone walls, and the stone chamber exterior of TT between May 2004 and February 2009 (Fig. 2). These were: 90 samples gathered from the stone chamber interior during May 2004 to December 2006; 19 from the adjacent space during May 2004 to July 2006; 50 from the joint space of the stone walls during April 2007 to August 2007; 66 from the stone chamber exterior during November 2006 to August 2007; and 11 from the mound during February 2008 to February 2009.

4-1-1. Changes in mycobiota of the stone chamber interior between May 2004 and December 2006. A total of 426 isolates were obtained from the stone chamber interior between May 2004 and December 2006. The succession of mycobiota of the stone chamber interior, mainly based on the major fungal genera, is summarized in Fig. 5. From May to September 2004, *Fusarium* (FSSC clade), *Trichoderma*, and *Penicillium* (mainly *Penicillium* sp. 1, subsequently identified as the known species *Penicillium paneum*; see An et al., 2009) were the predominant fungal genera in the stone chamber interior. After cooling of the TT mound and stone chamber starting from September 2005, *Fusarium* and *Trichoderma* species decreased in number, but *Penicillium* continued to be the major fungal

Fig. 5. Changes in mycobiota of the TT stone chamber interior (May 2004–Dec 2006) (modified from Sugiyama et al., 2009).

The graph indicates the proportions at the genus level among the samples collected during each sampling period.

colonizers. From May 2006, the temperature of the stone chamber interior was kept stable at approximately 10°C, and members of *Acremonium* sect. *Gliomastix*, the causal biodeteriogens of the “Black Spots on the Asuka Beauties (*Asuka Bijin*)”, were mainly isolated from the stone chamber interior together with *P. paneum* (Fig. 5; cf., Subsection 6-3). *Clonostachys* was the primary isolate in various other samples. In addition, ascomycetous yeasts, such as *Candida takamatsuzukensis*, *C. tumulicola*, *Pichia guilliermondii*, and *P. membranifaciens*, were isolated from viscous gels (biofilms) developed on the mural paintings and plaster walls. A report on *C. takamatsuzukensis* and *C. tumulicola* includes a brief discussion on the significance of the novel *Candida* species in the tumulus environment and related disinfectants (Nagatsuka et al., 2009).

Many fungal species were common to both mycobiotas of the TT and KT (Table 4, Table S2). *Penicillium paneum*, *Fusarium* (FSSC clade), *Trichoderma*, and *Clonostachys* cf. *rosea* were the major colonizers in the stone chamber of the TT. These fungal genera are reported as the major groups involved in the biodeterioration of mural paintings outside of Japan (Garg et al., 1995; Karbowska-Berent, 2003). *Acremonium* (sect. *Gliomastix*) *masseei* was predominantly isolated from the TT stone chamber interior. These species were reported for the first time, in this study, from the black stains/spots on the mural paintings (Figs. 1j and k); however, to date, there is no record of its occurrence outside of Japan. This major colonizer of the black

stains/spots is considered to be affected by environmental changes, such as temperature and humidity.

Arai (1984, 1987, 1990b) reported that *Doratomyces* sp., *Fusarium* sp., *Cladosporium* sp., and *Mucor* sp. were the predominant colonizers of the TT in March 1975, while a few *Trichoderma* sp. and *Penicillium* sp. were also present. The zygomycete genus *Mucor* was not encountered in our survey of either tumulus. The fungal isolates recovered between 1975 and the spring of 2001 (Arai, 1984, 1987, 1990b) were not available for study because they had not been deposited in any public culture collection. In addition, there were no illustrations or brief/complete descriptions of these fungi. Consequently, comparisons of fungal records between previous surveys and our survey are based on fragmentary data at the genus, but not the species, level. The genus *Cylindrocarpon*, a causal agent of the brown stains on the north and south walls of the TT in December 2001 (Kigawa et al., 2006b), was not isolated from the stone chamber interior between 2004 and 2006 in our survey. During the dismantling and relocation of the stone chamber between April and August 2007, *Cylindrocarpon* (taxonomically and ecologically similar to *Fusarium*) was isolated from samples recovered from the joint spaces between the stone walls and the stone chamber exterior. Therefore, the decline in occurrences of *Cylindrocarpon* since 2001 was considered to be affected by other factors, such as microbial behavior (e.g., the physiological decline of the fungus itself, invasion of other fungi such as dark *Acremonium*) or biocide treatment.

Table 4. List of fungal taxa isolated from the TT stone chamber interior and neighboring environments.

	Sampling area	Stone chamber interior	Adjacent space	Joint/Space of stone walls	Stone chamber exterior	Mound	Habitat cited in the literature ^{b)}				
Taxon	Sampling period	2004.5–2006.12	2004.5–2006.7	2007.4–8	2006.11–2007.8	2008–2009.2	Soil	Plant	Air	Arthro-pods	Others
Ascomycota											
<i>Acremonium</i> (sect. <i>Gliomastix</i>) <i>masseei</i>		+		+				●			●
<i>Acremonium</i> (sect. <i>Gliomastix</i>) <i>murorum</i>		+		+			●	●			
<i>Acremonium</i> spp.		+	+	+	+	+	●	●	●	●	●
<i>Alternaria</i> sp.					+		●	●			
<i>Aphanocladium</i> sp.				+			●				
<i>Arthrinium</i> sp.						+	●		●		●
<i>Arthrobotrys</i> sp.		+		+			●			●	●
<i>Aspergillus</i> spp. (incl. <i>A. niger</i> agg.)		+	+			+	●		●		●
Bionectriaceous group			+				●	●			●
<i>Cephalotrichum verrucisporum</i>				+			●	●			●
<i>Cladophialophora</i> spp.				+	+	+	●	●	●		●
<i>Cladosporium</i> spp.		+			+		●	●	●		
<i>Clonostachys</i> cf. <i>rosea</i>		+	+	+	+		●	●			●
<i>Cylindrocarpon</i> sp.				+	+	+	●	●			●
<i>Exophiala</i> sp.						+	●	●			●
<i>Fusarium</i> spp. (FSSC clade)		+	+	+	+	+	●	●	●		●
<i>Geomyces</i> cf. <i>pannorum</i>						+	●		●		●
<i>Gilmaniella humicola</i>						+	●				
<i>Kendrickiella phycomyces</i>		+		+	+		●	●			●
<i>Lecanicillium</i> spp.		+		+	+						
<i>Nodulisporium</i> sp.					+		●	●			
<i>Oidiodendron</i> spp.		+	+	+	+	+	●	●	●		
<i>Paecilomyces variotii</i> complex				+	+	+	●	●	●	●	●
<i>Papulaspora</i> sp.						+	●	●			●
<i>Penicillium paneum</i>		+	+	+	+	+	●	●	●		●
<i>Penicillium</i> spp.		+	+	+	+	+	●	●	●	●	●
<i>Phialocephala</i> spp.		+		+			●	●			●
<i>Phialophora</i> sp.				+			●	●	●		
<i>Purpureocillium lilacinum</i>		+	+				●		●		●
<i>Rhinocladiella</i> sp.		+		+			●	●			
<i>Sagenomella striatispora</i>				+	+	+	●	●			●
<i>Talaromyces minioluteus</i>		+	+	+	+	+	●				●
<i>Talaromyces</i> spp.						+	●	●			●
<i>Trichocladium opacum</i>				+		+	●	●			●
<i>Trichoderma</i> spp.		+	+	+	+	+	●	●	●		●
<i>Trichosporiella</i> sp.					+		●				●
<i>Tritiratum</i> sp.					+						
<i>Verticillium</i> sp.				+			●	●		●	●
Yeast spp. (incl. <i>Candida</i> , <i>Pichia</i>)		+	+	+	+	+	●	●	●		●
Basidiomycota											
<i>Cryptococcus</i> spp.						+	●	●			●
<i>Sporotrichum</i> sp.		+						●			●
Mucoromycotina											
<i>Cirrenalia</i> sp.						+	●				
<i>Gongronella butleri</i>						+	●				
<i>Mortierella</i> sp.				+	+		●			●	●
<i>Zygorhynchus</i> sp.				+			●	●			
Others											
Unidentified spp. (incl. sterile mycelium)		+	+	+	+	+					
Total no. of isolates		426	72	337	451	124					
No. of examined samples		90	19	50	66	11					

^{a)} + indicates that the taxon was isolated.

^{b)} ● indicates that the taxon has been recorded from the habitat on the basis of the literature information (e.g., Domsch et al., 2007; Seifert et al., 2011).

4-1-2. Mycobiota of samples collected from the TT stone chamber interior and neighboring environment. During the survey of mycobiota related to the biodeterioration of murals and plaster walls from May 2004 to February 2009, a total of 236 selected samples were subjected to fungal isolation. As previously mentioned in Subsection 3-1 of this review, these samples were categorized into five groups depending on the sampling area. The stone chamber interior contained 90 samples collected from May 2004 to December 2006: the adjacent space contained 19 samples collected from May 2004 to July 2006, the joint space of the stone walls contained 50 samples gathered from

April to August 2007, the stone chamber exterior contained 66 samples gathered from November 2006 to August 2007, and the mound contained 11 samples gathered from February 2008 to 2009.

From the total of 236 samples, 1410 fungal isolates (including unidentified groups) were isolated and identified at the genus/species level (Fig. S3, Table 4), including 426 from the stone chamber interior, 72 from the adjacent space, 337 from the joint space of the stone walls, 451 from the stone chamber exterior, and 124 from the mound. Members of the genus *Penicillium* (e.g., *P. paneum*, the predominant colonizer of the stone chamber interior) were

isolated from the adjacent space, the joint space of the stone walls, and the stone chamber exterior, but not from the mound. On the other hand, *Talaromyces minioluteus* ($\equiv$ *Penicillium minioluteum*) was one of the predominant species isolated from the joint space of the stone walls and the stone chamber exterior (see Subsection 6-3 of this review). Isolates of the FSSC clade were mainly isolated from the stone chamber interior and the joint space of the stone walls, and a few were found on the stone chamber exterior and within the mound. *Trichoderma* species were the predominant group isolated from some samples. *Acremonium* sect. *Gliomastix* spp. were mainly isolated from the stone chamber interior, but not from the adjacent space, the stone chamber exterior, or the mound. *Oidiodendron* spp., which accounted for most of the gray to black stains/spots in the adjacent space on May 2006, were isolated from the adjacent space and stone chamber exterior. The genus *Oidiodendron*, a causal agent of gray to blackish stains/spots in the adjacent space on May 2006, was only isolated from the adjacent spaces and the stone chamber exterior of the TT, but not the KT. The genus *Oidiodendron* contains species that are common soil- and plant-associated fungi (Domsch et al., 2007); therefore, members of this genus are considered to have invaded the adjacent spaces via plant roots and similar substrates from the neighboring environment of the stone chamber.

In Table 4, *Cephalotrichum verrucisporum* ($\equiv$ *Doratomyces verrucisporus*) was only isolated from the joint space between west wall 1 and the ceiling stone of wall 1 (Kiyuna et al., 2017). *Doratomyces* (*sic*) was recorded in the Agency's reports as the predominant fungus occurring in the stone chamber in the 1970–80s (Arai, 1984, 1987, 1990b; the Agency webpage, <http://www.bunka.go.jp/seisaku/bunkazai/takamatsu_kitora/>). The *Doratomyces* isolates and related vouchers were not preserved in any public culture collections; therefore, a comparison of the two is not possible. However, one of our *Doratomyces* isolates is believed to be the same species mentioned in Arai's reports (1987, 1990b). Hence, the *Doratomyces* isolate in question may have survived in the joint space between the plaster walls. Furthermore, various chaetotryalean black fungi (e.g., *Cladophialophora* and *Phialophora*) were isolated from the joint space of the stone walls and identified by direct observation. Various fungi were isolated from the joint space of the stone walls in addition to other areas of TT (Table 4). The joint spaces of the stone walls are located between the stone chamber interior and exterior, in both the stone chamber exterior and the burial mound. Therefore, these spaces were susceptible environments of the stone chamber interior and exterior and, for many years, were considered to be "accumulation spots" for various microbes from both of these environments. A good example is our detection of *Cephalotrichum verrucisporum* in the joint space, as described later.

Regarding *Fusarium* TT and KT isolates, haplotypes of the FSSC clade were detected (Kiyuna et al., 2008). A similar discovery was reported from the prehistoric painted cave of Lascaux in France (Dupont et al., 2007; Oriol et al., 2011). These fungal members are reported to deteriorate wall paintings (Dhawan et al., 1993; Garg et al., 1995).

Fusarium solani, with the ability to oxidize elemental sulfur, has been identified in deteriorated sandstones of Angkor monuments in Cambodia (Li et al., 2010).

Cladosporium, a ubiquitous fungal genus, has been observed in biodeteriorated samples outside of Japan (Garg et al., 1995; Karbowska-Berent, 2003). However, only a few *Cladosporium* spp. were isolated from the murals and plaster walls of both the TT (Table 4) and the KT (Table S2). The presence of this genus as a predominant fungus was observed in the TT stone chamber interior from 1972 to 2001 (ACA, Japan web page; http://www.bunka.go.jp/seisaku/bunkazai/takamatsu_kitora/hozon_kako.html).

Purpureocillium lilacinum ($\equiv$ *Paecilomyces lilacinus*) and *Lecanicillium* spp. were isolated from various samples from the TT (Table 4) and KT (Table S2) stone chambers. *Purpureocillium lilacinum* has been found in soil, insects, nematodes, clinical samples (Luangsa-Ard et al., 2011), and in numerous samples from the Altamira Cave in Spain (Novakova et al., 2014). The presence of members of the entomogenous genera *Purpureocillium* and *Lecanicillium* suggests that these fungi invaded the stone chamber through arthropods, such as mites and springtails (see Subsection 9-3 of this review).

Outbreaks of black stains/spots on the mural paintings were common problematic occurrences in TT, KT, and the Lascaux Cave (e.g., Ishizaki and Kigawa, 2011). After the outbreak of the FSSC in the Lascaux Cave, black spots/stains caused by *Gliomastix murorum* (*sic*) appeared on the cave walls and became problematic in December 2001 (Oriol et al., 2011). Occurrences of *Gliomastix murorum* (*sic*), treated as *Acremonium* (sect. *Gliomastix*) *murorum* (Kiyuna et al., 2011), were similar to those of KT. Since 2006, the novel black spot/stain phenomenon has been found on the cave walls, and the causal agents were identified as *Ulocaldium*, *Gliomastix*, *Scolecobasidium tshawytschae*, and *Verticillium* (Bastian and Alabouvette, 2009; Bastian et al., 2010). Subsequently, *Ochroconis lascauxensis*, *O. anomala*, *Acremonium nepalense*, and *Exophiala* were identified as the major causal agents of the black stain (Martin-Sanchez et al., 2012a, b). These fungal colonizers differed between the TT and the KT (*Acremonium* [sect. *Gliomastix*] *masseei* in TT; *Acremonium* [sect. *Gliomastix*] *tumulicola* in the KT). *Acremonium nepalense* is phylogenetically placed in the family Plectosphaerellaceae (Glomerellales), which differs from the section *Gliomastix* of *Acremonium* (Hypocreales) (Summerbell et al., 2011). *A. nepalense* develops white colonies on PDA and melanized colonies (gray-green color) on ECA medium (Martin-Sanchez et al., 2012a). This cultural characteristic on PDA differed from that of our isolates of *Acremonium* (sect. *Gliomastix*) spp.

4-2. Yeast biota

A total of 159 yeast strains were isolated from samples collected from the TT between 2004 and 2009. Subsequently, we selected 71 isolates as representatives based on their sources, their collection sites, and their colony characteristics, and the LSU gene sequences of these selected isolates were determined. The methods used to characterize the phenotypes and genotypes of these strains are

Fig. 6. Changes in the yeast biota of the TT stone chamber interior (May 2004–Dec 2006).

The graph indicates the number of isolates at the genus level among the samples collected during each sampling period. None of the isolates were obtained from samples collected in Oct 2006.

described in Kurtzman et al. (2011), Natastuka et al. (2002, 2005, 2009, 2016), and Yamada et al. (1999).

4-2-1. Changes in the yeast biota of the stone chamber interior between May 2004 and December 2006. The results of bootstrapped neighbor-joining (NJ) analysis of LSU gene sequences are summarized in Fig. S4 and Table S3. Changes in the yeast biota of the stone chamber interior, along with the number of samples, are shown in Fig. 6. Only four yeast strains were isolated from 22 samples collected from the TT chamber interior between May and September 2004, while 34 yeast strains were isolated from 68 samples from the TT chamber interior after mound cooling was started in September 2005. *Yamadazyma tumulicola*, the most frequent isolate in TT interior samples, including many biofilms, had not been isolated before the cooling of the burial mound (Table S3, Fig. 6). The cooling of the mound is thought to be one of the triggers of the change in yeast biota. *Meyerozyma guilliermondii* ($\equiv$ *Pichia guilliermondii*), *Pichia membranifaciens*, *Yamadazyma takamatsuzukensis* ($\equiv$ *Candida takamatsuzukensis*), and *Candida boidinii* were isolated before cooling of the burial mound, and after cooling as well, but at a lower frequency.

4-2-2. Yeast biota of samples collected from the TT stone chamber interior and neighboring environment. Figure S4 and Table S3 summarize the isolate taxon/phylogeny and the sampling area from where the isolates were collected. *Y. tumulicola*, *M. guilliermondii*, *Y.*

takamatsuzukensis, and *P. membranifaciens* were the most frequent species identified in the samples collected inside the stone chamber. A paper on *C. takamatsuzukensis* and *C. tumulicola* (both later designated as the genus *Yamadazyma*; Nagatsuka et al., 2016) briefly discusses the significance of these novel *Candida* species in the tumulus environment and related disinfectants (Nagatsuka et al., 2009). *Yamadazyma tumulicola*, *Y. takamatsuzukensis*, and *P. membranifaciens* were frequently isolated from biofilm samples on the walls in the stone chamber interior and the joints/spaces of stone walls, but not from the stone chamber exterior or mound. Only *M. guilliermondii* was found in samples from both the stone chamber interior and exterior. Many species in the *Yamadazyma* clade to which *Y. tumulicola* and *Y. takamatsuzukensis* belong, including *P. membranifaciens* and *M. guilliermondii*, were reported to be associated soil, insects, and plants (Ganter, 2006; Suh et al., 2005). *Y. tumulicola*, *M. guilliermondii*, *Y. takamatsuzukensis*, and *P. membranifaciens* are thought to have been brought into the stone chamber from the outside environment by transporters. Nine of 11 isolates from the mound were assigned to the genus *Cryptococcus* and were reported to be accompanied by many insect-/soil-associated yeast species (Fonseca et al., 2011). None of the species isolated from mound samples were found in any other area: namely, the stone chamber interior, adjacent space, joint/space of stone walls and stone chamber interior.

Fig. 7. Changes in bacteriobiota of the TT stone chamber interior (Sep 2005–Apr 2007).

The graph indicates the proportions at the phylum/class level among the samples collected during each sampling period. *Bacillus cereus* group included *B. cereus* and its phylogenetically related species, *B. thuringiensis*, *B. toyonensis*, and *B. pseudomycoides*, and three unknown species in this group. Other Alphaproteobacteria: isolates belonged to the Alphaproteobacteria except *Ochrobactrum*; Other Betaproteobacteria: isolates belonged to the Betaproteobacteria except *Bordetella*; Other Gammaproteobacteria: isolates belonged to the Gammaproteobacteria except *Stenotrophomonas*. Note: Quotation marks for names of the bacterial taxa above the rank of class are eliminated in appearance within all the figures of this review.

4-3. Bacteriobiota

In the survey of the TT bacteriobiota, we obtained a total of 516 strains from 177 samples collected from September 2005 to February 2009. We performed a 16S rRNA gene sequence analysis of 242 isolates, including 10 acetic acid bacteria, and we determined the bacterial community of TT based on these 242 isolates.

4-3-1. Bacteriobiota of the TT stone chamber interior.

The bacteriobiota of the TT was determined initially from results obtained from only 50 samples (Sugiyama et al., 2009). The bacteriobiota of the TT stone chamber interior was investigated during the period September 2005 to April 2007, when the dismantling work had just begun. Before the dismantling work started, the chamber interior was kept at about 10°C to control fungal activities (Inuzuka and Ishizaki, 2007). The temperature control started in September 2005 and was continued up until April 2007. The temperature of the chamber interior was stable, around 10°C, during 2006–2007, just before the beginning of the dismantling work. Before dismantling, the chamber was a dark, highly humid, enclosed space. The bacteriobiota survey of the stone chamber interior was carried out during this period.

Changes in the bacteriobiota of the TT stone chamber interior are shown in Fig. 7. The phylum-level taxa of these TT isolates include the “Proteobacteria” (including three

classes, Alpha-, Beta-, and Gamma-proteobacteria), “Bacteroidetes”, “Firmicutes”, and “Actinobacteria”. The samples in 2005 were collected immediately after cooling of the chamber interior. Initially, Gram-positive bacteria, including *Bacillus* (mainly the *B. cereus* group, see Subsection 6-6 of this review) and some “Actinobacteria”, accounted for more than half of the isolates; however, the ratio of Gram-negative bacteria in the isolated strains increased after cooling stabilized in the chamber in 2006. In particular, all isolates of *Bordetella* in the class Betaproteobacteria were only found in samples collected in 2006. The number of isolates belonging to *Stenotrophomonas* in the class Gammaproteobacteria was also higher at this time. Additionally, *Ochrobactrum* spp. in the class Alphaproteobacteria was one of the major taxa found. Therefore, the four genera *Bacillus*, *Stenotrophomonas*, *Bordetella*, and *Ochrobactrum* were considered to be predominant in the bacterial community on the plaster surface in this period. *Ochrobactrum* and *Bordetella* were only isolated from the TT samples; no isolates were obtained from the KT samples. As the dismantling work started, the inside of the chamber was exposed to the outside air. The microclimate of the plaster surface was warmer and drier than before. *Bordetella* spp. and *Ochrobactrum* spp. were not isolated from the plaster surface samples collected in 2007. While the ratio of *Bacillus* to “Proteobacteria” recovered to 1:1 in 2007, no

“*Actinobacteria*” were isolated at this time (Fig. 7). These results indicated that environmental conditions, such as temperature and humidity, affected the bacterial diversity in the stone chamber interior, especially on the plaster surface. Before the dismantling, the appearance of many viscous gels (biofilms) was observed on the stone chamber wall surface (Figs. 1h, i, t, and v); therefore, most bacteria were highly active at that time, even though the stone chamber interior was maintained at a low temperature.

4-3-2. Bacteriobiota of samples collected from the TT stone chamber interior and the neighboring environment.

Because of repeated disturbances of the TT by microorganisms, the stone chamber was dismantled and relocated for conservation work. The samples collected in this period were separated into five groups as previously mentioned (Section 3 of this review). A comparison of the bacterial diversity between the interior and exterior of the TT stone chamber is shown in Fig. S5. The *Gluconacetobacter* spp. were isolated using the enrichment culture method (see Subsection 7-8 of this review), which is a method not used very often in this study. The isolates represented six phyla/classes as determined by the 16S rRNA gene sequence. The ratio of each taxa in the total isolates was as follows: *Alphaproteobacteria*, 16.1%; *Betaproteobacteria*, 9.1%; *Gammaproteobacteria*, 14.9%; “*Bacteroidetes*”, 5.0%; “*Firmicutes*”, 28.5%; and “*Actinobacteria*”, 26.4% (Fig. S5). The proportion of “*Proteobacteria*” increased toward the interior from the exterior of the stone chamber. The converse result was observed for “*Actinobacteria*” and “*Firmicutes*”. In the class *Alphaproteobacteria*, *Ochrobactrum* spp. were isolated from the stone chamber exterior, the joint/space of the stone wall, and the stone chamber interior, while *Gluconacetobacter* spp. were not found in any of the stone chamber interior samples. In the class *Betaproteobacteria*, three *Bordetella* spp. were isolated from the stone chamber interior and the joint/space of the stone wall. In the class *Gammaproteobacteria*, the proportion of *Stenotrophomonas* spp. increased toward the interior from the exterior of the stone chamber, but no strains of this genus were isolated from the mound samples. Interestingly, members of the *Bacillus cereus* group were isolated from all sites except the stone chamber exterior, while the *B. simplex* group was not found inside the stone chamber. The phylum “*Firmicutes*” was the most predominant in the TT samples; in particular, the isolates of the *B. cereus* and *B. simplex* groups accounted for 21.5% of all isolates. These results suggest that the bacteria isolated from the stone chamber interior might have invaded the chamber from the surrounding area and adapted to the environment (see Subsections 6-6 and 9-3 of this review).

5. Dynamics of the Microbiota of the KT Stone Chamber Interior and Neighboring Environments from 2004 to 2013 Based on Culture-Dependent Methods

5-1. Mycobiota of the KT

A total of 198 samples collected from the KT stone chamber interior and exterior between June 2004 and February 2013 (Table S2) were used for mycobiota analyses. These were: 157 samples were gathered from the stone chamber

interior during June 2004 to September 2012, 9 from the thieving (thief) hole during February 2013, 25 from the adjacent small room during September 2004 to March 2006, 5 from the adjacent rooms in March 2006, 1 from the external air in March 2006, and 1 from the mound in June 2008.

5-1-1. Changes in the mycobiota of the KT stone chamber interior between June 2004 and September 2012.

From the 157 samples collected from the stone chamber interior, 832 isolates were isolated (Table S2). In June 2004, *Penicillium* (mainly *P. paneum*) and ascomycetous yeasts (*Candida* and *Pichia*, mainly isolated from a viscous gel) were predominant. Subsequently, *Fusarium* (FSSC clade), *Acremonium* cf. *strictum*, *Trichoderma*, *Acremonium* sect. *Gliomastix*, *Myxozyma* spp., and *Clonostachys* cf. *rosea* were the predominant fungi inside the stone chamber (Fig. 8). In April 2006, a basidiomycetous anamorphic fungus (*Burgoa*), denoted as “black particles” (see Subsection 7-2), was isolated only from the stone chamber interior (Fig. 8, Table S2).

After intermittent UV irradiation of the KT stone chamber started in April 2009, microbial appearances (e.g., mold spots and viscous gels (biofilms)) were effectively suppressed; this assessment was based on observations made with the naked eye (Kigawa et al., 2010c, 2012b). However, dematiaceous fungi, *Cladophialophora*, and *Phialophora* species were clearly isolated from the stone chamber interior, together with other fungi from various samples (Fig. 8).

Many fungal species were common to both the TT (Table 4) and KT mycobiotas (Table S2). *Penicillium paneum*, *Fusarium* (the FSSC clade), and *Trichoderma* were the major colonizers of the KT stone chamber interior. These fungal genera are reported as major contributors to the biodeterioration of mural paintings outside of Japan (Garg et al., 1995; Karbowska-Berent, 2003). *Acremonium* cf. *strictum*, *Acremonium* (sect. *Gliomastix*) *tumulicola*, *Burgoa anomala*, and *Kendrickiella phycomyces* are the predominant species isolated from the KT stone chamber interior. These species were reported for the first time in the biodeteriorated samples in this study. To date, there is no record of their occurrence outside of Japan.

KT isolates of *Acremonium* cf. *strictum* were re-identified as *A. killense* and *A. strictum*, known as clinically important species in molecular phylogenetic analyses. Recently, *A. killense* and *A. strictum* were reclassified into the genus *Sarocladium* (anamorphic Hypocrales; Giraldo et al., 2015; Summerbell et al., 2011). Detection of these species was recorded mainly from viscous substances on the plaster and stone walls in the KT stone chamber; therefore, they are thought to be involved in viscous gel (biofilm) formation on the mural paintings. Recently, *A. strictum* was identified in samples from the Altamira Cave, but was not discussed as a threat to the conservation of murals in the Altamira Cave (Novakova et al., 2014).

5-1-2. Mycobiota of the KT stone chamber interior and the neighboring environment.

While surveying mycobiota related to the biodeterioration of the murals and plaster walls from June 2004 to February 2013, a total of 198 samples were collected for fungal isolation. A total of

Fig. 8. Changes in mycobiota of the KT stone chamber interior (Jun 2004–Oct 2011).

The graph indicates the proportions at the genus level among the samples collected during each sampling period. Data modified and compiled from Kiyuna et al. (2008), Kigawa et al. (2009b, 2010b, 2011, 2012a), and the Agency for Cultural Affairs, Japan webpage (<<http://www.bunka.go.jp/>>).

157 samples were selected from the stone chamber interior (June 2004–September 2012), 25 from the adjacent small room (September 2004–March 2006), 5 from the adjacent rooms (March 2006), and 1 each from the mound (June 2008) and the external air (March 2006). From a total of 198 samples, 1092 fungal isolates (including unidentified groups) were isolated, of which 832 isolates were acquired from the stone chamber interior, 76 from the adjacent small room, 43 from the adjacent rooms, and 42 from the mound/external air (Table S2). KT samples for microbial surveys were mainly collected from the stone chamber interior; therefore, the comparison of mycobiotas between the stone chamber interior and exterior (including the adjacent small room and rooms, external air, and mound) was difficult. Core genera of mycobiota of the stone chamber interior, mainly *Penicillium*, dark *Acremonium*, *Fusarium*, and *Trichoderma*, were similar to those of the adjacent small room (Table S2).

5-2. Yeast biota of the KT samples

5-2-1. Changes in the yeast biota of the KT stone chamber interior between June 2004 and September 2012. We determined the phylogenetic positions of 62 yeasts isolated from samples collected from the KT stone chamber interior. Figure 9 summarizes the changes in the KT yeast biota over time. *Yamadazyma olivae* was the yeast pre-

dominant in the KT stone chamber interior before intermittent UV irradiation started in April 2009. After UV irradiation, the predominant species changed from *Y. olivae* to *Candida boidinii*, which was isolated from the sample collected in 2007 for the first time. The yeast temporarily named *Myxozyma cf. monticola* dominated between 2008 and 2011, and *M. guilliermondii* was continually isolated throughout the period, regardless of intermittent UV irradiation.

5-2-2. Comparison of the yeast biota of the stone chamber interiors of KT and TT. The phylogenetic position in the NJ tree of 38 TT and 62 KT yeast isolates from chamber interior samples was determined based on D1/D2 LSU rRNA gene sequences (Fig. 10). Subsequently, about 55% of the TT and 35% of the KT isolates were assigned to the *Yamadazyma* clade (Table S3, Fig. 10). However, one KT isolate was assigned to *Y. tumulicola*; the *Yamadazyma* species *Y. tumulicola* and *Y. takamatsuzukensis* were specifically isolated from the TT, and *Y. olivae*, *Y. kitorensis*, and *C. friedrichii* were specifically isolated from the KT. We do not have sufficient evidence to explain these isolate specificities at the moment. On the other hand, *Meyerozyma guilliermondii* and *Candida boidinii* were frequently isolated from both tumuli (Figs. 6, 9, and 10). Moreover, species of the *Yamadazyma* clade, *M. guilliermondii*, and *C. boidinii* were reportedly associated

Fig. 9. Changes in the yeast biota of the KT stone chamber interior (2004–2012) and exterior (2013).

2009 (1) and 2009 (2) includes the isolates from the sample collected before/after intermittent UV irradiation in 2009, respectively. The graph indicates the proportions at the genus level among the samples collected during each sampling period. None of the isolates were obtained from samples collected in 2004.

with various insects and/or natural environmental substrates, including water, atmosphere, and soil (Kurtzman, 2011; Lachance et al., 2011; Suh et al., 2005). The invasion of arthropods, including the spring tail *Porcellio* sp. and mites, plant roots, water, and traces of soil through narrow spaces between wall stones was observed in the TT stone chamber (ICECDMPPT, 2010). The presence of multiple yeast species of the *Yamadazyma* clade and *M. guilliermondii* in both the TT and KT stone chamber interiors may be associated with such phenomena. Minority yeast isolates include *Zygoascus polysorbophila* from TT (Fig. 6) and *Zygoascus biomembranicola* from KT (Fig. 9). A yeast-like microalga, *Prototheca* sp., which very recently has been identified as *P. tumulicola* sp. nov. (Fig. S4 and Fig. 6; Nagatsuka et al., 2017; cf., Subsection 7-6 of this review), was isolated from the TT, and *Myxozyma* sp., which formed slimy colonies, was isolated from the KT (Fig. 9).

5-3. Bacteriobiota

In the survey of KT bacteriobiota, we obtained a total of 257 isolates from a total of 99 samples collected during September 2005 to February 2013. The 16S rRNA gene sequence was analyzed in 164 selected isolates. Therefore, the determination of the KT bacterial community was based on these 164 isolates. The phylogenetic tree constructed from selected bacterial isolates obtained from both tumuli is shown in Fig. 11. All isolates represented six phyla/classes as determined by the 16S rRNA gene sequence divergence.

5-3-1. Bacteriobiota of the KT stone chamber interior.

As shown in Table 2, the mural paintings were detached from the walls inside the stone chamber and relocated to

the preservation facility soon after the discovery in 2004 that the wall plaster was loosely held to the stone. After all paintings were relocated, UV intermittent irradiation was carried out to control the activity of microorganisms (starting in March 2009) (Kigawa et al., 2009b, 2010c, 2011; Table 2).

The bacteriobiota of the KT stone chamber is shown in Fig. 12. The major taxa of the isolates from the KT stone chamber interior included the phyla “*Proteobacteria*” (*Rhizobium* in the class *Alphaproteobacteria*, *Achromobacter* in the class *Betaproteobacteria*, and *Stenotrophomonas* in the class *Gammaproteobacteria*), “*Bacteroidetes*”, “*Firmicutes*”, and “*Actinobacteria*”. Isolates from the KT chamber interior included more species of Gram-negative, than Gram-positive, bacteria. The genus *Gluconacetobacter* in the class *Alphaproteobacteria* was thought to have caused severe damage to the wall plaster. The samples in 2009 differed from those of other years, as no *Alphaproteobacteria* were isolated. At that time, UV irradiation had begun. Hence, UV irradiation likely affected the microbial community in the KT stone chamber interior. The phylogenetic tree based on 16S rRNA gene sequences of TT and KT isolates from viscous gel (biofilm) samples is shown in Fig. 11. A clear difference in bacterial diversity is seen between the two tumuli.

5-3-2. Bacteriobiota of the KT stone chamber interior before, and after, UV irradiation, and bacteriobiota of the stone chamber exterior.

After relocation of the mural paintings and the plaster inside the stone chamber, intermittent UV irradiation was begun in 2009 to prevent microorganism growth. In the samples collected before UV irradiation, more than 60% of the isolates comprised bacteria of the phylum “*Proteobacteria*”. Members of the

Fig. 10. Comparison of the yeast biota of the TT and KT chamber interiors.

The tree was constructed based on the neighbor-joining analysis of LSU gene sequences (446 nt). Bootstrap percentage values >50% derived from 1000 replications are shown above or below the corresponding branches. Numbers in parentheses indicate DDBJ accession numbers. Bar indicates 0.05 nucleotide substitutions per site. The initials T and K of strain numbers indicate isolates from the TT and KT, respectively; ○: isolates from the KT before intermittent UV irradiation; ●: isolates from the KT after intermittent UV irradiation.

Fig. 11. Molecular phylogeny placing selected 157 TT and KT bacterial isolates.

The tree was constructed based on the neighbor-joining analysis of 16S rRNA gene sequences (1300 nt). Bootstrap percentage values >70 % derived from 1000 replications are shown above or below the corresponding branches. The type strain of *Aquifex pyrophilus* is used as an outgroup. Bar indicates 0.02 nucleotide substitutions per site. The TT, KT and both in parentheses indicate the respective major taxa isolated from both tumuli. The *Bacillus cereus* group included *B. cereus* and its phylogenetically related species, *B. thuringiensis*, *B. toyonensis*, and *B. pseudomycoides*, and three unknown species in this group, whereas the *B. simplex* group included *B. simplex* and its related species, *B. butanolivorans*.

phyla “Actinobacteria” and “Bacteroidetes” increased after UV irradiation (Fig. S6). These results suggest that UV irradiation had an effect on the bacterial community.

Stenotrophomonas tumulicola (Handa et al., 2016), *Microbacterium shaanxiense* (Peng et al., 2015), and *Advenella* sp., were isolated from the TT stone chamber interior and from the stone chamber exterior and burial mound soil of the KT.

5-3-3. Comparison of the bacterial communities of the TT and KT. Viscous gels appeared on some parts of the wall surface inside the stone chamber starting in 2004 in the TT and in the summer of 2005 in the KT, respectively. These gels (biofilms) were aggregates of microorganisms, including mycelia, spores, yeasts, and bacterial cells, as observed through a light microscope (Figs. 1h, i, t, and v). Most of the isolates from these gels were *Ochrobactrum* spp., *Rhizobium* sp., *Gluconacetobacter* spp., *Advenella* sp., *Bordetella* spp., *Stenotrophomonas* spp., *Bacillus* spp., and several species of “Actinobacteria” (Fig. 11).

A clear difference in bacterial diversity is seen between the two tumuli. Alphaproteobacteria was more diverse in

the KT than in the TT. For instance, acetic acid bacteria were isolated from the stone chamber interior of the KT but not the TT. One of predominant TT isolates belonged to the genus *Ochrobactrum* in the class Alphaproteobacteria. *Ochrobactrum* members were not isolated from the KT. In the KT, *Rhizobium* aff. *radiobacter* was the most predominant bacterium in the class Alphaproteobacteria.

In the class Betaproteobacteria, *Bordetella* was isolated from the TT only, whereas *Advenella* and *Achromobacter* were isolated from both tumuli. The genus *Stenotrophomonas* in the class Gammaproteobacteria was predominant among isolates obtained from both tumuli. However, the representative species of this genus differed between tumuli. Most *Stenotrophomonas* isolates from the TT were of a novel taxon, recently proposed by Handa et al. (2016) as *S. tumulicola*, with the type strain T5916-2-1b^T (JCM 30961^T). In contrast, those from the KT belonged to *S. rhizophila*, which was not isolated from the TT. The major taxa of the bacterial communities isolated from the stone chamber interiors of both KT and TT were *Bacillus*, the *Rhizobiales*, and *Streptomyces*. Thus, environmental

Fig. 12. Changes in the bacteriobiota of the KT stone chamber interior (Sep 2005–Sep 2012).

The graph indicates the proportions at the phylum/class level among the samples collected during each sampling period. The *Bacillus cereus* group included *B. cereus* and its phylogenetically related species, *B. thuringiensis*, *B. toyonensis*, and *B. pseudomyoides*, and three unknown species in this group, whereas the *B. simplex* group was included its related species, *B. butanolivorans*. Other Alphaproteobacteria: isolates belonged to the Alphaproteobacteria except *Gluconacetobacter* and *Rhizobium*; Other Gammaproteobacteria: isolates belonged to the Gammaproteobacteria except *Stenotrophomonas*; Other “Firmicutes”: isolates belonged to the “Firmicutes” except the *Bacillus cereus* and *simplex* groups.

differences (cooling or not, dismantling or not, and UV intermittent or not) between the tumuli affected the bacterial taxa composition of the biofilms.

5-3-4. Comparison of the bacterial communities of TT, KT, and the Lascaux and Altamira Caves. The initial record of the bacterial community inside the TT stone chamber reported *Actinomycetes* along with some fungi (Arai, 1987). At the time of the discovery of the TT stone chamber, and at the first fungal outbreak, the investigation of bacteria was not as extensive as that of fungi. In addition, fungicide treatments (formaldehyde fumigation; ethanol) had been performed to prevent fungal outbreaks. Therefore, it is assumed that the original bacterial community in the TT stone chamber interior had already changed when our microbiological survey started. Our bacteriobiota survey for the KT stone chamber interior started from September 2005. At that time, viscous gels (biofilms) had been observed on the surface of stone walls, and several biocide treatments by paraformaldehyde fumigation had already been performed (Kigawa et al., 2005).

The most predominant bacteria in the Lascaux and Altamira Caves were of the phyla “Proteobacteria” and “Actinobacteria”. According to Bastian et al. (2010), the most abundant bacterial taxa in the Lascaux Cave, as determined by the cloning method, were “Proteobacteria”, such as *Ralstonia* in the class *Betaproteobacteria* and *Pseudomonas* in the class *Gammaproteobacteria*. Presumably, bacteria in these genera might have been selected as

a result of benzalkonium chloride treatment for the control of the microorganisms in the Lascaux Cave (Bastian et al., 2009c). On the other hand, neither *Ralstonia* nor *Pseudomonas* were isolated (or very few strains were isolated) from the TT and KT using the culture-dependent method. Additionally, neither of these two genera were detected in the TT and KT samples using the PCR-DGGE method. In the Altamira Cave, the major taxa of isolates were “Actinobacteria” (mostly *Streptomyces*), “Proteobacteria” (mostly *Gammaproteobacteria*), and “Firmicutes” (mostly *Bacillus*) (Gonzalez et al., 2008a; Laiz et al., 2003). The predominant airborne bacteria in this cave were *Micrococcus luteus* (85.3%), *Pseudomonas* spp. (6.8%), and *Bacillus* spp. (1.8%) (Garcia-Anton et al., 2014). Human disturbance (fungicide, artificial constructions) of this cave is considered to have strongly affected the balance of the bacterial community (Saiz-Jiménez, 2015).

6. Identification of the Major Microbial Colonizers of the Stone Chamber Interiors of Both Tumuli and Their Roles in Biodeterioration

This section reviews the major microbial colonizers of the TT and KT based on an integrated analysis of their phenotypes and genotypes and their related biological characteristics. Phenotypic (particularly cultural and morphological) characteristics of the major microorganisms of the TT and KT are shown in Fig. 13.

Fig. 13. Cultural and morphological characteristics of the major or noteworthy microorganisms of TT and KT.

(a) *Penicillium paneum* T5916-6-1 (JCM 15987) on PDA at 25°C for 2 weeks, (b) *Fusarium* sp. (*F. solani* species complex clade (FSSC)) T4716-1 on PDA at 25°C for 1 week, (c) *Trichoderma* sp. (*T. harzianum* species complex) T4519-9-7 on PDA at 25°C for 1 week, (d) *Acremonium maseeii* (sect. *Gliomastix*) T6517-1-1 (JCM 17165) on PDA at 25°C for 4 weeks, (e) *Talaromyces minioluteus* T4906-7-3 (JCM 28737) on YES at 25°C for 2 weeks, (f) *Cephalotrichum verrucisporum* T7530-12-1 (JCM 28755) on PDA at 25°C for 3 weeks, (g) *Kendrickella phycomyces* K5906-1-1 (JCM 18028) on PDA at 25°C for 2 weeks/on GYC (surface/reverse) at 25°C for 4 weeks/K4910-1 (JCM 18027); The calcium carbonate decomposition and white precipitants were observed around the colony, (h) *Sagenomella striatipora* T7521-8D-1 (JCM 28750) on PDA at 25°C for 8 weeks/on GYC (surface/reverse) at 25°C for 8 weeks; The calcium carbonate decomposition was observed beneath the colony, (i) *Burgoa anomala* K6421-1-1 (JCM 30265) on PDA at 25°C for 4 weeks, (j) *Cladophialophora* sp. 1 K6203-7-2^T (JCM 28766^T) on PDA at 25°C for 2 weeks/T81203-3-5 (JCM 28763), (k) *Cladophialophora* sp. 3 T7521-8B-3^T (JCM 28749^T) on PDA at 25°C for 2 weeks, (l) *Candida tumulicola* T6517-3-6 (JCM 15400), (m) *Prototheca tumulicola* T6713-13-10^T (JCM 31123^T) on YM at 25°C for 3 days, (n) *Bacillus toyonensis* T5916-8b (JCM 28785); The endospores are observed as a transparent spot in the cell, (o) *Stenotrophomonas tumulicola* T6517-1-2b (JCM 30962), (p) *Gluconacetobacter takamatsuzukensis* T61213-20-1a^T (JCM 19094^T), (q) *Ochrobactrum pituitosum* T6220-2-3b (JCM 28787) on GYC at 25°C for 1 week. Bars = a–m, 10 µm; n–p, 1 µm. For abbreviations of culture media, see An et al. (2009), Kiyuna et al. (2008), and Tazato et al. (2012).

6-1. *Fusarium solani* species complex (FSSC) clade

Members of the genus *Fusarium*, ca. 111 species, are common soil fungi (molds) and plant pathogens. They are saprobes or parasites and are widespread (Domsch et al., 2007; Kirk et al., 2008). The majority of the TT and KT

Fusarium isolates (e.g., Figs. 13 and 14) belong to FSSC clade 3 (O'Donnell, 2000), along with one phylogenetic species (isolates LSP 1-1 and -2) from the Lascaux Cave (Dupont et al., 2007). Members of FSSC clade 3 are well known as plant pathogens and mycotoxigenic saprobes

Fig. 14. Molecular phylogeny placing the major TT/KT fungal isolates and selected reference strains.

The tree was constructed based on the neighbor-joining analysis of 49 LSU gene sequences (417 nt). Numerals in parentheses are DDBJ/GenBank/EMBL accession numbers. Taxon names in bold in shaded boxes indicate the respective major/noteworthy fungal genera and groups; for the higher taxonomic assignments, see Supplementary Materials. Bootstrap percentage values >50% derived from 1000 replications are shown above or below the corresponding branches. Bar indicates 0.05 nucleotide substitutions per site. The initials T and K of isolate numbers indicate isolates from the TT and KT, respectively; isolates TBT-1 and TBK-22 were from the TT and KT, respectively; ■: isolates from the stone chamber interior; ●: isolates from the joint/space of stone walls. Strain with a superscript "T/NT" indicates that derived from the ex-type/ex-neotype, respectively.

mainly occurring in soil. Recently, at least 300 phylogenetically distinct species were estimated (O'Donnell et al., 2015). O'Donnell et al. (2004, 2007) and Zhang et al. (2006) reported that fusaria pathogenic to humans and other animals belong to either the FSSC or FOSC clade. In light of the conclusion by Dupont et al. (2007) regarding the Lascaux Cave, we suggested that most TT and KT fusaria in the FSSC clade 3 were also deeply involved in the deterioration of the murals (Kiyuna et al., 2008). Members of FSSC clade 3 are well known as colonizers of various plants, soil, mycetomas, and other substrates (Domsch et al., 2007; Zhang et al., 2006). Taxonomic and molecular phylogenetic studies of FSSC clade 3 have been performed by numerous authors (e.g., Schroers et al., 2016). A future study is needed to compare the living *Fusarium* isolates between the two tumuli in Japan and the Lascaux Cave in France, and to assign them to a common phylogenetic species in the FSSC.

The metabolic activities of fungi produce various organic acids (e.g., acetic, formic, gluconic, and citric) that are known to cause chemical deterioration of wall paintings (e.g., Gomez-Alarcon and de la Torre, 1994; Sterflinger, 2000). Strains of *Fusarium*, which produce high amounts of acetic acid, significantly decreased the pH of the liquid culture medium (Sano et al., 2010). *Fusarium solani* is known to produce organic acids, including acetic, citric, fumaric, glyoxylic, and oxalic acid (e.g., Sterflinger, 2000). Furthermore, biodeterioration of concrete was shown to be caused by organic acids produced by *Fusarium* sp. (Gu et al., 1998).

6-2. *Trichoderma*

The genus *Trichoderma*, comprising 254 species (Bissett et al., 2015), is one of the most common genera among soil and wood-decaying fungi (molds), and these organisms are widespread (Domsch et al., 2007; Kirk et al., 2008). The TT and KT isolates belonged to three clades (Fig. 14): the Harzianum-Virens clade (Catoptron/Lixii clade), the Viride clade (Rufa clade), and *Trichoderma* sect. *Longibrachiatum* (Chaverri and Samuels, 2003; Samuels, 2006). Most of the isolates (nine TT and four KT) were clustered in the Harzianum-Virens clade. These TT and KT strains were isolated from the stone chamber interior and the adjacent space or the small room (Table 4, Table S2). Members of this clade may cause most of the wall plaster and mural deterioration, particularly in the interior stone chamber of TT. The morphological species *Trichoderma harzianum* (Fig. 13c) was the most commonly encountered *Trichoderma* in direct isolations, but it probably represents multiple phylogenetically distinct species (Kiyuna et al., 2008). *Trichoderma harzianum* is commonly isolated from soil (Domsch et al., 2007) and occurs as a colonizer of diverse plant substrates (Chaverri and Samuels, 2002). Therefore, *Trichoderma* members are considered to be common fungal colonizers of the interior and exterior of both tumuli. *Trichoderma harzianum* is also known to produce acetic, citric, formic, and oxalic acids (Sterflinger, 2000). On the other hand, TT and KT isolates of *T. harzianum* produced small amounts of acetic acid and no formic acid (Sano et al., 2010).

6-3. *Penicillium*: *Penicillium paneum* and *Penicillium* spp.

Penicillium, comprising 354 species, is one of the most common genera in soil, food, and as saprobes in a variety of decaying materials; these organisms are widespread (Kirk et al., 2008; Visagie et al., 2014). Members of this genus are involved in the biodeterioration of cultural properties, such as paintings, throughout the world (e.g., Garg et al., 1995). In Japan, *Penicillium* isolates have been reported in tumuli (Arai, 1984, 1990a; Emoto and Emoto, 1974; Emoto et al., 1983). In most cases, however, the *Penicillium* isolates in these previous reports were identified only at the genera level. Therefore, detailed comparisons of *Penicillium* isolates between the TT, KT, and other tumuli in Japan are impossible because no voucher isolates or specimens were preserved.

Penicillium isolates, which comprised a predominant fungal group, were obtained from samples collected from the stone chamber interior and adjacent spaces or rooms of both tumuli. Of 2502 fungal isolates of the TT and KT, 699 were assignable to *Penicillium*, of which 236 were phenotypically assigned to *Penicillium* sp. 1. Subsequently, our molecular phylogenetic analysis (inferred from the LSU, ITS, β -tubulin, and *lys2* sequences) clearly indicates that the 15 selected *Penicillium* sp. 1 isolates of the TT and KT, as well as the ex-holotype and another strain of *Penicillium paneum* (Fig. 13a), form a monophyletic cluster together with *P. roqueforti* and *P. carneum* in sect. *Roqueforti* (An et al., 2009). The phylogeny also suggests that there are no haplotypes within *Penicillium* sp. 1 isolates. These TT and KT isolates of *Penicillium* sp. 1 were identified and re-described as the known species *P. paneum* Frisvad based on an integrated analysis of phenotypic and genotypic characters by An et al. (2009). As for the closely related species *P. roqueforti*, *P. carneum*, and *P. paneum* in the series *Roqueforti*, Frisvad and Samson (2004) have commented that these three species are predominant in growths on rye bread, blue cheeses, and silage. Members of this series are a special case, growing very well at low pH, often in conjunction with organic acids and high CO₂ content of the atmosphere. O'Brien et al. (2006, 2008) has added baled grass silage as a habitat of *P. paneum*.

These *P. paneum* isolates produce blue-green colored conidial mass and slightly yellowish soluble pigment in agar plates which seem to be involved in the formation of stain spots on the mural paintings and plaster. The finding by An et al. (2009) is the first account of *P. paneum* isolated from samples related to the biodeterioration of cultural properties. This species was consistently isolated from the stone chamber interior before and after the cooling operation began in May 2004. Therefore, the activity of *P. paneum* was not affected by lower temperatures. The latest data referring to the identity of other *Penicillium* TT and KT isolates will be published elsewhere by Kiyuna et al. in the near future.

6-4. *Acremonium* sect. *Gliomastix*

Members (*ca.* 117 species) of the genus *Acremonium* are common soil fungi and plant pathogens, growing on decaying materials as saprobes or parasites (Domsch et al., 2007; Gams, 1971; Kirk et al., 2008).

A total of 74 representative isolates assignable to dark *Acremonium* (viz., *Acremonium* sect. *Gliomastix*) were isolated from the TT and KT stone chamber interior and exterior and were culturally and morphologically characterized (Kiyuna et al., 2011). Using integrated phenotypic and genotypic analyses, these isolates were identified as taxa of the series *Murorum* validated by Kiyuna et al. (2011) in sect. *Gliomastix*, including *Acremonium masseei*, *A. murorum*, *A. felinum* comb. nov., *A. polychromum*, and *A. tumulicola* sp. nov., typified by the ex-type strain K6613-2 (JCM 17184) (Kiyuna et al., 2011; cf., Fig. 14). Recently, draft genome sequences of *A. tumulicola* K6613-2 (JCM 17184) were determined and released on the JCM web site: <http://www.jcm.riken.jp/cgi-bin/nbrp/nbrp_list.cgi>. In this study, *A. masseei* was isolated only from the stone chamber interior of both tumuli (Fig. 13d). However, this species was not detected in exterior samples of either the TT or KT.

In Europe, fungal biodeterioration is well known to affect items of cultural significance, such as murals (Berner et al., 1997; Dhawan et al., 1993; Guglielminetti et al., 1994; Karbowska-Berent, 2003). The role of fungi in the deterioration of murals and their decay mechanisms have been reviewed by Garg et al. (1995) and Caneva et al. (2008). Black stains (or spots) caused by dematiaceous anamorphic fungi (e.g., *Cladosporium*) on mural paintings often causes problems in their conservation (Arai et al., 1991; Caneva et al., 2008; Ciferri, 1999). Fungal stains (or spots) are primarily caused by the secretion of metabolites or pigmentation of the fungi, especially melanin (Diakumaku et al., 1995; Nieto-Fernández et al., 2003; Saiz-Jiménez, 1995). Even after the fungus is dead, the pigmented cell walls remain on the surface of the substratum. These substances are particularly resistant to chemical and enzymatic degradation (Nieto-Fernández et al., 2003). Species of dark *Acremonium* or *Gliomastix* have been implicated in the biodeterioration of wall paintings by several researchers. Examples include the cave wall paintings of Lascaux, France (Oriol and Mertz, 2006; Oriol et al., 2009), indoor mural paintings in Europe (Nugari et al., 1993), Ajanta wall paintings in India (Dhawan et al., 1993), and Ozuka Tumulus paintings in Japan (Emoto and Emoto, 1974).

In 2001, the white molds growing on the prehistoric paintings in the Lascaux Cave in France were identified as members of the FSSC clade (Dupont et al., 2007; Oriol and Mertz, 2006). The following year, an emergence of the black mold *Gliomastix murorum* (sic) was reported by Oriol and Mertz (2006) and Oriol et al. (2009). In our study, a few isolates of *A. murorum* were isolated from the stone chamber interiors of both tumuli.

A few species of dark *Acremonium* sect. *Gliomastix* were detected and recorded before September 2005. However, starting in May 2006, the temperature of the stone chamber interior was maintained at approximately 10°C, and the growth of the dark *Acremonium* sect. *Gliomastix* (*A. masseei*) drastically increased on the mural of the stone chamber interior. This outbreak of dark *Acremonium* is considered to have been promoted by relationships with other microbes and environmental conditions, such as the drop in temperature.

6-5. Yeast taxa

Information regarding the habitat and sources of yeast isolates is available from Kurtzman et al. (2011) and from the lists or catalogues of the respective public culture collections.

The predominant species in the TT stone chamber interior were *Yamadazyma tumulicola* and *Y. takamatsuzukensis*, which we initially described as the novel species *Candida tumulicola* and *C. takamatsuzukensis* (Nagatsuka et al., 2009) before reclassification to the genus *Yamadazyma* as new combinations (Nagatsuka et al., 2016; cf., Fig. 10). *Yamadazyma olivae* and *Y. kitorensis*, which we proposed as new combinations and new species (Nagatsuka et al., 2016; cf., Fig. 10), were the predominant species in the KT stone chamber interior. These four *Yamadazyma* species were frequently isolated from viscous gel (biofilm) samples collected inside the stone chambers. The minority yeast isolates *Zygoascus polysorbophila* from the TT, and *Z. biomembranicola* from KT, were also isolated from biofilm samples collected in the stone chamber interior. We proposed the new combination and new species, respectively, for these yeast (Nagatsuka et al., 2016). These four *Yamadazyma* species and two *Zygoascus* species formed abundant hyphae and pseudohyphae (Nagatsuka et al., 2009, 2016). Multiple medical studies have documented the close relationships between *Candida albicans* biofilm development and hyphal formation with extracellular substances (Hawser and Douglas, 1994; Nett et al., 2010). The hyphae and pseudohyphae of these isolates may also have played important roles as substructures in the developing biofilm in the TT and KT, leading to the observed contamination.

Ethanol and isopropanol were used as mild disinfectants on the mural pigments; however, these alcohols may have been diluted sufficiently to facilitate their use as a carbon source by microorganisms in the stone chamber interiors of the TT and KT (Ishizaki and Kigawa, 2011). The previously mentioned four *Yamadazyma* species and two *Zygoascus* species, in addition to *P. membranifaciens* T4716-3 and *Meyerozyma guilliermondii* T6517-3-4, were found to utilize ethanol (approx. 0.5% and 1%, v/v) but not isopropanol as a single carbon source (Kigawa et al., 2010b; Nagatsuka et al., 2016). *Y. takamatsuzukensis* T4922-1-1^T (JCM 15410^T), *Y. tumulicola* T6517-9-5^T (JCM 15403^T), *Y. olivae* K5916-7-4 (JCM 30999), *P. membranifaciens* T4716-3, and *M. guilliermondii* T6517-3-4 were tested for their ability to produce acetic acid, and formic acid (Sano et al., 2010). Yeast isolates were cultured in Czapek broth originally containing sucrose as the sole carbon source at 25°C for three or four days on a reciprocal shaker. The yeast isolates produced acetic acid and/or formic acid, and *P. membranifaciens* T4716-3 produced rather strong acetic acids that decreased the pH of the TT plaster. The production of acetic acids by *P. membranifaciens* T4716-3 in Czapek broth containing ethanol instead of sucrose was found to be about three times that in standard Czapek broth containing sucrose. On the other hand, *P. membranifaciens* T4716-3 showed no growth and no production of carboxylic acids in Czapek broth containing isopropanol as a sole carbon source. From these results, isopropanol was concluded to be better as a

disinfectant against the yeasts composing these biofilms. The CaCO₃ solubilization test on GYC agar plates of selected TT and KT isolates showed that CaCO₃ was clearly dissolubilized by *M. guilliermondii* T6517-3-4 and *Y. takamatsuzukensis* T4922-1-1^T (JCM 15410^T) and slightly dissolubilized by *P. membranifaciens* T4716-3 (Sano et al., 2010). These results suggest that the yeast in the biofilms played an important role in the deterioration of the plaster walls in the moist stone chamber interior.

6-6. *Bacillus*

The firmicute genus *Bacillus* comprises over 220 species of gram-positive, commonly endospore-forming bacteria. *Bacillus* is one of the most common bacterial genera in soil and water (fresh and sea water) and is found in decaying materials (saprophytes) (Logan and De Vos, 2009). A few species are known to be pathogenic, causing illnesses, such as anthrax (*B. anthracis*), food-borne illness and opportunistic infections (*B. cereus*), and insect diseases (*B. thuringiensis*) (Logan and De Vos, 2009). In the world of conservation science, members of the genus *Bacillus* are thought to be involved in biomineralization (Cañaveras et al., 1999; Cuezva et al., 2012; Laiz et al., 1999).

Bacillus species were the most predominant bacteria isolated from the TT and KT samples. A total of 78 isolates were obtained from both tumuli. The original sources of the isolates were viscous gels or biofilms on the wall surface inside the stone chamber. Thus, *Bacillus* is thought to be one of the predominant components of such biofilms. These isolates were categorized into several groups based on their 16S rRNA gene sequence divergence. The top two groups were *B. cereus* which included *B. thuringiensis*, *B. toyonensis*, *B. pseudomyoides*, and three unknown species in this group, and *B. simplex* which included *B. butanolivorans* (see Supplementary Materials 1, Handa et al., 2017). Interestingly, nearly all of the *B. cereus* group strains were isolated from the TT stone chamber interior. However, the *B. simplex* group was only isolated from the TT stone chamber exterior, although it was a predominant isolate from the KT stone chamber interior. *B. simplex* group strains were isolated from TT samples collected between the wall stone or space and from the stone chamber exterior during the dismantling work (Sugiyama et al., 2009). It was suggested that the *B. simplex* group isolates had not adapted to grow in the cooled TT stone chamber. The partial DNA gyrase B subunit gene (*gyrB* gene) sequence-based phylogenetic analysis was performed as a high resolution approach to distinguish *B. cereus* and *B. simplex* group isolates (Handa et al., 2017). Especially, the *B. cereus* group includes pathogenic bacteria for humans, such as *B. cereus*, *B. anthracis*, and *B. weihenstephanensis*. Most of isolates in the *B. cereus* group were not clinical species except one isolate belonged to *B. cereus* (Handa et al., 2017). On the other hand, *B. muralis*, which is closely related to *B. simplex*, was isolated from a deteriorated mural painting (Heyrman et al., 2005). Based on the results of the phylogenetic analysis of the *gyrB* gene sequence, all of our *B. simplex* group isolates, except strain T81203-9-1b (JCM 28850), were tentatively identified as *B. simplex* (Handa et al., 2017).

Bacillus cereus group strains were also isolated or detected in samples from the Altamira Cave in Spain (Laiz et al., 1999), the Kartchner Caverns in the USA (Ikner et al., 2007), and the Mogao Grottoes in China (Ma et al., 2015). In caves, many bacteria (particularly actinobacteria) cause CaCO₃ precipitation (Cañaveras et al., 2006; Cuezva et al., 2009, 2012; Diaz-Herrera et al., 2013). Some *Bacillus* spp. are thought to be involved in CaCO₃ precipitation along with these actinobacteria (Cañaveras et al., 1999; Cuezva et al., 2012; Laiz et al., 1999).

Recently, *Bacillus* and *Sporosarcina* strains causing CaCO₃ precipitation were isolated from concrete; one of which was closely related to *B. cereus* (Kim et al., 2016). Our isolates did not show CaCO₃ decomposition or precipitation on GYC medium (Kigawa et al., unpubl.), but two isolates produced acetic acid and decreased the pH of the culture broth (*B. toyonensis* T5916-8-1b [JCM 28785] and *Bacillus simplex* K6203-10-3b [JCM 28856]) (Sano et al., 2010). Therefore, these *Bacillus* isolates might play a role in the biodeterioration of plaster. Furthermore, isolates T5916-8-1b and K6203-10-3b were shown to utilize hydroxyl propyl cellulose and methyl cellulose, compounds used as consolidants in mural paintings (Kigawa et al., 2012a). The utilization of ethanol or isopropanol as a carbon source was tested for isolates T5916-8-1b and K6203-10-3b (Kigawa et al., 2010b). These organic solvents were used as fungicides, as they were thought to be mild compounds that would not affect the paints or humans but were still effective for removing fungal mycelia. Tested strains showed growth in a 1% concentration (V/V) solvent containing broth medium. Thus, when these solvents were applied as fungicides, at least some bacilli could grow if the solution was sufficiently diluted. Isolate K6203-10-3b was also tested for its response to treatment with sodium hypochlorite (NaClO). This strain showed no growth when exposed to 100 ppm NaClO for 5 min (Kigawa et al., 2010c).

After relocation of the mural paintings of the KT, the stone chamber was intermittently exposed to UV irradiation to control microbial activity (Kigawa et al., 2010c). Two isolates, *Bacillus simplex* K6203-10-3b (JCM 28856; isolated before UV irradiation) and K101008-7-3b (JCM 28869; isolated after UV irradiation), were tested for resistance to UV irradiation (Kigawa et al., 2013b). Although most strains did not show a noticeable tolerance to UV irradiation, the isolate that was obtained after UV irradiation grew slightly after 2 min of UV exposure (Kigawa et al., 2013b). Because *Bacillus* species are spore-forming bacteria (Fig. 13), they can survive even when the environmental conditions are not suitable for their growth. According to Riesenman and Nicholson (2000), the spore inner coat layer plays a role in spore resistance to environmentally relevant UV wavelengths. It is thought that some *Bacillus* members survived inside the stone chamber despite UV irradiation.

6-7. *Stenotrophomonas*

The proteobacterium genus *Stenotrophomonas*, comprising at least 11 species (Euzéby, 1997), is common in the environment and associates closely with plants (Ryan et al., 2009). *Stenotrophomonas maltophilia* is found in a

Fig. 15. Molecular phylogeny placing *Stenotrophomonas* isolates and their related clones obtained from the Lascaux Cave and isolates of the Altamira Cave and Collembola.

The tree was constructed based on the neighbor-joining analysis of partial 16S rRNA gene sequences (731 nt). Bootstrap percentage values >50% derived from 1000 replications are shown above or below the corresponding branches. *Escherichia coli* was used as an outgroup. Bar indicates 0.02 nucleotide substitutions per site. The initials T and K of isolate numbers indicate isolates from the TT and KT, respectively. Strain with a superscript “T” indicates the type strain.

variety of environments and is frequently isolated from clinical samples and nosocomial infections (Palleroni, 2005).

In our preliminary report, *Stenotrophomonas* species were isolated as one of the major taxa from the wall surface of the TT stone chamber interior (Sugiyama et al., 2009). In particular, they were obtained from viscous gel (biofilm)-like samples that were collected from September 2005 to April 2007. Finally, 51 *Stenotrophomonas* isolates in total were obtained from both the TT and KT. These isolates were identified as belonging to 4 groups, based on 16S rRNA gene sequences analysis (Fig. 15). One of these isolates (the major group of the TT) was proposed to be a novel species, *S. tumulicola* (Fig. 13o), with type strain T5916-2-1b^T (JCM 30961^T) (Handa et al., 2016). This novel species was closely related to *S. chelatiphaga* (Kaparullina et al., 2009) (Fig. 15). The second major group, but only isolated from the KT samples of *Stenotrophomonas* isolates, was *S. rhizophila*, and the third

one was related to *S. maltophilia*, which was isolated from both tumuli as a minor group. *S. maltophilia* is usually isolated from clinical samples. However, this bacterium has been isolated previously from cave samples, particularly from “show caves”, which are those accessible to the public (e.g., Bastian et al., 2010; Garcia-Anton et al., 2014). With many visitors entering a cave, microorganisms may be introduced into its interior. The invasion of such clinical bacteria from water, insects, roots, or human shoes with attached soil, has been suggested by Jurado et al. (2014). In the case of the TT and KT stone chambers, two strains of *S. maltophilia* have been isolated and identified by Handa et al. (2016). Additionally, two isolates, *S. maltophilia* T7214-8-1b and T6713-10-4b, were closely related to the Lascaux Cave clones and an Altamira Cave isolate (Fig. 15). In the Lascaux Cave, clones of *S. maltophilia* represented 18.1% of the total (Martin-Sanchez and Saiz-Jiménez, 2014). Interestingly, three *S. maltophilia*-like strains were isolated from the gut and

feces of collembolans, *Folsomia candida* (Hoffmann et al., 1998). The temperatures enabling growth of *S. rhizophila* (4–37°C) and *S. tumulicola* (10–37°C) were lower than that for *S. chelatiphaga* (25–40°C) (Handa et al., 2016; Kaparullina et al., 2009; Wolf et al., 2002), suggesting that *S. rhizophila* and *S. tumulicola*, but not *S. chelatiphaga*, could grow inside the chamber of both tumuli. The isolates, *S. tumulicola* T5916-2-1b^T (JCM 30961^T) and *S. rhizophila* K5916-3-1b produced acetic acid and caused a slight pH decrease of the culture broth, and the latter strain also produced formic acid (Sano et al., 2010). These results suggest that the isolates caused severe damage to the plaster walls. Furthermore, the *S. rhizophila* strains, K5916-3-1b and K6613-3b, were able to utilize hydroxyl propyl cellulose and methyl cellulose, which were used as consolidants of the mural paintings (Kigawa et al., 2012a).

Next, *S. tumulicola* T5916-2-1b^T and *S. rhizophila* K5916-3-1b were tested for the ability to utilize ethanol or isopropanol as a carbon source. Tested isolates grew in a 1% concentration (V/V) solvent containing broth medium. Isolate K5916-3-1b did not grow after exposure to 100 ppm of NaClO for 5 min (Kigawa et al., 2010c).

Three isolates, K5916-3-1b (before UV irradiation), K101008-2-1b (1 year after UV irradiation began), and K111013-5-1b (2 years after UV irradiation began), were tested for resistance to UV irradiation (Kigawa et al., 2013b). These isolates did not grow under UV irradiation (Kigawa et al., 2013b).

6-8. *Ochrobactrum*

The genus *Ochrobactrum* belongs to the order *Rhizobiales* in the class *Alphaproteobacteria*. The rhizobial genus *Ochrobactrum* (*Brucellaceae*) comprises 18 species that are isolated mainly from the environment (e.g., soil, water, sludge, plants, or rhizospheres), animals, or human clinical samples (Kämpfer et al., 2014). The isolation of *O. pseudogrignonense* has been recorded from an Altamira Cave sample by Jurado et al. (2014).

Samples of the TT stone chamber interior, and from the dismantling work from September 2005 to April 2007, yielded 17 *Ochrobactrum* isolates. These isolates were classified into 2 groups based on 16S rRNA gene sequence analysis (Handa et al., 2017). One group was closely related to *O. pecoris* (Kämpfer et al., 2011). This group contained the only two isolates from samples collected when the cooling of the chamber began, and seemed to be a novel species in the genus *Ochrobactrum* as determined by *groEL* and *gyrB* gene sequence analyses (Handa et al., 2017). The other 15 isolates were tentatively identified as *O. pituitosum* (Huber et al., 2010) based on 16S rRNA, *groEL*, and *gyrB* gene sequence analyses (Handa et al., 2017).

Nearly all of the TT isolates were obtained from samples collected when the stone chamber interior was kept at a stable, cool temperature, except for two isolates of novel species. The mean temperature inside the chamber was around 10°C (Inuzuka and Ishizaki, 2007). This data suggests that the *Ochrobactrum* isolates grow at lower temperatures than the other species. Thus, no isolates of the genus *Ochrobactrum* were obtained from KT samples that were not kept in the cooler environment. The isolate

O. pituitosum T6220-2-3b (JCM 28787) formed a gruel colony when grown on GYC plates (Fig. 13q), but it did not decompose CaCO₃. However, this isolate produced small amounts of acetic acid, thereby lowering the medium pH (Sano et al., 2010). Most of the samples were collected from the stone wall surface and from gel-like biofilms. These results suggest that the isolates played a large role in the plaster surface deterioration and the production of biofilms.

6-9. Actinobacterial genera *Microbacterium*, *Promicromonospora*, and *Streptomyces*

The genera *Microbacterium*, *Promicromonospora*, and *Streptomyces* are in the class *Actinobacteria* (phylum “*Actinobacteria*”). *Microbacterium* (*Microbacteriaceae*, *Actinomycetales*) comprises 98 species mainly isolated from soil, water (fresh and sea water), the plant phyllosphere, and rhizosphere (Euzéby, 1997 <<http://www.bacterio.net>>; Whitman et al., 2012). The genus *Promicromonospora* (*Promicromonosporaceae*, *Micrococcaceae*) comprises 12 non-pathogenic species found mainly in soil and air (Schumann and Stackebrandt, 2012). The genus *Streptomyces* (*Streptomycetaceae*, *Actinomycetales*) includes more than 780 species (Euzéby, 1997 <<http://www.bacterio.net>>; Whitman et al., 2012) found in various environments including soil, fresh water, and seawater. A few species are known plant pathogens, and many species are known to produce bioactive substances.

In the survey of biodeterioration caused by bacteria, 64 strains of actinobacteria were isolated from the TT, and 36 strains were isolated from the KT. These isolates represented 26.4% of the 242 TT isolates and 21.6% of the 164 KT isolates. The major taxa of the actinobacterial isolates were the genera *Streptomyces*, *Microbacterium*, and *Promicromonospora* (Fig. S7). The genus *Rhodococcus* was also major for the TT but not the KT. On the other hand, the genus *Herbiconiux* was only isolated from the KT. Several studies reported that *Actinobacteria* members cause biodeterioration of mural paintings in caves (Adetutu and Ball, 2014; Cuezva et al., 2012; Diaz-Herraiz et al., 2013; Urzi et al., 2010). The major taxa of actinobacterial colonizers in these caves belonged to the genera *Streptomyces*, *Nocardia*, and *Pseudonocardia*. *Actinobacteria* are reported to play an important role in biodeterioration inside caves, including mineral biodeterioration and bioprecipitation (Abdulla, 2009; Cuezva et al., 2012; Diaz-Herraiz et al., 2013). CaCO₃ deposits and calcite crystal products are thought to result from the activities of these actinobacteria in caves (Cañaveras et al., 2006; Cuezva et al., 2009, 2012; Diaz-Herraiz et al., 2013). Both the TT and KT mural paintings were painted directly onto the thin layer of plaster that contained CaCO₃ as one of the major components (Ishizaki and Kigawa, 2011; Kitada et al., 2015). Some samples collected from both tumuli contained white powdery or pigmented substances. Some parts of the plaster from the KT stone chamber seemed hollow or decomposed (Kigawa et al., 2008b, 2009b). This damage to the plaster might have been caused by microbial activity on the plaster surface. After the outbreak of fungi, diverse microorganisms, such as fungi, yeasts, and bacte-

ria, including actinobacteria, were present on the plaster surface.

Some species of *Nocardia*, *Pseudonocardia*, and *Rhodococcus* are known to be pathogenic or opportunistic pathogens. Such bacteria also have been detected in cave samples (Jurado et al., 2010), and it was thought that humans may have introduced these bacteria into the cave. No pathogenic *Nocardia* or *Pseudonocardia* members were detected in the KT samples. However, three isolates from the TT samples were closely related to *Nocardia nova*, which is designated to be risk group 2 (classification according to German TRBA, URL: <http://www.baua.de/n_n_15226/de/Themen-von-A-Z/Biologische-Arbeitsstoffe/TRBA/pdf/TRBA-466.pdf>) or biosafety level 2 (BSL, <http://jsbac.org/archive/img/bsl_level.pdf>). These isolates were obtained from the burial mound soil during the dismantling work. Isolates belonging to the genus *Rhodococcus* were obtained only from TT samples that were collected from the stone chamber interior and during the dismantling work. These isolates were categorized into two groups based on their 16S rRNA gene sequence divergence (Fig. S8). All *Rhodococcus* isolates from the TT were non-pathogenic species.

Most of the actinobacterial isolates from both tumuli were of the genus *Streptomyces*, and they showed nearly identical 16S rRNA gene sequences (Fig. S8a). This observation suggests that the species of the genus *Streptomyces* inhabited the area around both tumuli in Asuka village as ubiquitous actinobacteria. By the same token, the genera *Microbacterium* and *Promicromonospora* are also thought to be ubiquitous actinobacteria around both tumuli. Some of the microbacterial strains were isolated from samples collected during the dismantling of the TT stone chamber. They exhibited the same phylogenetic position as those strains isolated from the stone chamber interior before the dismantling work (Fig. S8b). This data suggests that the bacteria invaded the stone chamber interior from the surrounding soil. The second major taxon of actinobacteria isolates from TT samples is the genus *Microbacterium* (Fig. S8b). A total of 23 *Microbacterium* strains were isolated from both tumuli: 16 strains from the TT and 7 strains from the KT. These isolates were separated into 10 groups based on the 16S rRNA gene sequence divergence (Fig. S8b). One of these isolates, T7528-3-6b^T (JCM 28836^T), was proposed to be a novel species in the genus, designated '*Microbacterium tumbae*' by Nishijima et al. (2017b). The isolate *M. shaanxiense* T6220-7-3b (JCM 28790) could not utilize ethanol or isopropanol as carbon sources (Kigawa et al., 2010b), so ethanol or isopropanol might be effective for removing these microbacteria. Isolate *Microbacterium* sp. K6303-8-2b was observed to utilize hydroxyl propyl cellulose and methyl cellulose (Kigawa et al., 2012a).

Promicromonospora species were also isolated from both tumuli (Fig. S8b). And this bacterium was also detected by culture-independent (molecular) methods from both tumuli (see Section 9). One of these, strain T6220-5-2b^T (JCM 28789^T), was isolated as a novel lineage of the family *Promicromonosporaceae*. We propose that it is a novel genus and species in the family

Promicromonosporaceae, *Krasilnikovella mularis* (Nishijima et al., 2017a). As of 2016, the genus *Promicromonospora* comprises 12 species (Euzéby, 1997 <<http://www.bacterio.net>>). Most of these species were isolated from soil, the rhizosphere, or the surface of plants. *P. vindobonensis* and *P. aerolata* were isolated from indoor air samples (Busse et al., 2003), and *P. umidemergens* (Martin et al., 2010) was isolated from indoor moisture with mold. Recently, *P. alba* was isolated from ant cuticles (Guo et al., 2016). Thus, *Promicromonospora* species are ubiquitous actinomycetes in the soil environment and are distributed by air, water, molds, and insects. Our isolates were obtained from the surfaces of mural paintings as contaminant microorganisms. They may have inhabited the soil surrounding the stone chamber, invading the mural paintings via roots, water, insects, or molds.

The survey of microorganisms in the KT stone chamber interior after UV irradiation indicated that the growth of fungal mycelia was suppressed and bacterial growth decreased. The tolerance to UV irradiation was examined for three actinobacterial KT isolates, namely *Streptomyces* sp. K101008-5-3b and two *Promicromonospora* isolates, *Promicromonospora* sp. K111013-1-1b and K111013-4-2b (Kigawa et al., 2013b). These isolates were obtained from the samples collected after UV irradiation. However, the tested actinobacteria exhibited no resistance to UV irradiation (Kigawa et al., 2013b).

7. Identity of the Minority, but Noteworthy, Fungi and Bacteria in the Stone Chamber Interiors of Both Tumuli, and Their Involvement in Biodeterioration

7-1. *Kendrickiella phycomyces*

The genus *Kendrickiella*, which is currently treated as incertae sedis in the Pezizomycotina, Ascomycota, is monotypic with the type species *K. phycomyces* as its sole member (Kiyuna et al., 2012). It inhabits mainly wood, polypores, and soil and is distributed throughout Africa, Europe, and South America (Seifert et al., 2011).

A total of 31 isolates, classified as "bristle-like fungal colonizers", were collected from the stone chamber interior and exterior of the KT and TT. These isolates were identified as the anamorphic fungal species *K. phycomyces* based on molecular phylogenetic placement and its cultural and morphological characteristics (Fig. 13g; Kiyuna et al., 2012). This fungus constitutes a new addition to the mycobiota (fungal inventory) of Japan. In addition, this finding is the first case in which *K. phycomyces* was isolated from biodeteriorated stone, plaster walls, and murals in a tumulus. Our isolates, represented by *K. phycomyces* T61114-1-1 (JCM18031) and K5906-1-1 (JCM 18028), were inoculated on GYC agar plates and the cultural characteristics of CaCO₃ solubilization were observed (Kiyuna et al., 2012, Fig. 13g). In the liquid medium of *K. phycomyces* K5906-1-1 (JCM 18028), a significant pH decrease was observed (Sano et al., 2010). In another study, significant citric acid production by *K. phycomyces* was observed (Sano et al., unpubl.). It is thought, therefore, that the cause of its pH decrease is citric acid production. These physiological characteristics suggest that *K. phycomyces* may have been involved in

the deterioration of the plaster walls of the TT stone chamber interior.

Recently, draft genome sequences of *K. phycomyces* K4910-1 (JCM 18027) have been determined and released on the JCM web site; <http://www.jcm.riken.jp/cgi-bin/nbrp/nbrp_list.cgi>.

7-2. *Burgoa anomala*

The basidiomycete anamorph-genus *Burgoa* (class Agaricomycetes, order Cantharellales) comprises nine species, which mainly inhabit wood. These species are widely distributed throughout the northern temperate zone (Seifert et al., 2011).

A total of 20 representative isolates, denoted as “black particles” (*Kuro-tsubu* in Japanese), were isolated from samples collected over a period of six years from the KT stone chamber interior walls, including the star chart painting on the ceiling (Fig. 13i; Kiyuna et al., 2015). These isolates represented by K6421-1-1 (JCM 30265) and K7316-1-1 (JCM 30266) were identified as *Burgoa anomala* ($\equiv$ *Papulaspora anomala*) based on morphological characteristics and molecular phylogenetic assignment (Kiyuna et al., 2015). According to Goidànich (1937) and Hotson (1912), *Burgoa anomala* has been isolated from plant substrates. Therefore, this is the first record of isolation of *B. anomala* from biodeteriorated cultural heritage materials. Furthermore, this finding is noted as the first inventory record of *B. anomala* from Japan.

7-3. *Cephalotrichum* (syn. *Doratomyces*)

The dematiaceous hyphomycete genus *Doratomyces* comprises eight species, found on herbaceous stems, dead wood, dung, and soil, mainly in Europe and North America (Ellis, 1971, 1997; Sandoval-Denis et al., 2016). Taxonomically, this genus is heterotypically synonymous with *Cephalotrichum* Link (Hughes, 1958), but it is generally cited as *Doratomyces* Corda in the literature from the field of conservation science (Seifert et al., 2011). Phylogenetically, *Doratomyces* (e.g., *D. stemonitis*) is related to members of the order Microascales in Sordariomycetes (Zhang et al., 2006). The name *Cephalotrichum* is hereafter used instead of *Doratomyces*.

As shown in Table 4, only one strain (T7530-12-1 = JCM 28755) of *Cephalotrichum verrucisporum* ($\equiv$ *Doratomyces verrucisporus*) was isolated from the TT stone chamber; this isolate came from plaster pieces and soil/plant root mixtures in the joint spaces of stone walls sampled during the dismantling of the TT (Fig. 13f; Kiyuna et al., 2017). Interestingly, *Doratomyces* (*sic*) was recorded as a predominant fungal genus detected inside the stone chamber in the 1980s (Arai, 1984, 1987, 1990b). As briefly mentioned in Subsection 2-1, in those days, *Doratomyces* growth was rampant inside the stone chamber. The first finding of this genus was March 1975, about three years after the excavation of the tumulus, with samples found at fixed spots of the east wall (beside *Seiryu*) and west wall (the lower part of *Byakko*). This fungus was isolated from samples taken by the uncommon method of collection using sterile moist cotton swabs. These colonies were invisible to the naked eye (Arai, 1987, pers. comm.). In October 1976, *Doratomyces*, along with *Streptomyces*, was also

isolated at the same spot. In February and November 1980, *Doratomyces* (Fig. 13f; also see photographs 1–4 and figures 22–27 in Arai, 1987 and 1990b, respectively) was isolated from other spots, including the west wall (upper part of *Byakko*), north wall (*Genbu*), and others. In June 1981 (after nine years of excavation), *Doratomyces*, together with *Streptomyces*, was isolated from all the samples taken at the upper-right part of *Genbu* and the central part of the ceiling, in addition to the fixed spots (Arai, 1987; Arai, pers. comm.). At that time, the entire stone chamber interior had turned black (Arai, pers. comm.). Details on paraformaldehyde fumigation as a preventive measure performed between 1981 and 1986 have been noted in Arai (1987, 1990b). In July 1986, it was confirmed that all of the *Doratomyces* inside the stone chamber had been killed (Arai, pers. comm.).

Unfortunately, no voucher isolates or specimens from that time were preserved. Recently, two *Doratomyces* (*sic*) species have been reported from cave environments in the Altamira Cave in Spain, including the wall and ceiling, rodent feces, and rhizomorphs. However, these isolates were not discussed as a threat to conservation of the murals of the Altamira Cave (Novakova et al., 2014). Our *Doratomyces* (*sic*) isolate (Kiyuna et al., 2017) is considered to be the same species as that reported by Arai (1987, 1990b) and is presumably assignable to one of the dematiaceous (or dark) fungi surviving since the 1970s. To date, *Cephalotrichum verrucisporum* has been recorded from mountain soil (Jiang and Zhang, 2008), sand dune soil (Sandoval-Denis et al., 2016), and soil highly contaminated by PCBs (Mouhamadou et al., 2013). The TT isolate *C. verrucisporum* T7530-12-1 (JCM 28755) is presumed to have originated in soil. Therefore, this finding is the first reliable report of *C. verrucisporum* from a biodeteriorated stone wall in a special historic site and is a new addition to the fungal inventory in Japan.

7-4. *Sagenomella*

The 11 species of the trichocomaceous genus *Sagenomella* (Eurotiales) inhabit soil, peat, and dung, are found on plants and man, and are widespread in the Northern hemisphere (Samson et al., 2011; Seifert et al., 2011).

Four isolates, represented by isolate T7521-8D-1 (JCM 28750) of *Sagenomella striatispora* (Fig. 13h) came from samples collected during the dismantling of the TT stone chamber, and one *S. griseoviridis* isolate TBK-20 (JCM 15364), which was initially assignable to *Penicillium* sp. in Kiyuna et al. (2008), derived from the stone surface of KT (Kiyuna et al., 2017). We observed conidia characteristics of *S. striatispora* in many samples collected from the joint spaces between stone walls, suggesting that *S. striatispora* was a common fungus of the TT stone chamber interior. These isolates were inoculated onto GYC agar plates, and physiological characteristics, such as CaCO₃ solubilization, were observed along with those of *Kendrickiella phycomyces* (Kiyuna et al., 2012). In physiological studies of *Sagenomella striatispora* T7521-8D-1, the production of various organic acids (acetic, malic, lactic, succinic, and formic) was observed, together with a large decrease in the pH of the liquid media (Sano et al., unpubl.). These results suggest that *Sagenomella* species

may be involved in the deterioration of plaster walls of the TT stone chamber interior. This report is the first to associate *Sagenomella* species with the biodeterioration of cultural properties.

7-5. *Cladophialophora* and chaetothyrialean black fungi

The 22 known species of the chaetothyrialean genus *Cladophialophora* are parasitic on humans, other animals, and plants, and saprophytic in soil and juice; these species are cosmopolitan (Seifert et al., 2011).

While dismantling and relocating the TT stone chamber, many *Cladophialophora* and chaetothyrialean black fungi, including *Exophiala* and *Phialophora*, were isolated in the joint spaces between the stone walls. Furthermore, since the start of UV irradiation of the KT stone chamber in 2009, these black fungi were notably isolated from the stone walls. Recently, *Cladophialophora* and chaetothyrialean black fungi have been found in various rock substrates (e.g., Badali et al., 2008). *Cladophialophora* and chaetothyrialean black fungi have not been observed in biodeteriorated samples outside of Japan; therefore, our finding is the first to be reported.

Molecular phylogenetic analyses revealed that the isolates of *Cladophialophora* and chaetothyrialean black fungi differed between the two tumuli. In our ongoing study, two new species of *Cladophialophora* will be described elsewhere by Kiyuna et al. in the near future: *Cladophialophora* sp. 1 (Fig. 13j) from the viscous gels and various substrates on the stone walls of the TT and KT, and *Cladophialophora* sp. 3 (Fig. 13k) from black substances on the plastic cover over the thieving hole, soil, and plaster pieces in the TT.

7-6. *Prototheca*, an achlorophyllous, yeast-like microalga

The achlorophyllous algal genus *Prototheca* comprises eight species and is ubiquitous in nature (Pore, 2011). Some strains of *Prototheca* (e.g., *P. zopfii*, *P. wickerhamii*, *P. cutis*, and *P. miyajii*) have been reported as agents of infections (protothecosis) in humans and animals (Masuda et al., 2016; Pore, 2011; Satoh et al., 2010). Other strains have been isolated as saprobes from the natural environment, and whether they are pathogenic is unknown (e.g., *P. stagnora* and *P. ulmea*) (Pore, 2011).

Two achlorophyllous microalgal isolates of *Prototheca* were among the minority yeast-like strains (Nagatsuka et al., 2017; Figs. 6, 10, and 13m). These isolates originated in the soil and in a white moldy colony collected inside the TT stone chamber. Morphological and other phenotypic and genotypic (molecular) evidence indicates that these two isolates are a novel species of the Chlorellaceae genus *Prototheca* (Trebouxiophyceae, Chlorophyta). From the available data, Nagatsuka et al. (2017) proposed the name *Prototheca tumulicola* based on the two isolates T6713-13-10^T (JCM 31123^T) (Fig. 13m) and T61213-7-11 (JCM 31124). Many studies report that *Chlorella* is involved in the biodeterioration of cultural assets (Dakal and Cameotra, 2012; Macedo et al., 2009). However, reports regarding an association between the biodeterioration of cultural assets and *Prototheca* are rare. The colonies of two of the isolates, *P. tumulicola* T6713-13-10^T and

T61213-7-11, were very slimy (Fig. 13m), and the cells and sporangia in both isolates were surrounded by capsules. Whether extracellular substances of these isolates participated in biofilm formation is an interesting question.

7-7. *Bordetella*

The proteobacterium genus *Bordetella* comprises nine species, most of which are pathogens of humans (*B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*), other mammals, and birds (*B. hinzii* and *B. avium*); the exception is *B. petrii* in the environment (von Wintzingerode et al., 2001).

Since 2006, when the stone chamber was kept at a stable 10°C and an RH of 97% (Inuzuka and Ishizaki, 2007), ten *Bordetella* strains were isolated from a large number of samples from the TT stone chamber. Most of the strains were isolated from the surface of the mural paintings (Tazato et al., 2015). Our ten isolates are closely related to *B. petrii* at the molecular and phylogenetic level. Based on the taxonomic characterization of the isolates, we proposed three novel species in the genus *Bordetella*: *B. muralis*, *B. tumulicola*, and *B. tumbae* (Tazato et al., 2015).

As of April 2016, eleven species of the genus *Bordetella* have been reported (Fig. S9) with *B. pertussis* as the type species. Most of these species are known to be pathogenic, since they were isolated from human and animals (e.g., Sanden and Weyant, 2005; Vandamme et al., 2015; Weiss, 2006; Weyant et al., 1996). Only four species, *B. petrii* (von Wintzingerode et al., 2001) and the three novel species mentioned above, were isolated from the environment. However, whole genome analysis verified that *B. petrii* has pathogenic genes in its genome (Gross et al., 2008). Interestingly, *B. petrii* has been isolated from clinical samples since the initial finding by von Wintzingerode et al. (2001) (e.g., Fry et al., 2005; Le Coustumier et al., 2011; Stark et al., 2007). One species, '*B. ansorpii*', which has not been validated and is closely related to *B. petrii* (Fig. S9), has been isolated from clinical samples and is known to be pathogenic (Ko et al., 2005). At present, it is unknown whether our isolates are pathogenic. In general, pathogenic *Bordetella* species are able to grow at temperatures around 37°C and are fastidious, which is likely due to host-parasite interactions. Based on the whole genome sequence of *B. petrii*, Gross et al. (2008) suggested that this bacterium might grow in a variety of environments because it has remarkable metabolic versatility. Most pathogenic species of the genus *Bordetella* lack metabolic versatility, so they are host-restricted pathogens. Gross et al. (2008) also proposed that *B. petrii* represents an evolutionary link between free-living environmental bacteria and the host-restricted obligate pathogenic species of *Bordetella*.

Our isolates can grow on a standard culture medium and at 10°C. Therefore, they might have similar gene sequences that confer metabolic versatility. The *Bordetella* isolates were growing on the plaster wall surface of mural paintings that is presumed to provide poor nutrient support for bacterial growth, and were kept at approximately 10°C.

These *Bordetella* species were not found during other periods of the microbiological survey of the TT stone

chamber and were not isolated in samples from the KT stone chamber, which was not kept under cool conditions.

One of the isolated strains, *B. tumulicola* T7410-1-3b (JCM 30939), was isolated from plaster between the north wall and the ceiling at the beginning of the dismantling of the TT (Tazato et al., 2015). This finding indicates that the *Bordetella* isolates had invaded the chamber through the surrounding soil and that these isolates might be predominant because the temperature condition was suitable for their growth rather than the growth of other microorganisms during the stone chamber cooling period.

In the Lascaux cave in France, paleolithic paintings were surveyed for clinical bacteria using a culture-independent method, and few *Bordetella*-related clones were detected (Bastian et al., 2009a). The partial sequences of the 16S rRNA gene of the clones were more closely related to '*B. ansorpii*' (Fig. S9) and the TT isolates than to other pathogenic species in the genus *Bordetella*.

7-8. *Gluconacetobacter*

Until our proposal of two novel species (Tazato et al., 2012) mentioned below, the acetobacteriaceae genus *Gluconacetobacter* comprised only five species with the type species *G. liquefaciens*. These species have been isolated from dried fruits, the rhizosphere of coffee plants, sugarcane, and the pink sugar cane mealybug (Komagata et al., 2014). Species of the genus *Gluconacetobacter* are known to produce acetic acid from ethanol and gluconic acid from glucose (Sievers and Swings, 2005).

During the microbiological surveys of the KT mural paintings, a few small holes (ca. 10 mm diam; see Subsection 2-2) on the plaster ceiling wall were observed in the stone chamber interior in September 2005 (Fig. 1s; figure 1c in Kigawa et al., 2013a). These small holes were filled with a black gel or viscous materials, and increased in size with time (Kigawa et al., 2006a). We isolated six acetic-acid-producing bacteria from these black gels and viscous materials (Tazato et al., 2012).

According to 16S rRNA gene sequence analysis, these isolates formed a novel lineage in the genus *Gluconacetobacter*. We designated these novel species as *G. asukensis* and *G. tumulicola* (Tazato et al., 2012). Initially, these acetic-acid-producing bacteria were not isolated from the TT samples by the cultivation method we used but were identified using a molecular phylogenetic approach using denaturing gradient gel electrophoresis (DGGE). This result suggested that the acetic-acid-producing bacteria might have been present in the samples collected during the dismantling or in the TT burial mound soil (see Subsection 8-2 of this review; Nishijima et al., 2013). Therefore, we have used the enrichment culture method on samples from which the DGGE results suggested the existence of acetic-acid-producing bacteria. We obtained 10 isolates from seven TT samples and performed polyphasic taxonomic analysis (Nishijima et al., 2013). These 10 isolates were separated into four groups based on 16S rRNA gene sequence similarity, and one species, *G. asukensis*, was also isolated from the KT. The other three groups belonged to a novel lineage in the genus *Gluconacetobacter*. These three novel species of the genus *Gluconacetobacter* were designated as *G. tumulisoli*,

G. aggeris, and *G. takamatsuzukensis* (Fig. 13p). A total of 16 strains were isolated from both tumuli, representing minority taxa in the contaminant bacteria. However, these strains, particularly the KT isolates, caused severe damage to the plaster wall interior.

Our isolated strains also produce acetic acid from ethanol (Kigawa et al., 2013a; Sano et al., 2010). In addition, our previous experiments (Kigawa et al., 2009b) clearly demonstrated that *G. tumulicola* K5929-2-1b^T (JCM 17774^T) dissolved calcium carbonate (CaCO₃), the primary component of the plaster, and produced a water-soluble brownish pigment in the GYC medium that contained CaCO₃ (Kigawa et al., 2013a). As a result of acid production, these isolates cause a large decrease in the pH of the culture broth (Kigawa et al., 2013a; Sano et al., 2010). During the September 2005 survey, the pH of the black substance collected from the KT stone chamber was approximately 6 (by pH indicator paper) or 6.6–6.8 (by a pH meter) (Kigawa et al., 2013a). The pH of the surface of the surrounding plaster was approximately 7 by pH indicator paper. In contrast, the pH of a section of plaster adjacent to the "White Tiger" painting, which was relocated in October 2004, was 7.1–8.8 (by a pH meter). This result suggests that the pH of the plaster surface is decreased by the acids produced by microorganisms and that *Gluconacetobacter* isolates in particular cause severe damage to the plaster. Because the TT and KT murals were painted directly onto the thin calcium-carbonate-containing plaster (e.g., Kigawa et al., 2009a), these results strongly suggest that the isolates cause severe damage, including the small, brown-colored holes on the plaster surface of the KT stone chamber interior (Fig. 1s).

The *Gluconacetobacter* isolates cannot grow in an isopropanol-containing medium and produce no acid (Kigawa et al., 2013a; Sano et al., 2010). *Gluconacetobacter* species have not been isolated since the fungicide treatment was changed to isopropanol. *G. tumulicola* K5929-2-1b^T (JCM 17774^T) was also tested for its sensitivity to NaClO. This isolate showed no growth when exposed to NaClO at a concentration of 1000 ppm for 10 s (Kigawa et al., 2010c). No *Gluconacetobacter* species survived under UV irradiation (Kigawa et al., 2013b). Thus, no such bacteria were isolated after treatment of the tumulus with intermittent UV irradiation. An example of biodeterioration caused by acid-producing microorganisms is the sulfate-producing *Mycobacterium* strains isolated from the sandstone in Angkor Wat in Cambodia (Kusumi et al., 2011). These bacteria produce sulfuric acid, causing severe damage to the historical sandstone constructions.

Gluconacetobacter strains were not isolated from the TT stone chamber interior. However, they were present in the environment surrounding the stone chamber and might have invaded the stone chamber if countermeasures had been delayed. One novel species *G. asukensis* isolated from the KT (Tazato et al., 2012) was also isolated from the samples collected from joints/space of stone walls of the TT. This finding is interesting because it suggests that these acetic-acid-producing bacteria might be ubiquitously distributed in the area of Asuka village in Nara. One of these isolates, *G. aggeris* T6203-4-1a^T (JCM 19092^T), was derived from a soil sample collected from the TT mound

before relocation of the stone chamber. Of the seven samples from the TT investigated for isolates, just one provided all four group isolates (Nishijima et al., 2013): *Gluconacetobacter sacchari*, which is closely related to our isolates, from the rhizosphere of sugar cane, soil rhizosphere, or the pink sugarcane mealy bug (Franke et al., 1999). Ants, collembolans, mites, and spiders were found in the stone chambers of both tumuli (Kigawa et al., 2006a, 2007a). These results suggest that the ubiquitous acetic-acid-producing bacteria invaded the tumulus either directly or through soil surrounding the walls of the stone chamber.

Many acetic acid bacteria are aerobic and form biofilms (pellicle) on the surface of culture broth (Kersters et al., 2006; Komagata et al., 2014; Mamlouk and Gullo, 2013). Cellulose produced by *Komagataeibacter xylinus* ($\equiv$ *G. xylinus*; Yamada et al., 2013) is a food known as “nata de coco”. Strains isolated from both tumuli also produce a film on the broth surface, and KT strains were isolated from biofilms or viscous gels. Thus, these isolates are most likely involved in the formation of the biofilm.

On the GYC medium used for the cultivation of acetic acid bacteria, a clear zone is produced around colonies of bacteria that produce acetic acid by the oxidation of glucose. In addition, overoxidation of acetic acid by strains of *Acetobacter*, *Gluconacetobacter*, and *Acidomonas* is indicated by re-precipitation of CaCO_3 (Kersters et al., 2006). The isolates, *G. takamatsuzukensis* T61213-21-1a (JCM 19095) and *G. aggeris* T6203-4-1a^T (JCM 19092^T), both caused CaCO_3 re-precipitation. Calcite crystal precipitation is caused by microorganisms in caves (e.g., Altamira, Spain) (Cañaveras et al., 2006). The bioprecipitation of minerals in caves is thought to be caused by actinobacteria (Cañaveras et al., 2006; Cuezva et al., 2009, 2012; Diaz-Herrera et al., 2013). The *Gluconacetobacter* isolates might have caused not only the decay of the plaster wall surface but also the hollows in the plaster. Additionally, three species of *Gluconacetobacter* (*G. diazotrophicus*, *G. azotocaptans*, and *G. johanae*) are known as nitrogen-fixing bacteria (Fuentes-Ramírez et al., 2001; Gillis et al., 1989). These bacteria could be used as some root-nodule bacteria or alternatives to avoid or reduce the use of nitrogen fertilizers in fields (Pedraza, 2008). Although we did not confirm the nitrogen fixing ability of our isolates using the acetylene reduction method, our isolates grew on a nitrogen-free medium described in Dutta and Gachhui (2007) (Nishijima et al., 2013; Tazato et al., 2012). This observation suggests that these bacteria are capable of fixing nitrogen from the air and may supply it as a nutrient source to other microorganisms in the nutrient-poor environment of the stone chamber.

8. Comparison of the TT and KT Microbial Communities Using Culture-Independent (Molecular) Methods

Recently, microbial communities are often characterized without the use of culture-dependent (cultivation) methods because cultivation conditions, such as the medium, pH, temperature, and aerobic or anaerobic environment, can impose a selective pressure (bias) toward some mi-

croorganisms. However, to investigate the biological properties of causal microorganisms, the use of purified microbial isolates is very important. Culture-independent methods do not impose such a bias and can be used to identify uncultured or difficult-to-culture microorganisms. Therefore, the joint use of culture-dependent and culture-independent methods for the analysis of microbial communities complements the respective weak points of each method and gives more accurate, comprehensive results. In this section, we describe the fungal and bacterial communities of both tumuli (TT and KT) using culture-independent methods and compare the results of the two methods. Based on the data obtained using both methods, the origins of the causal microorganisms and related invasion routes will be discussed in Section 9. The following results in this section were obtained from samples collected under restricted conditions because both tumuli are special historic sites, and, in particular, because the mural paintings of the TT are designated as a national treasure. Therefore, in general, samples from sites of historical heritage may not be collected according to the usual experimental strategy and methodologies (see Section 3). Hence, for example, we could not examine absolute microbial populations by direct cell counting or measuring the biomass, and the obtained phylotype data were qualitative not quantitative.

8-1. Fungal community

8-1-1. Analysis of the TT fungal community. A total of 50 samples were selected for culture-independent (primarily DGGE) analysis. All but four of these samples were collected during the dismantling of the tumulus. These samples were categorized into five groups according to the collection site (see Subsection 3-1, Fig. S1).

Culture-dependent method: A total of 397 fungal strains were isolated from the same samples used for DGGE analysis using the culture-dependent method. Figure S10 shows the proportions of organisms at the phylum/order or family level at each collection site. The most abundant taxa isolated from the stone chamber interior were the orders Hypocreales (mostly *Gliocladium* [*Clonostachys* spp.]), Eurotilales (mostly *Penicillium* spp., with *P. paneum* predominant), and Saccharomycetales (the major yeast order) (Fig. S10). The order Chaetothyriales (mostly *Cladophialophora*) was isolated from four collection sites, but not the chamber interior. The diversity of isolated taxa was lower in the chamber interior and adjacent space than at other collection sites. Further details of the fungal community in the TT acquired using the culture-dependent methods are described in Section 4.

DGGE: A total of 50 samples were analyzed by DGGE profiling of partial SSU rRNA gene sequences, and a total of 129 fungal partial SSU gene sequences were identified. BLAST (Altschul et al., 1997) analysis revealed a total of 41 different fungal genera along with other eukaryotes, such as Collembola, Cercozoa, and Labyrinthulida. The major fungal phyla of the community were Ascomycota (74%), Basidiomycota (6%), and Zygomycota (4%) (Fig. S10). The ascomycete orders Eurotilales, Helotiales, Hypocreales, Chaetothyriales, and Saccharomycetales rep-

represented a large fraction of the fungal community, as observed using the cultivation method. The predominant fungal genera observed as a percentage of the total detected band sequences were *Exophiala* (11%), *Verticillium* (9%), *Aspergillus* (5%), *Mortierella* (4%), *Penicillium* (3%), *Phialocephala* (3%), *Candida* (3%), *Thermomyces* (3%), and *Fusarium* (2%).

The Saccharomycetales (ascomycetous yeasts) were prevalent in the stone chamber interior samples (viscous gels/biofilms). The Orbiliales were only detected in the stone chamber interior. The Helotiales were detected in the burial mound, the stone chamber exterior, and the adjacent space. The Hypocreales were detected in the burial mound, the stone chamber exterior, the adjacent space, and the joint/space of the stone walls. *Penicillium*, *Fusarium*, and *Trichoderma* were the major fungal colonizers as determined by the culture-dependent method, whereas *Exophiala*, *Verticillium*, and *Aspergillus* were also major fungal colonizers according to the DGGE method. In addition, collembolans were detected in the adjacent space, the stone chamber exterior, and the joint/space of the stone walls.

Clone library analysis: A total of 22 DGGE samples were also analyzed by clone libraries of the partial SSU gene. 1201 clones were analyzed, and their closest relatives were identified by BLAST. The results according to the collection site are shown in Fig. S10. A total of 46 different fungal genera were identified, along with other eukaryotes such as Collembola and Alveolata. The fungal groups identified include the phyla Ascomycota (83%), Basidiomycota (5%), and Zygomycota (0.1%) (Fig. S10). The predominant fungal genera observed as a percentage of the total clones were *Exophiala* (19%), *Pichia* (11%), *Phialocephala* (11%), *Penicillium* (8%), *Candida* (6%), *Hyaloscypha* (4%), *Scytalidium* (3%), and *Orbilia* (3%). The orders Eurotiales and Helotiales were detected at all five collection sites, whereas Chaetothyriales was not detected at the burial mound or the stone chamber interior.

The ascomycete yeasts (Saccharomycetales) represented more than 70% of the stone chamber interior fungi according to DGGE and clone library analyses. In contrast, ascomycete yeasts represented only 16% according to the culture-dependent method. The phylum Basidiomycota was detected at all sites except the chamber interior using clone library analysis, but this fungus was only isolated from mound samples. Comparison of culture-dependent methods to culture-independent (community analysis) of fungi using SSU (Fig. S10) revealed that *Penicillium* in the Eurotiales was detected at all sites, from the burial mound to the stone chamber exterior. The joint/space of stone walls is considered to be the boundary between the stone chamber interior and the external environment, which includes the stone chamber exterior, the burial mound, and the habitation environment. This region was influenced by microorganisms from both environments for many years. The wide variety of fungi detected in the joint/space of stone walls supports this notion.

Using clone library analysis, collembolans were also detected in the adjacent space, the joint/space of stone walls, and the stone chamber exterior. In the survey of the TT stone chamber interior, arthropods, such as mites, ants,

and collembolans, were detected (Figs. 1e, f, g, and u). Collembolans are thought to be one of the transporters of fungal propagules and bacterial cells involved in the biodeterioration of the stone chamber interior. The primers used for clone analysis might be not suitable for mites and other arthropods, since they were not detected. Collembola is discussed in Subsubsection 9-1-3.

8-1-2. KT samples.

Culture-dependent method: Microbial surveys of the KT samples (Fig. S2) were carried out from 2004 to 2013, mainly using mycological methods. The results are summarized by Kigawa et al. (2015) and in Section 5. After the excavation of the KT in 2004, the genera *Trichoderma*, *Penicillium*, and *Fusarium* were isolated as the major fungal colonizers inside the KT stone chamber. Later, the genera *Burgoa* and *Acremonium* (sect. *Gliomastix*), members of black fungi/molds, were also detected with *Penicillium* and yeasts from the samples collected in 2006. Further species of the black molds, including *Phialophora* sp., *Ophiostoma* sp., and *Cladosporium* sp., were also predominant isolates. The diversity of the fungal community increased with time after the excavation, and similar trends were shown in the case of the TT and the Lascaux cave (Kigawa et al., 2015). The dark-colored fungi (e.g., *Cladophialophora*, *Arthrimum*, and *Exophiala*) were the most abundant isolates after intermittent UV irradiation carried out in 2009.

Culture-independent method: Analysis of the KT fungal community using the culture-independent method was carried out only once, using next generation sequence (NGS) analysis (Sato et al., 2015). The last samples collected before the stone chamber was buried in 2013 were used for the NGS analysis. The fungal communities of these samples consisted predominantly of the genus *Farlowiella* in the class Dothideomycetes, the genera *Mariannaea*, *Beauveria*, and *Acremonium* (*Sarocladium*-clade) in the class Sordariomycetes, the genus *Thelebolus* in the class Leotiomycetes, and the genera *Phialophora* and *Exophiala* in the class Eurotiomycetes (Sato et al., 2015). These KT stone chamber interior samples were collected after intermittent UV irradiation. The major taxa detected coincided closely to those identified by the culture-dependent method (Kigawa et al., 2015). In particular, the black mold genera *Phialophora* and *Exophiala* were identified as being predominant by both methods. However, the genera *Farlowiella*, *Mariannaea*, *Thelebolus*, and *Beauveria* were not isolated using the culture-dependent method. The genus *Beauveria* is known mainly as the insect pathogenic fungus commonly called Chinese Caterpillar Fungus. Therefore, Sato et al. (2015) suggested that invading insects played an important role as vectors in the distribution of these fungi.

8-2. Bacterial community analysis

8-2-1. TT.

Culture-dependent method: The major taxa (phyla/classes) of the TT bacterial community, as determined by the culture-dependent method, are the phyla “*Proteobacteria*” (classes *Alpha*-, *Beta*-, *Gamma*-*proteobacteria*), “*Bacteroidetes*”, “*Firmicutes*” (class *Bacilli*), and

“*Actinobacteria*”.

The most dominant was the *Bacillus cereus* group (see also Subsubsection 6-3-1). The next predominant bacteria inside the stone chamber included *Ochrobactrum* spp. (*Alphaproteobacteria*), *Bordetella* spp. (the *Betaproteobacteria*), and *Stenotrophomonas* spp. (the *Gammaproteobacteria*), along with *B. cereus* (Figs. 7 and 11, in Subsections 4-3 and 5-3). “*Actinobacteria*” isolates increased, moving from the stone chamber interior toward the stone chamber exterior (Fig. S5).

DGGE: A total of 109 TT samples were used for bacterial community analysis using the culture-independent method (PCR-DGGE). The TT samples were classified according to the sampling area described in Subsection 3-1 (Fig. 2, Fig. S1). The number of samples were as follows: 4 samples collected in 2006 (before the dismantling work) from the stone chamber interior, 36 samples from the joint/space of the wall stone, 10 samples from the adjacent room, 45 samples from the stone chamber exterior, and 14 burial mound samples.

The bands derived from *Ochrobactrum* were detected in samples from both the stone chamber interior and exterior. *Stenotrophomonas* was identified in samples from the stone chamber interior and the interspace of the east wall. *Bordetella* was detected in the stone chamber interior, whereas *Bacillus* was detected in the interspace of the stone wall. These bacteria were also identified by the isolation method as being predominant. As summarized here, these four bacterial genera were detected as the major bands using DGGE and as the predominant TT isolates.

In the isolation method results, the most abundant bacterial isolates were members of the “*Actinobacteria*” and the “*Firmicutes*”, such as *Bacillus* and *Streptomyces*. The bands of *Bacillus* and “*Actinobacteria*” were also detected in the PCR-DGGE analysis of burial mound soil samples collected during the dismantling work. Bands derived from the “*Acidobacteria*” were also abundant in the mound soil samples, although “*Acidobacteria*” were not isolated (see Subsubsection 9-1-5).

The population dynamics of bacterial diversity in the TT, according to the culture-independent method, are shown in Fig. S11. The DGGE results indicate that *Alpha*-, *Beta*-, and *Gamma*-*proteobacteria* were the predominant bacteria in the stone chamber interior. This result is consistent with the isolation results. The presence of *Alphaproteobacteria* increased, moving from the stone chamber exterior toward the interior. However, the sequences derived from *Alphaproteobacteria* were assignable not only to *Ochrobactrum* spp., but also to the other order, *Rhizobiales*. The finding that the phylum “*Acidobacteria*” was detected as the major band in the samples collected from outside the stone chamber is intriguing. Species of “*Acidobacteria*” were not isolated by the method we used (see Subsection 3-3). The phylum “*Chloroflexi*” was detected in the stone chamber exterior, but this phylum was not isolated using our methods.

8-2-2. KT.

Culture-dependent method: The major taxa of isolates were the phylum “*Proteobacteria*”, including *Rhizobium* (*Alphaproteobacteria*), *Achromobacter* (*Betaproteobacteria*), and *Stenotrophomonas*

(*Gammaproteobacteria*). The samples taken from inside the KT chamber in 2009 differ from other samplings, as *Alphaproteobacteria* were not isolated (Fig. 12). At that time, UV irradiation had just begun. Members of the phylum “*Proteobacteria*” decreased a little after UV irradiation (Fig. S6). Hence, it is considered that UV irradiation affected the microbial community of the KT stone chamber interior (Subsection 5-3).

DGGE: A total of 15 samples were analyzed by the PCR-DGGE method. The DGGE band patterns from the five samples (four west wall, and one northwest ceiling, samples) were similar, and the genus *Gluconacetobacter* was detected as one of the major bands (Fig. S12). The sample in lane No. 15 was collected from the east wall after UV irradiation, but a band from *Gluconacetobacter* was also detected. After UV irradiation was routinely carried out, *Gluconacetobacter* spp. were not isolated from samples inside the stone chamber. These data suggest that *Gluconacetobacter* spp. were widely distributed inside the stone chamber.

DGGE results for the KT show that bands derived from gram-negative bacteria represent more than 70% of all bands from samples collected inside the stone chamber interior (Fig. S13). The predominant genera were the same as those identified by isolation. *Betaproteobacteria* was recognized as the second most common genera in the stone chamber interior. However, the detected genera differed from the isolates; for example, the genus *Achromobacter* was not detected, but several strains were isolated. Remarkably, a bacterium thought to be myxobacteria in the class *Deltaproteobacteria* was detected in the stone chamber interior. This bacterium was also detected in the TT dismantling work samples derived from the interspace of the stone wall using DGGE, but was not isolated from either tumuli using the isolation method. Interestingly, the phyla “*Acidobacteria*”, “*Cyanobacteria*”, and “*Chlorobi*” were detected in the stone chamber interior samples before UV irradiation. “*Acidobacteria*” were also detected in the TT samples but not in those from the TT stone chamber interior. The KT stone chamber was usually kept under dark conditions. A band derived from “*Cyanobacteria*” was detected in a sample collected from the south side floor stone. Very weak exposure to light might have occurred during the preservation treatments, or when checking the stone chamber interior, since the south side was the entrance to the chamber interior (Figs. 1p and 3). Because this floor was once covered with inflowing soil, another possibility is that cyanobacteria entered the stone chamber in soil and survived there. Before UV irradiation, the various taxa were detected as similar to the isolation results. After UV irradiation, however, the bacterial community was less diverse, and the predominant species changed. In the burial mound soil, uncultured or difficult-to-cultivate bacteria, such as the phyla “*Acidobacteria*”, “*Chloroflexi*”, and “*Nitrospirae*”, were detected with an abundance similar to that of TT soil samples.

Clone library: The bacterial community under intermittent UV irradiation was surveyed using culture-independent methods (Clone library and NGS) (Sato et al., 2013, 2015). Clone library analysis revealed that the genus

Promicromonospora (class *Actinobacteria*) was the most predominant bacteria in all four samples collected from the stone chamber interior in 2010 (Sato et al., 2013). This result is consistent with the isolation result (Kigawa et al., 2013b). The major species of isolated bacteria belonged to *Actinobacteria*, including *Promicromonospora*, *Streptomyces*, and other genera. Strains of the genus *Promicromonospora* were isolated from samples collected before UV irradiation, but they were minor. These results suggest that the genus *Promicromonospora* inhabited the inside of the stone chamber but did not become predominant until the UV irradiation. While this result was based on only one sample, a clone that was closely related to *Gluconacetobacter tumulicola* was detected. Interestingly, the site from which the clone library analysis sample was collected (east wall, center of the upper side, near the ceiling) was near the site from which *Gluconacetobacter* members were isolated.

Next generation sequence (NGS): Based on the bacterial community analysis by NGS, the genera *Jiangella*, *Promicromonospora*, and *Streptomyces* in the phylum “*Actinobacteria*” were of high abundance in some samples (Sato et al., 2015). This result supports the isolation and clone library analyses. The intermittent UV irradiation affected the bacterial community of the KT stone chamber interior. *Gluconacetobacter*-related sequences were also faintly detected in four samples. No strains of the genus *Gluconacetobacter* were isolated from the samples collected after 2009 (Fig. 12). In contrast, using culture-independent methods (PCR-DGGE, clone library, and NGS analyses) *Gluconacetobacter* was detected in samples collected during intermittent UV irradiation (Sato et al., 2013, 2015). A clone derived from *G. tumulicola* was obtained from a sample collected very near the site from which *G. tumulicola* K8617-7-3b (JCM 17777) was isolated (Tazato et al., 2012). Additionally, *Gluconacetobacter* spp. were faintly detected in four of the eight samples tested by NGS analysis (Sato et al., 2015). These four samples were collected from the east wall, ceiling (north side and west side), and west wall. In the PCR-DGGE analysis, one band was detected from a floor sample. These findings suggest that *Gluconacetobacter* was possibly distributed throughout the KT stone chamber interior.

9. General Discussion

In this general discussion, we focus on the following four points: (1) the upsides and downsides of culture-dependent and culture-independent methods; (2) substrate preference; (3) mapping (based on the detection sites) of the major microbial colonizers involved in the deterioration of mural paintings and plaster walls; and (4) invasion routes as suggested by substrate preference and mapping the locations of detected TT isolates.

9-1. The upsides and downsides of culture-dependent and culture-independent methods

Many studies have investigated the causes of biodeterioration of cultural heritage sites using culture-independent methods, e.g., 16S rRNA gene sequence,

PCR-DGGE, clone library, and NGS analyses (Caneva et al., 2008; Cheeptham, 2013; Engle, 2015; Gurtner et al., 2000; Li et al., 2016; Saiz-Jiménez, 2003, 2014, 2015; Schabereiter-Gurtner et al., 2002a; Zengler, 2008). In particular, culture-independent methods have been used to analyze the microbial communities of the Lascaux Cave in France, the Altamira Cave in Spain, and the necropolis in Italy (Bastian et al., 2009a, 2009c, 2010; Diaz-Herrera et al., 2013; Garcia-Anton et al., 2014; Martin-Sanchez and Saiz-Jiménez, 2014; Saiz-Jiménez, 2014; Schabereiter-Gurtner et al., 2002b). Many of these surveys and investigations were carried out using both culture-dependent and culture-independent methods. In our survey, both methods were used to analyze the TT and KT samples (Sato et al., 2013, 2015; Sugiyama et al., 2009). We also conducted tests to determine the effect of fungicides, utilization of compounds, materials, production of acid, and UV tolerance by examined intended for our isolates. Using culture-independent methods, we were able to identify uncultured bacteria. These methods were also very useful for avoiding selective pressure in the cultivation conditions, such as the medium type, temperature, and air conditions. As an example, we could not culturally isolate *Gluconacetobacter* spp. from TT samples but did detect it using DGGE analysis (Nishijima et al., 2013). The isolation of *Gluconacetobacter* usually requires the use of a special method employing an acetic acid bacterial isolation medium (e.g., Yamada, 1986). On the other hand, proper use of culture-independent methods requires avoiding bias introduced by the choice of PCR primers.

In previous reports using PCR-DGGE, many results regarding the presence or predominance of microbes in the community differed depending upon the method used (González and Saiz-Jiménez, 2005; Gurtner et al., 2000; Portillo et al., 2008; Schabereiter-Gurtner et al., 2002a, 2002b, 2004). However, in the case of the TT and KT samples, the major taxa in the microbial communities were mostly similar in these two methods. For example, the major taxa in the fungal community were the orders Eurotiales, Hypocreales, Chaetothyriales, Helotiales, and Saccharomycetales in the phylum Ascomycota as determined by the culture-independent method (see Subsubsection 8-1-1). These results are consistent with those of the isolation method. On the other hand, the genera *Farlowiella*, *Mariannaea*, *Thelebolus*, and *Beauveria* were not isolated by culture-dependent methods but were detected by the culture-independent method. Similar results were observed in the case of the bacterial community analysis. The phyla “*Acidobacteria*”, “*Cyanobacteria*”, “*Chlorobi*”, and “*Chloroflexi*” were known as unculturable taxa or microorganisms that are different to cultivate and isolate using a conventional culture medium; therefore, they were detected using the culture-independent method (see Subsection 8-2). Some differences in the detected taxa in the fungal community were observed between the PCR-DGGE and clone library methods. For example, Hypocreales was detected using both the cultivation method and DGGE, but not using clone analysis. In the burial mound, Xylariales was detected by DGGE, whereas Pleosporales was detected using clone analysis. In addition, clones were obtained from

collembolans but not mites, because the primer sets used may not have been suitable for the SSU rRNA gene sequences of mites (Nishijima et al., unpubl.). To more accurately reveal the biological community/biota, multiple methods are needed.

Members of the domain “*Archaea*” were reported from a microbiological survey in some Caves (Saiz-Jiménez, 2015), especially, uncultured low-temperature “*Crenarchaeota*” were detected from the Altamira Cave as metabolically active archaea (Gonzalez et al., 2006). In this study, “*Archaea*” was not detected from either tumuli by the culture-independent method we used. It might be that the primers used for the tests were not suitable to detect “*Archaea*”.

9-1-1. Gram-positive bacteria: the phyla “*Proteobacteria*” vs. “*Actinobacteria*” and “*Firmicutes*”.

Bastian et al. (2009c) reported that the almost exclusive presence of “*Proteobacteria*” could be considered a marker of a human-impacted cave. Similar results were reported in the Kartchner Caverns in Arizona, USA, by Ikner et al. (2007). In this cave, “*Actinobacteria*”, “*Firmicutes*”, and “*Proteobacteria*” were the three primary phyla detected in samples exposed to different levels of human impact. The “*Proteobacteria*” were predominant (>77%) in high impact areas, while “*Firmicutes*” predominated in the low (66%) and medium (52%) impact areas. Ikner et al. (2007) also proposed that the predominance of the phylum “*Proteobacteria*” was a consequence of increased organic matter availability in lint and other organics brought into the cave by visitors.

In the case of the Lascaux Cave, “*Proteobacteria*” was predominant in the bacteriobiota. Human clinical bacteria such as *Pseudomonas* and *Ralstonia* appeared after the introduction of selective pressure by benzal chloride treatments (Bastian et al., 2009c). In the case of the TT and KT stone chamber interiors, “*Proteobacteria*” appeared as the predominant bacteria after 2004. After UV irradiation was begun in the KT stone chamber interior, the prevalence of “*Proteobacteria*” decreased and “*Bacteroidetes*” or “*Actinobacteria*” became the major bacteria.

9-1-2. Deltaproteobacteria.

The class *Deltaproteobacteria* has been detected in the bacterial community of some caves, especially the Altamira Cave and the Niu Cave (Saiz-Jiménez, 2015; Zhou et al., 2007). However, most of the *Deltaproteobacteria* in cave bacteriobiota are sulfur- or sulfate-reducing bacteria, most of which are anaerobic. According to the report by Zhou et al. (2007) (for the Altamira cave, see Schabereiter-Gurtner et al., 2002b), the slime bacterial order *Myxococcales* in the class *Deltaproteobacteria* has been detected only in the Altamira Cave samples. In our DGGE survey, we obtained a few bands derived from *Myxococcales* in both the TT and KT samples (see Subsubsection 8-2-2). Those from KT were collected mainly from gel-like (biofilm) substances in the stone chamber interior, while, in contrast, those from the TT samples were collected from the interspace of wall stone. Members of the genus *Myxobacteria* feed other microorganisms and inhabit various environments; however, their cultivation is known to be difficult, especially in medium

conditions and long-term cultivation (Shimkets et al., 2006). Many microorganisms and biofilms were found on the plaster surface inside the stone chamber, particularly in the KT. Therefore, a plaster surface might be suitable for the growth of myxobacteria.

9-1-3. Arthropods.

Many arthropods, including mites, spiders, centipedes, and collembolans were observed on the surface of the plaster walls of the TT and KT chamber interiors (Figs. 1e–g, u). The results of culture-independent methods suggested that these arthropods were present both inside and around the outside of the chamber. These arthropods might have played a role in distributing the contaminating microorganisms to the inside of the chamber, also providing a good source of nutrition once dead. Dromph (2003) reported that collembolans are vectors of entomopathogenic fungi. Greif and Currah (2007) isolated species of the fungal genera *Acremonium*, *Beauveria*, *Cladosporium*, *Cryptendoxyla*, *Geomyces*, *Gliocladium*, *Hormiactis*, *Leptographium*, *Oidiodendron*, *Penicillium*, and *Verticillium* from collembolans. In the case of the Lascaux Cave, collembolans fed on the mycelia/hyphae of *Fusarium* and *Ochroconis* and carried conidia on their bodies (Bastian et al., 2009b, 2010). In the case of the TT, our observations indicate that mites fed on fungal bodies and colonies and then walked on the collected samples and the surface of the culture medium in the plates.

9-1-4. Effects of UV irradiation on the microbial community.

Unfortunately, culture-independent analysis was not carried out on the KT samples before intermittent UV irradiation began. However, the results of next generation sequence (NGS) analysis by Sato et al. (2015) were consistent with those of the culture-dependent method using the same samples. In addition, some uncultured fungi, such as *Beauveria*, were only detected using culture-independent methods. These data suggest that more comprehensive biota analysis using both culture-dependent and culture-independent methods should be performed. The mycobiota after UV irradiation in the KT stone chamber interior was composed of predominant black or dark-colored fungi, including *Cladophialophora*, *Arthrimum*, and *Exophiala*, which showed a relatively high tolerance for UV irradiation (Kigawa et al., 2013b). However, there was no significant increase in UV tolerance of taxa/isolates when comparing isolates before and after UV irradiation.

As for bacteriobiota, “*Proteobacteria*”, accommodating acetic acid bacteria, were detected as being predominant in the samples collected before UV irradiation; however, the phyla “*Bacteroidetes*” and “*Actinobacteria*” predominated after UV irradiation. To investigate the effects of UV irradiation on the microbiome, representative isolates of major microorganisms (fungi, yeasts, and bacteria) in the KT stone chamber interior were tested for UV tolerance (Kigawa et al., 2010c). Dark-colored fungi (presumably melanin-rich) and actinomycete were comparatively tolerant (slightly tolerant, in the case of the latter) to UV irradiation (Kigawa et al., 2013b).

Fungal mycelia were not observed after UV irradiation in the KT stone chamber interior, and it was considered that microbial activities overall were inhibited within the

stone chamber. Therefore, it was suggested that the difference or change in the proportion of taxa appearing after UV irradiation was caused by the selective growth of UV-tolerant microorganisms, presumably survivors (Kigawa et al., 2015).

9-1-5. “*Acidobacteria*”. Using the culture-independent methods, the phylum “*Acidobacteria*” was detected as being predominant, especially in the burial mound soil of both the TT and KT. In particular, these bacteria were detected from the KT stone chamber interior. In other caves, these bacteria were commonly retrieved but not cultured (Zimmermann et al., 2005). In the Spanish caves (viz., La Garma Cave, Tito Bustillo Cave, and Altamira Cave), this bacterium was predominant along with “*Proteobacteria*” (Schabereiter-Gurtner et al., 2003). Some band sequences detected from both tumuli showed a similar phylogenetic position to those of bacteria detected in the Spanish caves (Fig. S14). According to Saiz-Jiménez (2015), it is possible that the abundance of the “*Acidobacteria*” in the Spanish caves might be biased by sampling, and/or by the low number of sequences studied. Based on the results of a molecular survey of 60 caves, “*Acidobacteria*” represented as low as 3% of the population (Lee et al., 2012). In our survey, we might not have obtained enough samples and sequences (see Subsection 8-2). It is interesting that similar bacteria were detected at sites in Europe and Japan. In addition, “*Acidobacteria*” were detected as the second major bacteria after the phylum “*Proteobacteria*” in the Niu Cave, Southwest of China (Zhou et al., 2007). On the other hand, no “*Acidobacteria*” and only rare “*Actinobacteria*” were detected, while more than 98% of the bacteria detected in the Lascaux Cave were assignable to the “*Proteobacteria*” (Bastian et al., 2009c). Biocide treatment and human disturbance are thought to have had a strong impact on the microbial community, especially that of the Lascaux Cave (Bastian et al., 2009c).

9-2. Substrate preference of the major and noteworthy TT microbial species

Primary references concerning substrate and habitat data for fungi (Domsch et al., 2007; Ellis, 1971, 1976, 1997; Samson et al., 2010; Seifert et al., 2011) have been used here. Ecological substrates or habitats were categorized as soil, on living or dead plants, on insects, in the air, and in/on others. The substrate preferences of the fungal species/isolates of the TT have been assigned according to these categories (Table 4). Similarly, bacteria were categorized based on substrate preferences. The major bacterial genera *Bacillus*, *Ochrobactrum* and *Stenotrophomonas*, and a few actinobacteria (*Microbacterium*, *Promicromonospora*, and *Streptomyces*), to which the TT and KT bacterial isolates were assigned, are soil-inhabiting members (Kämpfer et al., 2014; Logan and De Vos, 2009; Ryan et al., 2009; Whitman et al., 2012). *Bordetella* from the TT samples and *Gluconacetobacter* from both the TT and KT samples have been cited as noteworthy genera. Most species of *Bordetella* are mammalian and avian parasites and pathogens. They cause respiratory infections in humans and a variety of warm-blooded animals (Sanden and Weyant, 2005; Weiss, 2006; Weyant et al., 1996; cf., Subsection 7-7 mentioned above). The

closest related species found in TT isolates, *B. petrii*, was isolated from river sediment (von Wintzingerode et al., 2001). *Gluconacetobacter* species have been isolated from dried fruits, the rhizosphere of coffee plants, sugarcane, and the pink sugarcane mealybug. Thus, most of these bacteria prefer plant substrates (Komagata et al., 2014; cf., Subsection 7-8 mentioned above). Overall, the substrate preference data indicate that the fungal and bacterial species detected in the TT and KT depend on soil and/or plant substrates.

9-3. Mapping of the major fungal and bacterial colonizers detected from the TT samples

Mapping (plotting) data from two fungal colonizers (*Penicillium* and dark *Acremonium*) and three bacterial colonizers (*Bacillus*, *Ochrobactrum*, and *Stenotrophomonas*) are illustrated, respectively, in Figs. S15 and S16). The remaining maps will be published by Kiyuna et al. in the near future.

(1) *Penicillium paneum* (Fig. S15a): The 141 isolates of *Penicillium paneum*, in which the 25 isolates were molecular phylogenetically characterized by LSU gene sequences, from the stone chamber interior and the neighboring environment of the TT were used for the mapping analysis. *Penicillium paneum* was consistently isolated from the stone chamber interior before and after cooling of the stone chamber since May 2004. *P. paneum* was isolated from various samples collected from the stone chamber interior and neighboring environment, including the mural paintings and plaster surface of the stone walls, the joint space between the stone walls, and the stone chamber exterior. Only three isolates were isolated from the mound. Therefore, this species is thought to be an indigenous mold inside the stone chamber. Furthermore, this genus was isolated from the stone chamber interior before 2004 (Arai, 1984, 1987, 1990b; Kigawa et al., 2006b); however, it is uncertain whether these Arai isolates are assignable to *P. paneum*. *Penicillium paneum* has been isolated from moldy rye bread and bakers yeast, silage, and cassava chips in northern Europe and Canada (Frisvad and Samson, 2004). These isolation records suggest that this mold invaded the stone chamber through plant-associated substrates such as roots.

(2) *Acremonium* sect. *Gliomastix* spp. (Fig. S15b): The 51 isolates of the dark *Acremonium* species (46 *Acremonium masseei*, in which 32 isolates were molecular phylogenetically characterized by LSU gene sequences, three *Acremonium murorum*, and two *Acremonium polychromum* isolates) from the stone chamber interior and the neighboring environment of the TT were used for mapping analysis. Dark *Acremonium* sect. *Gliomastix* spp. were mainly isolated from the stone chamber interior but not from the adjacent space, the stone chamber exterior, or the mound soil. From May 2006, the temperature of the stone chamber interior was maintained at about 10°C, and the two species of *Acremonium* sect. *Gliomastix* (*A. masseei* and *A. murorum*) appeared mainly on the mural of the stone chamber interior and the joint space between the stone walls. On the other hand, *A. polychromum* was only detected on the exterior of the stone chamber as an airborne fungus, with no appearance inside the stone cham-

ber.

(3) *Bacillus* spp. (Fig. S16a): A total of 52 *Bacillus* spp. isolates identified by 16S rRNA gene sequences were obtained from TT samples. Isolates of the *B. cereus* group (27) and the *B. simplex* group (25) from the original source sampling sites, with 64 isolates which were tentatively identified on the basis of morphological and cultural characteristics, are shown in Fig. S16a. Members of the *B. cereus* group were isolated throughout the chamber interior, and especially the northwest, north, and northeast walls. This result suggests that the *B. cereus* group was predominant and distributed throughout the chamber interior. The *B. cereus* group was also present at the chamber exterior, the interspace of the wall stone, and the burial mound. While no *B. simplex* group was isolated from the chamber interior, it was isolated from the adjacent space (cover of thieving/thief hole), the joint space of the stone wall, the stone chamber exterior, and the mound soil. These observations suggest that these two groups of *Bacillus* spp. are ubiquitous microorganisms in the environment surrounding the TT. During our survey (September 2005–April 2007), the TT chamber interior was maintained at around 10°C and at a very high humidity (nearly 100% RH). These microclimates are within the allowed growth range of these two *Bacillus* isolates. The *B. cereus* group might have adapted faster to the low temperature and high humidity than did the *B. simplex* group isolates. Alternatively, the differences in the habitats of these two *Bacillus* groups may have been caused by other factors, such as competition in the community. *Bacillus* spp. were abundant and frequent in the TT samples. This genus is known to form endospores (Fig. 13n), which would have allowed it to survive and distribute throughout the chamber interior.

(4) *Ochrobactrum* spp. (Fig. S16b): A total of 42 isolates belonging to the genus *Ochrobactrum* were obtained from TT samples collected from September 2005 to April 2007. In these isolates, 17 isolates were identified by 16S rRNA, *gyrB*, and *groEL* genes sequence analyses (Handa et al., 2017), and the remaining isolates were tentatively identified on the basis of morphological and cultural characteristics. Interestingly, two isolates were taken from samples collected just as the cooling of the chamber interior began (*Ochrobactrum* sp. 1), but fifteen isolates of *O. pituitosum* were obtained when the temperature was stable, or from dismantling samples. Before chamber dismantling, these isolates were found mainly on the plaster surface of the west wall inside the stone chamber (Fig. S16b). However, after dismantling, *O. pituitosum* was isolated from joint/space stone wall samples (Fig. S16b). This result suggests that these isolates invaded from the surrounding environment to the inside of the stone chamber. *Ochrobactrum* spp. were not isolated from burial mound samples. Their optimum growth temperature is 20–37°C, but environmental isolates will grow at 4°C. Therefore, these isolates might be predominant in a cool environment.

9-4. Origins and invasion routes of the major microbial colonizers into the stone chamber interior

Our mycobiota and bacteriobiota surveys detected various taxonomical and ecological microbial groups from

samples of the stone chamber interior and the neighboring environments of both tumuli (Table 4, Table S2). Based mainly on substrate preference and mapping data analyses, the possible origins of these microorganisms are considered to be the surrounding soil (carried mainly by water), plant substrates (e.g., roots), air, water, and small animals (e.g., mites, collembolans, and sow bugs). In the Altamira Cave, fungal colonies have three well-defined origins: plant saprophytes introduced from the exterior, entomopathogenic fungi introduced by arthropods, and coprophilous fungi growing on rodent feces (Jurado et al., 2009; Saiz-Jiménez et al., 2011).

Water, air currents, and micro-animals are also thought to play roles in the invasion of species into the stone chamber. For example, in the case of the Lascaux cave, *Fusarium solani* species complex (FSSC) are thought to have been carried by water from soils above the cave (Dupont et al., 2007). Furthermore, the dispersal of fungi on the mural paintings is reported to be mainly caused by arthropods (mites or collembolans) (e.g., Gorbushina and Petersen, 2000; Guglielminetti et al., 1994; Jurado et al., 2009; Fig. 1g). Bastian et al. (2009b) reported that the composition of the fungal community in the Lascaux cave is largely influenced by arthropod colonization and activity, with the collembolans being of primary significance. They concluded that “entomophilous fungi play an important role in the cave and arthropods contribute to the dispersion of spores and fungal development.” Our direct observations of collected samples and the official records of ACA, Japan (<<http://www.bunka.go.jp/>>), report the presence of arthropods such as centipedes, millipedes, springtails (Collembola), common rough woodlice, carabids, earwigs, spiders, and ants. Living mites and springtails were observed in fungal colonies on plaster walls inside the TT stone chambers. Although further studies on associated microorganisms and arthropods are needed, it is easy to imagine that these arthropods, especially mites and springtails, were involved in the expansion of microbial (bacteria, molds, and yeasts) colonization and disturbances in the stone chamber interior. As pointed out by Bastian et al. (2009b), in the Lascaux Cave, the role of arthropods as vectors is great within the tumulus stone chamber. The major bacterial members, such as *Bacillus*, *Stenotrophomonas*, *Ochrobactrum*, and a few actinobacterial genera were colonized inside the stone chamber by the same vectors that introduced the fungi.

Inside the KT stone chamber, the bristle-like (penicillate) mold *Kendrickiella phycomyces* was first observed on the surface of the stone ceiling in the adjacent small room in August 2004 (Fig. 13g; Kiyuna et al., 2012). Subsequently, this fungus was continuously observed in the stone chamber (Table S2). Conidial masses produced on the apex of conidiophores in *Kendrickiella phycomyces* are considered suitable for dispersal by small animals (e.g., arthropods), similar to species of the ophiostomatoid anamorphic genus *Leptographium* (Jacobs and Wingfield, 2001). Therefore, the invasion of *K. phycomyces* into the stone chamber is considered to have occurred via arthropods (e.g., mites or collembolans). In the TT, this fungus was observed on plant roots in a hole beside the stone wall above the adjacent space (Kiyuna et

al., 2012; e.g., isolate T61213-27-1). Thus, another possible route of invasion of *K. phycomyces* into the stone chamber is via plant roots.

Further possible major invasion routes into the TT stone chamber are considered to be the big hole on the south wall made by thieves (during the Kamakura period, in AD 1185–1333), the adjacent space/room, the joint/space of the stone walls, and cracks in the stone walls (Figs. S12 and S13). Our microbial community analyses suggest that a variety of ecological groups inhabited the stone chamber and its environs (cf., Table 4 for fungal isolates). The proposed pathways of entry of the bacteria and fungi that participated in the deterioration of the wall paintings include several pathways from the soil environment of the stone chamber entrance and the neighboring environment of the TT. In addition, also possibly involved are the surrounding soil (mainly by water), plant substrates (for details of identification, Noshiro et al., unpublished), air, and arthropods (e.g., mites and collembolans; Figs. 1e–g, u). In these various ways, it is presumed that various bacteria and fungi were able to enter the stone chamber interior, and those microorganisms suited to the high humidity inhabited the wall paintings of the stone chamber interior. The results of bacterial community analysis by culture-dependent and culture-independent methods suggest that the bacteria entered the interior from the stone chamber exterior, for example via the burial mound. Evidence for such entry includes the decreasing abundance of some types of bacteria as sampling moved from the stone chamber exterior to the interior. In particular, the phylum *Acidobacteria*, detected in the TT samples, was predominant in the burial mound and decreased from the exterior to the interior. Such observations are of interest in speculating on the invasion routes of microbial contaminants.

10. Conclusions, Lessons, and Future Perspectives

The advances of our microbiological survey work (2004–2016) of the TT and KT stone chambers in Asuka village, Nara Prefecture, Japan, to reveal the cause of biodeterioration of the 1300-year-old mural paintings are briefly summarized below.

Using culture-dependent (cultivation) methods, the predominant microorganisms relating to the deterioration inside the stone chambers of both the TT and KT were fungi (molds and yeasts) and bacteria. These predominant fungi of the TT samples collected from May 2004 to February 2005 were assigned to the phialidic anamorph genera *Fusarium*, *Trichoderma*, and *Penicillium*, which grow rapidly and produce numerous conidia. However, soon after cooling of the mound began in September 2005, *Fusarium* spp. and *Trichoderma* spp. decreased and were replaced by dark *Acremonium* (*Acremonium* sect. *Gliomastix*). When the temperature was maintained at about 10°C, one of the dark *Acremonium* (*Acremonium masseei*, a heterotypic synonym of *Gliomastix masseei*) appeared as the major cause of the black stains/spots on the painting of a group of four women (called “Asuka beauties” or “Asuka Bijin”) on the west wall. These so-called common molds grow rapidly and produce numerous dry or slimy conidia (Mason, 1937) as propagules, and are ubiquitous in the

soil. The top three common bacterial genera, *Bacillus*, *Ochrobactrum*, and *Stenotrophomonas*, were detected during the same period. Similar changes in bacteriobiota were seen after the cooling of the mound; members of *Bordetella*, which have been identified and proposed as three novel species (see Supplementary Materials) became predominant. Viscous gels that developed on the stone walls of both the TT and KT stone chamber interiors were biofilms comprising bacteria, yeasts, and molds. The identities of these major microbial colonizers of the TT and KT samples, including those from biofilms, were determined using integrated analysis of phenotypic and genotypic characteristics. In addition, the identity of noteworthy microorganisms, including the TT and/or KT isolates of the fungal genera *Cephalotrichum* (= *Doratomyces*), *Kendrickiella*, and *Burgoa* and the bacterial genera *Gluconacetobacter* and *Bordetella* were identified at the species level based on their phenotypic and genotypic characteristics, and novel species have been proposed (see Supplementary Materials). In addition, SEM observations of plaster and biofilm samples revealed fungal hyphae and bacterial/microbial cells deeply penetrating the plaster texture (Figs. 1l and m; cf., figures 15 and 16 in Sugiyama et al., 2009). Culture-independent DGGE, clone library, and pyrosequencing methods were applied to analyze the bacterial and fungal communities of the TT and KT. For the major microbial colonizers, the results of the cultivation and DGGE methods were consistent. However, the results obtained using culture-independent methods indicated a greater diversity of microorganisms. Consequently, the latter method is essential to reliably reveal the species composition of microbiota and microbial communities, as recommended by Martin-Sanchez and Saiz-Jiménez (2014) in their studies of the air of the Lascaux Cave. As demonstrated by Heyrman and Swings (2003), as well as in this review, all applicable synthetic approaches in modern microbiology and related areas help to elucidate the causes of biodeterioration of cultural treasures such as the TT and KT mural paintings and walls.

Based on substrate preference (ecological groups) and mapping (plotting the detection sites of the isolates) of selected major microbial colonizers, their possible origins and invasion routes into the TT stone chamber interior are speculated. Comprehensive analyses of data obtained from these mycological surveys identified multiple invasion routes, including a large hole on the south wall made by thieves, spaces adjacent to the stone chamber, spaces between the stone walls, and cracks in the stone walls. These bacteria and fungi were brought into the stone chamber from the outside through wall cracks and spaces between stone walls by soil, rain water, small animals (e.g., mites and collembolans), plant roots, and air. In addition, human factors are considered to be a means of invasion. In conclusion, the microbial biodeterioration and contamination inside the stone chamber was caused by multiple factors, as suggested by the ICECDMPTT (2010). Further studies are needed to elucidate the mechanisms of biodeterioration.

In closing, lessons learned and future perspectives based on our microbiological surveys to date are:

- (1) When sampling sites and materials that are national

historic sites and national treasures or suchlike, such as the Takamatsuzuka and Kitora Tumuli, non-destructive sampling methods are applied with some limitations (e.g., time, space, and sample amounts) under related laws. As a result, small amounts and numbers of samples frequently are used for the isolation of microorganisms. The acquired data (microbiota or microbial diversity) from such sampling using culture-dependent (cultivation) methods should be carefully analyzed and accessed, and the conclusions should be drawn after careful consideration. Culture-independent (molecular) methods complement traditional culture-dependent methods. A good example is *Gluconacetobacter*, one of the acetic acid bacteria genera. These bacteria were not isolated from the initial TT samples using the conventional isolation method. The DGGE analysis suggested the presence of acetic acid bacteria from the samples taken during the dismantling work and in the burial mound soil. Consequently, we used the enrichment culture method to isolate the bacteria in question from these samples. As a result, we obtained 10 isolates of the acetic acid bacteria from seven samples (Nishijima et al., 2013; Tazato et al., 2012). Recently, Solden et al. (2016) stated: “Yet, in many environments, only 0.1–1% of them have been cultivated, greatly hindering our understanding of the microbial world”. We should therefore pay more attention to the diversity of uncultured microorganisms (Kamagata, 2007; Lewis et al., 2010; Solden et al., 2016; Stackebrandt and Embley, 2000), even within a tumulus stone chamber which has a volume of only 3.13 m³, as in the case of the TT stone chamber interior.

(2) Accurate species identification, descriptions, and records of species with complete bioprofiling (or biological characterization) are essential for monitoring anything from global climate change (Systematics Agenda 2000, 1994) to a small tumulus stone chamber (this review). These data, namely information and knowledge of the identity of microbial contaminants/disturbances, are helpful for planning strategies for conservation measures (e.g., biocide treatments).

(3) The interior of a tumulus stone chamber is a small, narrow living environment and has a delicate ecosystem and enough nutrients for subterranean organisms in spite of the lack of light. In general, therefore, one might think that a stone chamber interior lacks diversity; however, a variety of life forms live even in such an oligotrophic environment. We must observe/detect not only the visible and invisible microbial diversity but also that of other organisms, especially arthropods, such as mites, collembolans (springtails), and other small animals. It is easy to imagine that these arthropods play a great role as vectors of microbial propagules, and as nutrients once dead. In short, “a compound eye” is needed when searching microbiota and/or biota concerning the cause of biodeterioration.

(4) Careful observations on site for the materials are recommended, including a loupe and possibly a portable microscope in addition to the naked eye. Microscopic observations of slide preparations of a material provide a great deal of vivid information at the sampling site; for example, if moldy or viscous gel (biofilm) samples are

observed through a microscope, its major microorganisms can be roughly identified immediately, and additional information acquired will be useful for mapping out the strategy for isolation in a laboratory.

(5) As for airborne fungi and an index for risk management, a few comments may be made. In the survey for airborne fungal contamination in show caves, Porca et al. (2011) proposed an index for cave disturbance risk over five categories, ranging from a cave with no problem to a cave with irreversible ecological disturbance. They said this index was not applicable to huge caves with large halls, but no comment was made regarding small stone chambers. The size/volume of stone chambers of both tumuli was too small to compare with show caves, such as the Altamira or Lascaux Cave. Therefore, the index was not adapted directly to either tumuli, but it provided us with an indication of disturbance. The fungal population of the air inside the TT stone chamber was more than 500 cfu/m³ in December 2003 and 420 cfu/m³ in the adjacent room (the room in front of the stone chamber, Fig. 2) (Sano et al., 2004). These were risk level 4 (already affected by fungi) and risk level 3 (threatened by fungi), respectively. In contrast, only two colonies were detected from the air of the KT stone chamber interior before excavation (January 2004) (Sano et al., 2005). After the excavation and relocation work was carried out, approximately 60 colonies maximum were detected in the air of the stone chamber interior between 2005 and 2006, although the detection method differed (Mabuchi and Sano, 2007). Fungal colonies in the KT air were not so abundant, and the most prevalent fungus detected was *Penicillium*, but many fungi and bacteria were isolated from the plaster surface of the stone chamber interior at the same time. It was thought that the predominant fungal genera, dark *Acremonium*, *Cylindrocarpon*, *Phialocephala*, and *Fusarium*, produce slimy numerous conidia, so they were not distributed by air. Therefore, the fungal concentration of air in the KT stone chamber was low, even though many fungal colonies were observed on the plaster walls. The maximum fungal concentration of air outside the KT was around 100 cfu (colony forming unit)/m³ in winter and over 6000 cfu/m³ in summer (Mabuchi and Sano, 2007). The predominant fungal genera in the air of the KT stone chamber exterior were *Cladosporium*, *Arthrimum*, and *Fusarium*; however, *Penicillium* was predominant inside both the conservation facility (Fig. 3) and the stone chamber (Mabuchi and Sano, 2007). In both tumuli, the results described above were obtained using methods that differed from those of Porca et al. (2011). In addition, these interiors differed with respect to chamber size and the presence of human visitors (the TT and KT were never open to the public). The fungal threat level was 3 in the Altamira Cave, and members of *Cladosporium* and *Epicoccum* were abundant, possibly caused by air exchange and ventilation from the outside air (Cuezva et al., 2015). For the future, consideration should be given to measuring the number of microorganisms and creating an index for the management of tumulus stone chamber interiors.

(6) In relation to the above-mentioned risk management, development of sophisticated 21st century systems for biomonitoring (Woodward et al., 2013) is needed to pro-

vide non-destructive methods for exploring microbial activities and changes in microbiota/microbial communities (e.g., a few methods in Sterflinger and Piñar, 2013 and Microbial Volatile Organic Compounds [MVOCs] in Pócsi, 2012; Suzuki et al., 2012; Takeuchi et al., 2012). At the same time, there is an urgent need to develop capable young “multifaceted” microbiologists (Bastian and Alabouvette, 2009) who can investigate biodeterioration and related implications for cultural properties and cultural heritage.

(7) The major and noteworthy microbial isolates (with the full strain data) that are identified at the species or genus level should be deposited in public culture collections as vouchers to further scientific studies. They should also be distributed to the science community inside and outside the country. In addition, the acquired data and knowledge should be published rapidly. These actions contribute to the progress of science. The majority of our 730 TT and KT microbial isolates (molds, yeasts, and bacteria) (Anonymous, 2016; cf., <http://www.riken.jp/en/pr/topics/2016/20160719_1>) in addition to our previous depositions closely related to 16 original papers (An et al., 2009; Handa et al., 2016, 2017; Kiyuna et al., 2008, 2011, 2012, 2015, 2017; Nagatsuka et al., 2009, 2016, 2017; Nishijima et al., 2013, 2017a, 2017b; Tazato et al., 2012, 2015; also see Supplementary Materials) are available to researchers in microbiology, conservation science, and related fields around the world through the Japan Collection of Microorganisms (JCM), operated by the RIKEN BioResource Center in Tsukuba, Japan.

(8) Age-related deterioration of material is inevitable. In the case of the TT mural paintings, it is estimated that these were 60–70% deteriorated from the initial state (0%) at the moment of excavation (ICECDMPTT, 2010). The microbial balance and ecosystem within the tumulus stone chamber are delicate. Changes in temperature and humidity, increases in CO₂, and the invasion of new biodeteriogens are triggers for the destruction of that delicate microbial balance. Disturbances originated by human visits and activities (especially restoration work) should be as limited as possible, as suggested by Saiz-Jiménez (2013) regarding the Lascaux Cave in France and Cuezva et al. (2015) regarding the Altamira Cave in Spain. To save valuable historical cultural properties from serious biodeterioration, we should gather knowledge using an effective multidisciplinary survey team.

Acknowledgments

A series of our microbiological investigations into the causes of biodeterioration of mural paintings and stone chambers of the Takamatsuzuka and Kitora Tumuli was approved by the Agency for Cultural Affairs, Japan. The funding was provided in part by Grants-in-Aid in Scientific Research (A) (No. 17206060 to Sadatoshi Miura, 2005–2007; No. 19200057 to Chie Sano, 2007–2011) from the Japan Society for the Promotion of Science (JSPS), and by a Research Grant from the Institute for Fermentation, Osaka (IFO) (to Kwang-Deug An, 2008–2010). The reproduction of several photographs shown in this review (for details, see figure legends) was permitted by the Office for the Protection of Tumuli and Murals, the Agency for Cultural Affairs, Japan, and also by the Asuka Village Board of Education. One of the authors (J.S.) thanks Dr. Hideo Arai, formerly at the National Research Institute for Cultural Properties, Tokyo, who kindly provided information on the *Doratomyces* problems of the Takamatsuzuka Tumulus stone

chamber interior. The writing of this review in part by one of the authors (J.S.) was done at the Department of Botany, National Museum of Nature and Science in Tsukuba as a Visiting Fellow. We thank the Editor and three anonymous reviewers for critical comments and helpful suggestions to improve the manuscript. Finally, we thank the very kind invitation from Dr. Kan Tanaka (Editor-in-Chief), Dr. Hiromi Nishida (Editor), and other editorial members of JGAM, to write and publish this comprehensive review.

Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

References

- Abdulla, H. (2009) Bioweathering and biotransformation of granitic rock minerals by actinomycetes. *Microb. Ecol.*, **58**, 753–761.
- Adetutu, E. M. and Ball, A. S. (2014) Microbial diversity and activity in caves. *Microbiology Australia*, **35**, 192–194.
- Adetutu, E. M., Thorpe, K., Bourne, S., Cao, X., Shahsavari, E. et al. (2011) Phylogenetic diversity of fungal communities in areas accessible and not accessible to tourists in Naracoorte Caves. *Mycologia*, **103**, 959–968.
- Adl, S. M., Simpson, A. G. B., Lane, C. E., Lukeš, J., Bass, D. et al. (2012) The revised classification of eukaryotes. *J. Eukaryot. Microbiol.*, **59**, 429–493.
- Agency for Cultural Affairs, Japan (ed.) (1987) National Treasures, the Takamatsuzuka Tumulus Mural Paintings: Conservation and Repair, 205 pp. (in Japanese).
- Allsopp, D., Seal, K., and Gaylarde, C. (2004) Introduction to Biodeterioration, 2nd ed., Cambridge University Press, U.K., 237 pp.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z. et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.*, **25**, 3389–3402.
- An, K.-D., Kiyuna, T., Kigawa, R., Sano, C., Miura, S. et al. (2009) The identity of *Penicillium* sp. 1, a major contaminant of the stone chambers in the Takamatsuzuka and Kitora Tumuli in Japan, is *Penicillium paneum*. *Antonie van Leeuwenhoek*, **96**, 579–592.
- Anonymous (2016) Microorganisms from ancient tombs. *RIKEN Research*, Fall 2016, p. 8.
- Arai, H. (1984) Microbiological studies on the conservation of mural paintings in tumuli. In Conservation and Restoration of Mural Paintings (1) (Proceedings of the International Symposium on the Conservation and Restoration of Cultural Properties, November 17–21, 1983, Tokyo, Japan), ed. by Ito, N., Emoto, Y., and Miura, S., Tokyo National Institute of Cultural Properties, Tokyo, Tokyo, pp. 117–124.
- Arai, H. (1987) Microbiological environments and the counterplan for the Takamatsuzuka Tumulus mural paintings. In National Treasures, the Takamatsuzuka Tumulus Mural Paintings: Conservation and Repair, The Agency for Cultural Affairs, Japan, pp. 186–196 (in Japanese).
- Arai, H. (1990a) The environmental analysis of archaeological sites. *Trends Analyt. Chem.*, **9**, 213–216.
- Arai, H. (1990b) Biodeterioration of Cultural Properties and Its Control, Tokyo National Research Institute of Cultural Properties, Tokyo, Tokyo, 93 pp. (in Japanese).
- Arai, H., Kenjo, T., Nakasato, T., Miura, S., Mori, H. et al. (1991) Studies on the conservation of Shinto and Buddhist buildings in Nikko designated as national treasure and important cultural property. *Sci. Conserv.*, **30**, 65–128 (in Japanese).
- Aubert, M., Brumm, A., Ramli, M., Sutikna, T., Saptomo, E. W. et al. (2014) Pleistocene cave art from Sulawesi, Indonesia. *Nature*, **514**, 223–227.
- Badali, H., Gueidan, C., Najafzadeh, M. J., Bonifaz, A., Gerrits van den Ende, A. H. G. et al. (2008) Biodiversity of the genus *Cladophialophora*. *Stud. Mycol.*, **61**, 175–191.
- Bahn, P. (2008) Killing Lascaux. *Archaeology*, **61**, 18–19, 66–70.
- Bastian, F. and Alabouvette, C. (2009) Lights and shadows on the conservation of a rock art cave: The case of Lascaux Cave. *Int. J. Speleol.*, **38**, 55–60.

- Bastian, F., Alabouvette, C., and Saiz-Jiménez, C. (2009a) Bacteria and free-living amoeba in the Lascaux Cave. *Res. Microbiol.*, **160**, 38–40.
- Bastian, F., Alabouvette, C., and Saiz-Jiménez, C. (2009b) The impact of arthropods on fungal community structure in Lascaux cave. *J. Appl. Microbiol.*, **106**, 1456–1462.
- Bastian, F., Alabouvette, C., Jurado, V., and Saiz-Jiménez, C. (2009c) Impact of biocide treatments on the bacterial communities of the Lascaux Cave. *Naturwissenschaften*, **96**, 863–868.
- Bastian, F., Jurado, V., Nováková, A., Alabouvette, C., and Saiz-Jiménez, C. (2010) The microbiology of Lascaux Cave. *Microbiology*, **156**, 644–652.
- Berner, M., Wanner, G., and Lubitz, W. (1997) A comparative study of the fungal flora present in medieval wall paintings in the chapel of the castle Herberstein and in the parish church of St Georgen in Styria, Austria. *Int. Biodeterior. Biodegrad.*, **40**, 53–61.
- Bissett, J., Gams, W., Jaklitsch, W., and Samuels, G. J. (2015) Accepted *Trichoderma* names in the year 2015. *IMA Fungus*, **6**, 263–295.
- Busse, H. J., Zlamala, C., Buczolits, S., Lubitz, W., Kämpfer, P. et al. (2003) *Promicromonospora vindobonensis* sp. nov. and *Promicromonospora aerolata* sp. nov., isolated from the air in the medieval 'Virgilkapelle' in Vienna. *Int. J. Syst. Evol. Microbiol.*, **53**, 1503–1507.
- Cañaveras, J. C., Hoyos, M., Sanchez-Moral, S., Sanz-Rubio, E., Bedoya, J. et al. (1999) Microbial communities associated with hydromagnesite and needle-fiber aragonite deposits in a Karstic Cave (Altamira, Northern Spain). *Geomicrobiol. J.*, **16**, 9–25.
- Cañaveras, J. C., Sanchez-Moral, S., Soler, V., and Saiz-Jiménez, C. (2001) Microorganisms and microbially induced fabrics in cave walls. *Geomicrobiol. J.*, **18**, 223–240.
- Cañaveras, J. C., Cuezva, S., Sanchez-Moral, S., Lario, J., Laiz, L. et al. (2006) On the origin of fiber calcite crystals in moonmilk deposits. *Naturwissenschaften*, **93**, 27–32.
- Caneva, G., Nugari, M. P., and Salvadori, O. (eds.) (2008) Plant Biology for Cultural Heritage: Biodeterioration and Conservation, The Getty Conservation Institute, Los Angeles, 408 pp.
- Carlile, M. J. and Watkinson, S. C. (1994) *The Fungi*, Academic Press, San Diego, 301 pp.
- Chaverri, P. and Samuels, G. J. (2002) *Hypocrea lixii*, the teleomorph of *Trichoderma harzianum*. *Mycol. Prog.*, **1**, 283–286.
- Chaverri, P. and Samuels, G. J. (2003) *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Stud. Mycol.*, **48**, 1–116.
- Cheeptham, N. (ed.) (2013) *Cave Microbiomes: A Novel Resource for Drug Discovery*, Springer, New York, 130 pp.
- Ciferri, O. (1999) Microbial degradation of paintings. *Appl. Environ. Microbiol.*, **65**, 879–885.
- Coye, N. (ed.) (2011) *Lascaux and Preservation Issues in Subterranean Environments: Proceedings of the International Symposium Paris, February 26 and 27, 2009*, Éditions de la Maison des sciences de l'homme, Paris, 357 pp.
- Cuezva, S., Sanchez-Moral, S., Saiz-Jiménez, C., and Cañaveras, J. C. (2009) Microbial communities and associated mineral fabrics in Altamira Cave, Spain. *Int. J. Speleol.*, **38**, 83–92.
- Cuezva, S., Fernandez-Cortes, A., Porca, E., Pasic, L., Jurado, V. et al. (2012) The biogeochemical role of *Actinobacteria* in Altamira Cave, Spain. *FEMS Microbiol. Ecol.*, **81**, 281–290.
- Cuezva, S., Jurado, V., Fernandez-Cortes, A., Garcia-Anton, E., Rogerio-Candelera, M. A. et al. (2015) Scientific data suggest Altamira cave should remain closed. In *Microbial Life of Cave Systems*, ed. by Annette, S. E., Walter de Gruyter GmbH, Berlin/Boston, pp. 303–320.
- Dakal, T. C. and Arora, P. K. (2012) Evaluation of potential of molecular and physical techniques in studying biodeterioration. *Rev. Environ. Sci. Bio/Technol.*, **11**, 71–104.
- Dakal, T. C. and Cameotra, S. S. (2012) Microbially induced deterioration of architectural heritage: routes and mechanisms involved. *Environ. Sci. Eur.*, **24**, 1–13.
- Deacon, J. W. (1997) *Modern Mycology*, 3rd ed., Blackwell Science, Oxford, p. 7.
- Dhawan, S., Garg, K. L., and Pathak, N. (1993) Microbial analysis of Ajanta wall paintings and their possible control *in situ*. In *Biodeterioration of Cultural Property 2* (Proceedings of the 2nd International Conference October 5–8, 1992, Yokohama, Japan), ed. by Toishi, K., Arai, H., Kenjo, T., and Yamano, K., International Communications Specialists, Tokyo, pp. 245–262.
- Diakumaku, E., Gorbushina, A. A., Krumbein, W. E., Panina, L., and Soukharjevski, S. (1995) Black fungi in marble and limestones: an aesthetical, chemical and physical problem for the conservation of monuments. *Sci. Total Environ.*, **167**, 295–304.
- Diaz-Herrera, M., Jurado, V., Cuezva, S., Laiz, L., and Pallecchi, P. et al. (2013) The Actinobacterial colonization of Etruscan paintings. *Scientific Reports*, **3**, 1440; doi:10.1038/srep01440.
- Domsch, K. H., Gams, W., and Anderson, T.-H. (2007) *Compendium of Soil Fungi*, 2nd ed., IHW-Verlag, Eching, 672 pp.
- Dromph, K. M. (2003) Collembolans as vectors of entomopathogenic fungi. *Pedobiologia*, **47**, 245–256.
- Dupont, J., Jacquet, C., Denetiere, B., Lacoste, S., Bousta, F. et al. (2007) Invasion of the French Paleolithic painted cave of Lascaux by members of the *Fusarium solani* species complex. *Mycologia*, **99**, 526–533.
- Dutta, D. and Gachhui, R. (2007). Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea. *Int. J. Syst. Evol. Microbiol.*, **57**, 353–357.
- Ellis, M. B. (1971) *Dematiaceae Hyphomycetes*, Commonwealth Mycological Institute, Kew, Surrey, U.K., 608 pp.
- Ellis, M. B. (1976) *More Dematiaceae Hyphomycetes*, Commonwealth Mycological Institute, Kew, U.K., 507 pp.
- Ellis, M. B. (1997) *Microfungi on Land Plants: An Identification Handbook*, New Enlarged edition, The Richmond Publishing Co., Slough, 868 pp.
- Emoto, Y. and Emoto, Y. (1974) Microbiological investigation of ancient tombs with paintings: Ozuka tomb in Fukuoka and Chibusan tomb in Kumamoto. *Sci. Conserv.*, **12**, 95–102 (in Japanese).
- Emoto, Y., Kadokura, T., Kenjo, T., and Arai, H. (1983) Surveys related to the preservation of murals in Torazuka ancient burial mound. *Sci. Conserv.*, **22**, 121–146 (in Japanese).
- Engle, A. S. (ed.) (2015) *Microbial Life of Cave Systems*, De Gruyter, Berlin/Boston, 335 pp.
- Euzéby, J. P. (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet. *Int. J. Syst. Bacteriol.*, **47**, 590–592; List of Prokaryotic names with Standing in Nomenclature <<http://www.bacterio.net>>.
- Fonseca, A., Boekhout, T., and Fell, J. W. (2011) *Cryptococcus* Vuillemin (1901). In *The Yeasts, A Taxonomic Study*, 5th ed., ed. by Kurtzman, C. P., Fell, J. W., and Boekhout, T., Elsevier, Amsterdam, pp. 1661–1737.
- Fox, J. L. (2008) Some say Lascaux cave paintings are in microbial "Crisis" mode. *Microbe*, **3**, 110–112.
- Franke, I. H., Fegan, M., Hayward, C., Leonard, G., Stackebrandt, E. et al. (1999) Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. *Int. J. Syst. Bacteriol.*, **49**, 1681–1693.
- Frisvad, J. C. and Samson, R. A. (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*—A guide to identification of food and air-borne terverticillate *Penicillia* and their mycotoxins. *Stud. Mycol.*, **49**, 1–173.
- Fry, N. K., Duncan, J., Malnick, H., Warner, M., Smith, A. J. et al. (2005). *Bordetella petrii* clinical isolate. *Emerg. Infect. Dis.*, **11**, 1131–1133.
- Fuentes-Ramírez, L. E., Bustillos-Cristales, R., Tapia-Hernández, A., Jiménez-Salgado, T., Wang, E. T. et al. (2001) Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. *Int. J. Syst. Evol. Microbiol.*, **51**, 1305–1314.
- Gams, W. (1971) *Cephalosporium-artige Schimmelpilze* (Hyphomycetes), Gustav Fischer Verlag, Stuttgart, 262 pp.
- Gams, W., van der Aa, H. A., van der Plaats-Niterink, A. J., Samson, S. A., and Stalpers, J. A. (1987) *CBS Course of Mycology*, 3rd ed., Centraalbureau voor Schimmelfcultures, Baarn, 136 pp.
- Ganter, P. F. (2006) Yeast and invertebrate associations. In *Biodiversity and Ecophysiology of Yeasts*, ed. by Rosa, C. A. and Gábor, P., Springer, Heidelberg, pp. 303–370.
- Garcia-Anton, E., Cuezva, S., Jurado, V., Porca, E., Miller, A. Z. et al. (2014) Combining stable isotope ($\delta^{13}\text{C}$) of trace gases and aerobiological data to monitor the entry and dispersion of microor-

- ganisms in caves. *Environ. Sci. Pollut. Res.*, **21**, 473–484.
- Garg, K. L., Jain, K. K., and Mishra, A. K. (1995) Role of fungi in the deterioration of wall paintings. *Sci. Total Environ.*, **167**, 255–271.
- Geneste, J.-M. (2011) The major phases in the conservation of Lascaux Cave. In *Lascaux and Preservation Issues in Subterranean Environments: Proceedings of the International Symposium Paris, February 26 and 27, 2009*, ed. by Coye, N., Éditions de la Maison des sciences de l'homme, Paris, pp. 51–71.
- Gillis, M., Kersters, K., Hoste, B., Janssens, D., Kroppenstedt, R. M. et al. (1989) *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. *Int. J. Syst. Bacteriol.*, **39**, 361–364.
- Giraldo, A., Gené, J., Sutton, D. A., Madrid, H., de Hoog, G. S. et al. (2015). Phylogeny of *Sarocladium* (Hypocreales). *Persoonia*, **34**, 10–24.
- Goidanich, G. (1937) Studi sulla microflora fungina della pasta di legno destinata alla fabbricazione della carta. *Bollettino della Stazione di Patologia vegetale Roma*, **17**, 305–399.
- Gomez-Alarcon, G. and de la Torre, M. A. (1994) The effect of filamentous fungi on stone monuments: the Spanish experience. In *Building Mycology*, ed. by Singh, J., Chapman & Hall, London, pp. 295–309.
- Gonzalez, J. M. and Saiz-Jiménez, C. (2005) Application of molecular nucleic acid-based techniques for the study of microbial communities in monuments and artworks. *Int. Microbiol.*, **8**, 189–194.
- Gonzalez, J. M., Portillo, M. C., and Saiz-Jiménez, C. (2006) Metabolically active *Crenarchaeota* in Altamira Cave. *Naturwissenschaften*, **93**, 42–45.
- Gonzalez, J. M., Portillo, M. C., and Saiz-Jiménez, C. (2008a) Bacterial diversity in the cave of Altamira. *Coalition*, **15**, 2–6; <www.rtphc.csic.es/boletin.htm>.
- Gonzalez, J. M., Portillo, M. C., and Saiz-Jiménez, C. (2008b) Microbes pose a risk to prehistoric cave paintings. *Microbe*, **3**, 72–77.
- Gorbushina, A. A. and Petersen, K. (2000) Distribution of microorganisms on ancient wall paintings as related to associated faunal elements. *Int. Biodeterior. Biodegrad.*, **46**, 277–284.
- Graff, J. (2006) Saving beauty. *TIME Eur.*, **167**, 36–42.
- Greif, M. D. and Currah, R. S. (2007) Patterns in the occurrence of saprophytic fungi carried by arthropods caught in traps baited with rotted wood and dung. *Mycologia*, **99**, 7–19.
- Gross, R., Guzman, C. A., Sebaihia, M., Martins dos Santos, V. A. P., Pieper, D. H. et al. (2008). The missing link: *Bordetella petrii* is endowed with both the metabolic versatility of environmental bacteria and virulence traits of pathogenic *Bordetellae*. *BMC Genomics*, **9**, 449.
- Groth, I. and Saiz-Jiménez, C. (1999) Actinomycetes in hypogean environments. *Geomicrobiol. J.*, **16**, 1–8.
- Groth, I., Vettermann, R., Schuetze, B., Schumann, P., and Saiz-Jiménez, C. (1999) Actinomycetes in Karstic caves of northern Spain (Altamira and Tito Bustillo). *J. Microbiol. Methods*, **36**, 115–122.
- Gu, J.-D., Ford, T. E., Berke, N. S., and Mitchell, R. (1998) Biodeterioration of concrete by the fungus *Fusarium*. *Int. Biodeterior. Biodegrad.*, **41**, 101–109.
- Guglielminetti, M., Morghen, C. G., Radaelli, A., Bistoni, F., Carruba, G. et al. (1994) Mycological and ultrastructural studies to evaluate biodeterioration of mural paintings: detection of fungi and mites in frescos of the Monastery of St Damian in Assisi. *Int. Biodeterior. Biodegrad.*, **33**, 269–283.
- Guo, L., Liu, C., Zhao, J., Li, C., Guo, S. et al. (2016) *Promicromonospora alba* sp. nov., an actinomycete isolated from the cuticle of *Camponotus japonicus* Mayr. *Int. J. Syst. Evol. Microbiol.*, **66**, 1340–1345.
- Gurtner, C., Heyrman, J., Pinar, G., Lubitz, W., Swings, J. et al. (2000) Comparative analyses of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis. *Int. Biodeterior. Biodegrad.*, **46**, 229–239.
- Handa, Y., Tazato, N., Nagatsuka, Y., Koide, T., Kigawa, R. et al. (2016) *Stenotrophomonas tumulicola* sp. nov., a major contaminant of the stone chamber interior in the Takamatsuzuka Tumulus. *Int. J. Syst. Evol. Microbiol.*, **66**, 1119–1124.
- Handa, Y., Tazato, N., Sato, Y., Kigawa, R., Sano, C. et al. (2017) Molecular phylogenetic placements of *Bacillus* and *Ochrobactrum* isolates from the Takamatsuzuka and Kitora Tumuli. *Sci. Conserv.*, **56** (in press) (in Japanese).
- Hawser, S. P. and Douglas, L. J. (1994) Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect. Immun.*, **62**, 915–921.
- Heyrman, J. and Swings, J. (2003) Modern diagnostic techniques on isolates. *Coalition*, **6**, 9–13.
- Heyrman, J., Logan, N. A., Rodríguez-Díaz, M., Scheldeman, P., Lebbe, L. et al. (2005) Study of mural painting isolates, leading to the transfer of '*Bacillus maroccanus*' and '*Bacillus carotarum*' to *Bacillus simplex*, emended description of *Bacillus simplex*, re-examination of the strains previously attributed to '*Bacillus macroides*' and description of *Bacillus muralis* sp. nov. *Int. J. Syst. Evol. Microbiol.*, **55**, 119–131.
- Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F. et al. (2007) A higher-level phylogenetic classification of the Fungi. *Mycol. Res.*, **111**, 509–547.
- Hoffmann, A., Thimm, T., Dröge, M., Moore, E. R. B., Munch, J. C. et al. (1998) Intergeneric transfer of conjugative and mobilizable plasmids harbored by *Escherichia coli* in the gut of the soil microarthropod *Folsomia candida* (Collembola). *Appl. Environ. Microbiol.*, **64**, 2652–2659.
- Hotson, J. W. (1912) Cultural studies of fungal producing bulbils and similar propagative bodies. *Proc. Am. Acad. Arts Sci.*, **48**, 227–306.
- Huber, B., Scholz, H. C., Kämpfer, P., Falsen, E., and Langer, S. et al. (2010) *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int. J. Syst. Evol. Microbiol.*, **60**, 321–326.
- Hughes, S. J. (1958) Revisions of hyphomycetum aliquot cum appendice de nominibus rejiciendis. *Can. J. Bot.*, **36**, 727–836.
- ICECDMPPT (Investigation Committee for Elucidating the Cause of Deterioration of the Mural Paintings of Takamatsuzuka Tumulus) (2010) Report on Investigation of the Cause of Deterioration of the Mural Paintings of Takamatsuzuka Tumulus, Agency for Cultural Affairs, Japan (in Japanese); the pdf file available at <http://www.bunka.go.jp/takamatsu_kitora/kentokaito/pdf/rekka_houkokusho_ver02.pdf>.
- Ikner, L. A., Toomey, R. S., Nolan, G., Neilson, J. W., Pryor, B. M. et al. (2007) Culturable microbial diversity and the impact of tourism in Kartchner Caverns, Arizona. *Microbial Ecol.*, **53**, 30–42.
- Inuzuka, M. and Ishizaki, T. (2007) Environmental measurement for dismantling the stone chamber of Takamatsuzuka Tumulus. *Sci. Conserv.*, **46**, 221–226 (in Japanese).
- Ishizaki, T. and Kigawa, R. (2011) Conservation of mural paintings of Takamatsuzuka and Kitora Tumuli in Japan. In *Lascaux and Preservation Issues in Subterranean Environments*, Proceedings of the International Symposium Paris, February 26 and 27, 2009, ed. by Coye, N., Éditions de la Maison des sciences de l'homme, Paris, pp. 261–274.
- Jacobs, K. and Wingfield, M. J. (2001) *Leptographium* Species: Tree Pathogens, Insect Associates, and Agents of Blue-stain, APS Press, St. Paul, Minnesota, 220 pp.
- Jiang, Y.-L. and Zhang, T.-Y. (2008) Two new species of *Doratomyces* from soil. *Mycotaxon*, **104**, 131–134.
- Jurado, V., Sanchez-Moral, S., and Saiz-Jiménez, C. (2008) Entomogenous fungi and the conservation of the cultural heritage: A review. *Int. Biodeterior. Biodegrad.*, **62**, 325–330.
- Jurado, V., Fernandez-Cortes, A., Cuezva, S., Laiz, L., Canaveras, J. C. et al. (2009) The fungal colonisation of rock-art caves: experimental evidence. *Naturwissenschaften*, **96**, 1027–1034.
- Jurado, V., Laiz, L., Rodriguez-Nava, V., Boiron, P., Hermosin, H. et al. (2010) Pathogenic and opportunistic microorganisms in caves. *Int. J. Speleol.*, **39**, 15–24.
- Jurado, V., Laiz, L., Sanchez-Moral, S., and Saiz-Jiménez, C. (2014) Pathogenic microorganisms related to human visits in Altamira Cave, Spain. In *The Conservation of Subterranean Culture Heritage*, ed. by Saiz-Jiménez, C., Taylor & Francis Group, London, pp. 229–238.
- Kamagata, Y. (2007) What are uncultured microbes? *J. Environ. Biotechnol.*, **7**, 69–73 (in Japanese).
- Kämpfer, P., Huber, B., Busse, H. J., Scholz, H. C., Tomaso, H. et al. (2011) *Ochrobactrum pecoris* sp. nov., isolated from farm animals. *Int. J. Syst. Evol. Microbiol.*, **61**, 2278–2283.
- Kämpfer, P., Wohlgemuth, S., and Scholz, H. (2014). The Family *Brucellaceae*. In *The Prokaryotes—Alphaproteobacteria* and

- Betaproteobacteria*, 4th ed., ed. by Rosenberg, E. et al., Springer, Berlin/Heidelberg, pp. 155–178.
- Kaparullina, E., Doronina, N., Chistyakova, T., and Trotsenko, Y. (2009) *Stenotrophomonas chelatiphaga* sp. nov., a new aerobic EDTA-degrading bacterium. *Syst. Appl. Microbiol.*, **32**, 157–162.
- Karbowska-Berent, J. (2003) Microbiobiodeterioration of mural paintings: a review. In *Art, Biology, and Conservation: Biodeterioration of Works of Art*, ed. by Koestler, R. J., Koestler, V. H., Charola, A. E., and Nietro-Fernandez, F. E., The Metropolitan Museum of Art, New York, pp. 267–301.
- Kerstens, K., Lisdiyanti, P., Komagata, K., and Swings, J. (2006) The family *Acetobacteraceae*: The genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. In *Prokaryotes, A Handbook on the Biology of Bacteria*, 3rd ed., ed. by Dworkin, M. et al., Springer, New York, pp. 163–200.
- Kigawa, R., Sano, C., and Miura, S. (2004) Past and present situation of microorganisms in Takamatsuzuka Tumulus. *Sci. Conserv.*, **43**, 79–85 (in Japanese).
- Kigawa, R., Sano, C., Mabuchi, H., and Miura, S. (2005) Investigation of moulds in Kitora Tumulus during its excavation and restoration works. *Sci. Conserv.*, **44**, 165–171 (in Japanese).
- Kigawa, R., Mabuchi, H., Sano, C., and Miura, S. (2006a) Investigation of biological issues in Kitora Tumulus during its restoration work (2). *Sci. Conserv.*, **45**, 93–105 (in Japanese).
- Kigawa, R., Sano, C., Ishizaki, T., and Miura, S. (2006b) Concept and measures of the conservation of Takamatsuzuka Tumulus for thirty years and the present situation of biodeterioration. *Sci. Conserv.*, **45**, 33–58 (in Japanese).
- Kigawa, R., Sano, C., Ishizaki, T., and Miura, S. (2007a) Circumstances of microorganisms in Takamatsuzuka Tumulus in 2006. *Sci. Conserv.*, **46**, 209–219 (in Japanese).
- Kigawa, R., Sano, C., Mabuchi, H., and Miura, S. (2007b) Investigation of biological issues in Kitora Tumulus during its restoration work (3). *Sci. Conserv.*, **46**, 227–233 (in Japanese).
- Kigawa, R., Sano, C., Tazato, N., Kiyuna, T., Koide, T. et al. (2007c) The effect of a biocide (Kathon CG) on bacterial and fungi isolated from biofilms on the plaster paintings of Kitora Tumulus and its direct effect on the biofilms. *Sci. Conserv.*, **46**, 39–50 (in Japanese).
- Kigawa, R., Sugiyama, J., Takatori, K., Mabuchi, H., and Sano, C. et al. (2008a) Overview of biological survey at Takamatsuzuka Tumulus during the excavation and relocation of the stone chamber in 2007. *Sci. Conserv.*, **47**, 121–128 (in Japanese).
- Kigawa, R., Mabuchi, H., Sano, C., and Miura, S. (2008b) Biological issues in Kitora Tumulus during relocation work of the mural paintings (2007). *Sci. Conserv.*, **47**, 129–133 (in Japanese).
- Kigawa, R., Sano, C., Ishizaki, T., Miura, S., and Sugiyama, J. (2009a) Biological issues in the conservation of mural paintings of Takamatsuzuka and Kitora Tumuli in Japan. In *International Symposium on the Conservation and Restoration of Cultural Property—Study of Environmental Conditions Surrounding Cultural Properties and Their Protective Measures*, ed. by Sano, C., National Research Institute of Cultural Properties, Tokyo, pp. 43–50.
- Kigawa, R., Sano, C., Mabuchi, H., Kiyuna, T., Tazato, N. et al. (2009b) Biological issues in Kitora Tumulus during restoration works of the mural paintings (2008). *Sci. Conserv.*, **48**, 168–174 (in Japanese).
- Kigawa, R., Sano, C., Takatori, K., Kiyuna, T., Sugiyama, J. et al. (2010a) Fungal growth tests of materials used in Takamatsuzuka Tumulus and for protective facing of the mural paintings in the recent relocation. *Sci. Conserv.*, **49**, 61–71 (in Japanese).
- Kigawa, R., Sano, C., Kiyuna, T., Tazato, N., and Sugiyama, J. (2010b) Use of ethanol and isopropanol as carbon sources by microorganisms isolated from Takamatsuzuka and Kitora Tumuli. *Sci. Conserv.*, **49**, 231–238 (in Japanese).
- Kigawa, R., Sano, C., Kiyuna, T., Tazato, N., Sugiyama, J. et al. (2010c) New measure to control microorganisms in Kitora Tumulus: Effects of intermittent UV irradiation (2009). *Sci. Conserv.*, **49**, 253–264 (in Japanese).
- Kigawa, R., Sano, C., Kiyuna, T., Tazato, N., Sugiyama, J. et al. (2011) Microorganisms in Kitora Tumulus during intermittent UV irradiation between restoration works (2010). *Sci. Conserv.*, **50**, 191–195 (in Japanese).
- Kigawa, R., Sano, C., Kiyuna, T., Tazato, N., Sugiyama, J. et al. (2012a) Microbial growth tests with consolidations of mural paintings: A case study of bacterial and yeast strains isolated from Kitora Tumulus. *Sci. Conserv.*, **51**, 157–166 (in Japanese).
- Kigawa, R., Sato, Y., Kiyuna, T., Tazato, N., Sugiyama, J. et al. (2012b) Microorganisms in Kitora Tumulus during intermittent UV irradiation (2011). *Sci. Conserv.*, **51**, 167–171 (in Japanese).
- Kigawa, R., Sano, C., Nishijima, M., Tazato, N., Kiyuna, T. et al. (2013a) Investigation of acetic acid bacteria isolated from the Kitora Tumulus in Japan and their involvement in the deterioration of the plaster of the mural paintings. *Stud. Conserv.*, **58**, 30–40.
- Kigawa, R., Kiyuna, T., Tazato, N., Sato, Y., and Sugiyama, J. (2013b) Tolerance test of microorganisms isolated from Kitora Tumulus against UV irradiation. *Sci. Conserv.*, **52**, 91–105 (in Japanese).
- Kigawa, R., Kiyuna, T., Tazato, N., Sato, Y., Sano, C. et al. (2015) Summary of the microbial surveys of the Kitora Tumulus from 2004 to 2013. *Sci. Conserv.*, **54**, 83–109 (in Japanese).
- Kim, H. J., Eom, H. J., Park, C., Jung, J., Shin, B. et al. (2016) Calcium carbonate precipitation by *Bacillus* and *Sporosarcina* strains isolated from concrete and analysis of the bacterial community of concrete. *J. Microbiol. Biotechnol.*, **26**, 540–548.
- Kirk, P. M., Cannon, P. F., Minter, D. W., and Stalpers, J. A. (2008) *Dictionary of the Fungi*, 10th ed., CAB International, Wallingford, 771 pp.
- Kitada, M., Kohzuma, Y., and Tateishi, T. (2015) Microstructure of surface contaminant layer of joint stucco between stone walls of Takamatsuzuka Tumulus. *J. Jpn. Inst. Met. Mater.*, **79**, 404–412 (in Japanese).
- Kiyuna, T., An, K.-D., Kigawa, R., Sano, C., Miura, S. et al. (2008) Mycobiota of the Takamatsuzuka and Kitora Tumuli in Japan, focusing on the molecular phylogenetic diversity of *Fusarium* and *Trichoderma*. *Mycoscience*, **49**, 298–311.
- Kiyuna, T., An, K.-D., Kigawa, R., Sano, C., Miura, S. et al. (2011) Molecular assessment of fungi in “black spots” that deface murals in the Takamatsuzuka and Kitora Tumuli in Japan: *Acremonium* sect. *Gliomastix* including *Acremonium tumulicola* sp. nov. and *Acremonium felinum* comb. nov. *Mycoscience*, **52**, 1–17.
- Kiyuna, T., An, K.-D., Kigawa, R., Sano, C., Miura, S. et al. (2012) Bristle-like fungal colonizers on the stone walls of the Kitora and Takamatsuzuka Tumuli are identified as *Kendrickiella phycomyces*. *Mycoscience*, **53**, 446–459.
- Kiyuna, T., An, K.-D., Kigawa, R., Sano, C., Miura, S. et al. (2015) “Black particles”, the major colonizers on the ceiling stone of the stone chamber interior of the Kitora Tumulus, Japan, are the bulbiliferous basidiomycete fungus *Burgoa anomala*. *Mycoscience*, **56**, 293–300.
- Kiyuna, T., An, K.-D., Kigawa, R., Sano, C., and Sugiyama, J. (2017) Noteworthy anamorphic fungi, *Cephalotrichum verrucisporum*, *Sagenomella striatispora*, and *Sagenomella griseoviridis*, isolated from samples collected while dismantling the Takamatsuzuka Tumulus stone chamber, Nara, Japan. *Mycoscience* (in press); dx.doi.org/10.1016/j.myc.2017.02.003.
- Ko, K. S., Peck, K. R., Oh, W. S., Lee, N. Y., Lee, J. H. et al. (2005) New species of *Bordetella*, *Bordetella ansorpilii* sp. nov., isolated from the purulent exudate of an epidermal cyst. *J. Clin. Microbiol.*, **43**, 2516–2519.
- Komagata, K., Iino, T., and Yamada, Y. (2014) The family *Acetobacteraceae*. In *The Prokaryotes—Alphaproteobacteria and Betaproteobacteria*, 4th ed., ed. by Rosenberg, E. et al., Springer, Berlin Heidelberg, pp. 1–78; doi:10.1007/978-3-642-30197-1_396.
- Krug, J. C. (2004) Moist chambers for the development of fungi. In *Biodiversity of Fungi, Inventory and Monitoring Methods*, ed. by Muller, G. M., Bills, G. F., and Foster, M. S., Elsevier Academic Press, Amsterdam, pp. 589–593.
- Kurtzman, C. P. (2011) *Meyerozyma Kurtzman & M. Suzuki* (2010). In *The Yeasts, A Taxonomic Study*, 5th ed., ed. by Kurtzman, C. P., Fell, J. W., and Boekhout, T., Elsevier, Amsterdam, pp. 621–624.
- Kurtzman, C. P., Fell, J. W., Boekhout, T., and Robert, V. (2011) Methods for the isolation, phenotypic characterization and maintenance of yeasts. In *The Yeasts, A Taxonomic Study*, 5th ed., ed. by Kurtzman, C. P., Fell, J. W., and Boekhout, T., Elsevier, Amsterdam, pp. 87–110.
- Kusumi, A., Li, X. S., and Katayama, Y. (2011) Mycobacteria isolated from Angkor monument sandstones grow chemolithoautotrophically

- by oxidizing elemental sulfur. *Front. Microbiol.*, 2:104 PMID 21747806.
- Lachance, M. A., Boekhout, T., Scorzetti, G., Fell, J. W., and Kurtzman, C. P. (2011) *Candida* Berkhout. In *The Yeasts, A Taxonomic Study*, 5th ed., ed. by Kurtzman, C. P., Fell, J. W., and Boekhout, T., Elsevier, Amsterdam, pp. 987–1278.
- Laiz, L., Groth, I., Gonzalez, I., and Saiz-Jiménez, C. (1999) Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). *J. Microbiol. Methods*, **36**, 129–138.
- Laiz, L., Gonzalez, J. M., and Saiz-Jiménez, C. (2003) Microbial communities in caves: Ecology, physiology, and effects on paleolithic paintings. In *Art, Biology, and Conservation: Biodeterioration of Works of Art*, ed. by Koestler, R. J., Koestler, V. H., Charola, A. E., and Nietro-Fernandez, F. E., The Metropolitan Museum of Art, New York, pp. 210–215.
- Lasheras, J. A., Sánchez-Moral, S., Sáiz-Jiménez, C., Cănaveras, J. C., and de las Heras, C. (2011) The conservation of Altamira Cave: a comparative perspective. In *Lascaux and Preservation Issues in Subterranean Environments*, Proceedings of the International Symposium Paris, February 26 and 27, 2009, ed. by Coye, N., Éditions de la Maison des sciences de l'homme, Paris, pp. 169–182.
- Le Coustumier, A., Njamkepo, E., Cattoir, V., Guillot, S., and Guiso, N. (2011). *Bordetella pertussis* infection with long-lasting persistence in human. *Emer. Infect. Dis.*, **17**, 612–618.
- Lee, N. M., Meisinger, D. B., Aubrecht, R. et al. (2012) Caves and karst environments. In *Life at Extremes: Environments, Organisms and Strategies for Survival*, ed. by Bell, E. M. et al., CAB International, Wallingford, pp. 320–344.
- Lefevre, M. (1974) La 'Maladie Verte' de Lascaux. *Stud. Conserv.*, **19**, 126–156.
- Lewis, K., Epstein, S., D'Onofrio, A., and Ling, L. I. (2010) Uncultured microorganisms as a source of secondary metabolites. *J. Antibiot.*, **63**, 468–476.
- Li, Q., Zhang, B., He, Z., and Yang, X. (2016) Distribution and diversity of bacteria and fungi colonization in stone monuments analyzed by high-throughput sequencing. *PLoS ONE*, **11**, e0163287.
- Li, X. M., Sato, T., Ooiwa, Y., Kusumi, A., Gu, J.-D. et al. (2010) Oxidation of elemental sulfur by *Fusarium solani* strain THIF01 harboring endobacterium *Bradyrhizobium* sp. *Microbial Ecol.*, **60**, 96–104.
- Logan, N. A. and De Vos, P. (2009) Genus I. *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*. Volume 3: The *Firmicutes*, ed. by Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W. et al., Springer, New York, pp. 21–128.
- Luangsa-Ard, J. I., Houbraken, J., van Doorn, T., Hong, S. B., Borman, A. M. et al. (2011) *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. *FEMS Microbiol. Lett.*, **321**, 141–149.
- Ma, Y., Zhang, H., Tian, T., Xiang, T., Liu, X. et al. (2015) The community distribution of bacteria and fungi on ancient wall paintings of the Mogao Grottoes. *Sci. Rep.*, **5**, 7752; doi:10.1038/srep07752.
- Mabuchi, H. and Sano, C. (2007) Investigation for evaluation of dynamic indoor environment in terms of airborne mold measurement—A model case at the conservation facility of Kitora Tumulus. *Sci. Conserv.*, **46**, 27–37 (in Japanese).
- Macedo, M. F., Miller, A. Z., Dionísio, A., and Saiz-Jiménez, C. (2009) Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview. *Microbiology*, **155**, 3476–3490.
- Mamlouk, D. and Gullo, M. (2013) Acetic acid bacteria: Physiology and carbon sources oxidation. *Indian J. Microbiol.*, **53**, 377–384.
- Martin, K., Schäfer, J., and Kämpfer, P. (2010) *Promicromonospora umidemergens* sp. nov., isolated from moisture from indoor wall material. *Int. J. Syst. Evol. Microbiol.*, **60**, 537–541.
- Martin-Sanchez, P. M. and Saiz-Jiménez, C. (2014) Contribution of culture-independent methods to cave aerobiology: the case of Lascaux Cave. In *The Conservation of Subterranean Cultural Heritage*, ed. by Saiz-Jiménez, C., Taylor & Francis Group, London, pp. 215–222.
- Martin-Sanchez, P. M., Nováková, A., Bastian, F., Alabouvette, C., and Saiz-Jiménez, C. (2012a) Use of biocides for the control of fungal outbreaks in subterranean environments: The case of the Lascaux cave in France. *Environ. Sci. Technol.*, **46**, 3762–3770.
- Martin-Sanchez, P. M., Nováková, A., Bastian, F., Alabouvette, C., and Saiz-Jiménez, C. (2012b) Two new species of the genus *Ochroconis*, *O. lascauxensis* and *O. anomala* isolated from black stains in Lascaux Cave, France. *Fung. Biol.*, **116**, 574–589.
- Martin-Sanchez, P. M., Miller, A. Z., and Saiz-Jiménez, C. (2015) Lascaux Cave: An example of fragile ecological balance in subterranean environments. In *Microbial Life in Cave Systems*, ed. by Engel, A. S., De Gruyter, Berlin/Boston, pp. 279–301.
- Mason, E. W. (1937) Annotated account of fungi received at The Imperial Mycological Institute, List 2, fasc. 3 (general part), 68–99.
- Masuda, M., Hirose, N., Ishikawa, T., Ikawa, Y., and Nishimura, K. (2016) *Prototheca miyajii* sp. nov., isolated from a patient with systemic protothecosis. *Int. J. Syst. Evol. Microbiol.*, **66**, 1510–1520.
- Mohri, M. (2007) Takamatsuzuka-Kofun wa Mamoreruka? The Challenge from Science in Conservation', NHK Books 1082, Japan Broadcasting Corporation, Tokyo, 251 pp.
- Mouhamadou, B., Faure, M., Sage, L., Marçais, J., Souard, F. et al. (2013) Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. *Fung. Biol.*, **117**, 268–274.
- Nagatsuka, Y., Kawasaki, H., Limtong, S., Mikata, K., and Seki, T. (2002) *Citeromyces siamensis* sp. nov., a novel halotolerant yeast isolated in Thailand. *Int. J. Syst. Evol. Microbiol.*, **52**, 2315–2319.
- Nagatsuka, Y., Kawasaki, H., Mikata, K., and Seki, T. (2005) *Candida khmerensis* sp. nov., a novel cation-tolerant yeast isolated from dry salted shrimp and sewage in Cambodia. *J. Gen. Appl. Microbiol.*, **51**, 235–243.
- Nagatsuka, Y., Kiyuna, T., Kigawa, R., Sano, C., Miura, S. et al. (2009) *Candida tumulicola* sp. nov. and *Candida takamatsuzukensis* sp. nov., novel yeast species assignable to the *Candida membranifaciens* clade, isolated from the stone chamber of the Takamatsuzuka tumulus. *Int. J. Syst. Evol. Microbiol.*, **59**, 186–194.
- Nagatsuka, Y., Ninomiya, S., Kiyuna, T., Kigawa, R., Sano, C. et al. (2016) *Yamadazyma kitorensis* f.a., sp. nov. and *Zygoascus biomembranicola* f.a., sp. nov., novel yeasts from the stone chamber interior of the Kitora Tumulus, and five novel combinations in *Yamadazyma* and *Zygoascus* for species of *Candida*. *Int. J. Syst. Evol. Microbiol.*, **66**, 1692–1704.
- Nagatsuka, Y., Kiyuna, T., Kigawa, R., Sano, C., and Sugiyama, J. (2017) *Prototheca tumulicola* sp. nov., a novel achlorophyllous algal species isolated from the stone chamber interior of the Takamatsuzuka Tumulus. *Mycoscience*, **58**, 53–59.
- Nara National Research Institute for Cultural Properties (ed.) (2006) An Investigation of the Takamatsuzuka Tumulus: A Report of Excavation Survey in 2004 for the Purpose of the Long-term Committee for the Mural Paintings of Takamatsuzuka Tumulus, Nara National Institute for Cultural Properties, Nara, 62 pp.+16 pls. (in Japanese).
- Nara National Research Institute for Cultural Properties (ed.) (2008) The Special Historic Site—Report on Investigation of Excavation and Research of the Kitora Tumulus, Agency for Cultural Affairs, Nara National Research Institute for Cultural Properties, Archaeological Institute of Kashihara, Nara Prefecture and Asuka Village Board of Education, Nara, 101 pp.+31 pls. (in Japanese).
- Nett, J. E., Marchillo, K., Spiegel, C. A., and Andes, D. R. (2010) Development and validation of an in vivo *Candida albicans* biofilm denture model. *Infect. Immun.*, **78**, 3650–3659.
- Nieto-Fernández, F. E., Centeno, S. A., Wypyski, M. T., Di Bonaventura, M. P., Baldwin, A. M. et al. (2003) Enzymatic approach to removal of fungal spots from drawings on paper. In *Art, Biology, and Conservation: Biodeterioration of Works of Art*, ed. by Koestler, R. J., Koestler, V. H., Charola, A. E., and Nietro-Fernandez, F. E., The Metropolitan Museum of Art, New York, pp. 111–127.
- Nishijima, M., Tazato, N., Handa, Y., Tomita, J., Kigawa, R. et al. (2013) *Gluconacetobacter tumulisoli* sp. nov., *Gluconacetobacter takamatsuzukensis* sp. nov. and *Gluconacetobacter aggeris* sp. nov., isolated from Takamatsuzuka Tumulus samples before and during the dismantling work in 2007. *Int. J. Syst. Evol. Microbiol.*, **63**, 3981–3988.
- Nishijima, M., Tazato, N., Handa, Y., Umekawa, N., Kigawa, R. et al. (2017a) *Krasilnikoviella muralis* gen. nov., sp. nov., a new member of the family *Promicromonosporaceae*, isolated from the

- Takamatsuzuka Tumulus stone chamber interior and reclassification of *Promicromonospora flava* as *Krasilnikoviella flava* comb. nov. *Int. J. Syst. Evol. Microbiol.* (in press); published ahead of print: 26 October, 2016; doi:10.1099/ijsem.0.001618.
- Nishijima, M., Tazato, N., Handa, Y., Umekawa, N., Kigawa, R. et al. (2017b) *Microbacterium tumbae* sp. nov., an actinobacterium isolated from the stone chamber of ancient tumulus. *Int. J. Syst. Evol. Microbiol.* (accepted).
- Novakova, A., Jurado, V., and Saiz-Jiménez, C. (2014) Are fungi a real threat for the conservation of Altamira Cave? In *The Conservation of Subterranean Cultural Heritage*, ed. by Saiz-Jiménez, C., CRC Press, Leiden, pp. 223–228.
- Nugari, M. P., Realini, M., and Roccardi, A. (1993) Contamination of mural paintings by indoor airborne fungal spores. *Aerobiologia*, **9**, 131–139.
- O'Brien, M., Nielsen, K. F., O'Kiely, P., Forristal, P. D., Fuller, H. T. et al. (2006) Mycotoxins and other secondary metabolites produced in vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. *J. Agr. Food Chem.*, **54**, 9268–9276.
- O'Brien, M., Egan, D., O'Kiely, P., Forristal, P. D., Doohan, F. M. et al. (2008) Morphological and molecular characterization of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. *Mycol. Res.*, **112**, 921–932.
- O'Donnell, K. (2000) Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia*, **92**, 919–938.
- O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Magnon, K. C., Cox, P. A. et al. (2004) Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: Evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. *J. Clin. Microbiol.*, **42**, 5109–5120.
- O'Donnell, K., Sarver, B., Brandt, M., Chang, D. C., Nobel-Wang, J. et al. (2007) Phylogenetic diversity and microsphere array-based genotyping of human pathogenic Fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. *J. Clin. Microbiol.*, **45**, 2235–2248.
- O'Donnell, K., Ward, T. J., Robert, V. A. R. G., Crous, P. W., Geiser, D. M. et al. (2015) DNA sequence-based identification of *Fusarium*: Current status and future directions. *Phytoparasitica*, **43**, 583–595.
- Orial, G. and Mertz, J.-D. (2006) Étude et suivi des phénomènes microbiologiques. *Monumental-Dossier Les grottes ornées-2006 Semestriel*, **2**, 76–87.
- Orial, G., Bousta, F., and François, A. (2009) Lascaux cave: monitoring of microbiological activities. In *International Symposium on the Conservation and Restoration of Cultural Property—Study of Environmental Conditions Surrounding Cultural Properties and Their Protective Measures*, ed. by Sano, C., National Research Institute for Cultural Properties, Tokyo, Tokyo, pp. 31–40.
- Orial, G., Bousta, F., François, A., Pallot-Frossard, I., and Warscheid, T. (2011) Managing biological activities in Lascaux: Identification of microorganisms, monitoring and treatments. In *Lascaux and Preservation Issues in Subterranean Environments*, Proceedings of the International Symposium Paris, February 26 and 27, 2009, ed. by Coye, N., Éditions de la Maison des sciences de l'homme, Paris, pp. 219–251.
- Palleroni, N. (2005) Genus IX. *Stenotrophomonas* Palleroni and Bradbury 1993, 608VP*. In *Bergey's Manual of Systematic Bacteriology*. Vol. 2. The *Proteobacteria*, Part B, The *Gamma-proteobacteria*, ed. by Garrity, G., Brenner, D. J., Krieg, N. R., and Staley, J. R., Springer, New York, pp. 107–115.
- Pallot-Frossard, I., Orial, G., Bousta, F., and Mertz, J.-D. (2009) Lascaux cave (France): A difficult problem of conservation. In *Lascaux and Preservation Issues in Subterranean Environments*, Proceedings of the International Symposium Paris, February 26 and 27, 2009, ed. by Coye, N., Éditions de la Maison des sciences de l'homme, Paris, pp. 7–14.
- Pedraza, R. O. (2008) Recent advances in nitrogen-fixing acetic acid bacteria. *Int. J. Food Microbiol.*, **125**, 25–35.
- Peng, S., Dongying, L., Bingxin, Y., Mingjun, L., and Gehong, W. (2015) *Microbacterium shaanxiense* sp. nov., isolated from the nodule surface of soybean. *Int. J. Syst. Evol. Microbiol.*, **65**, 1437–1443.
- Pike, A. W. G., Hoffmann, D. L., García-Diez, M., Pettitt, P. B., Alcolea, J. et al. (2012) U-series dating of paleolithic art in 11 caves in Spain. *Science*, **336**, 1409–1413.
- Pinna, D. and Salvadori, O. (2008) Process of biodeterioration: General mechanisms. In *Plant Biology for Cultural Heritage: Biodeterioration and Conservation*, ed. by Caneva, G., Nugari, M. P., and Salvadori, O., The Getty Conservation Institute, Los Angeles, pp. 15–34.
- Pinzari, F., Montanari, M., Michaelsen, A., and Piñar, G. (2010) Analytical protocols for the assessment of biological damage in historical documents. *Coalition*, **19**, 6–13.
- Pócsi, I. (2012) Volatile organic compounds of fungal origins—a potential future tool in the prevention and healing of microbial infections in artworks. In *Proceedings of Chemical and Ecological Researches on the Microorganisms for Preserving Cultural Properties in both Japan and Hungary (JSPS International Scientific Exchange: Joint Seminar between Japan Hungary, July 7 and 8, 2012)*, ed. by Suzuki, T., Nara Women's University, Nara, pp. 70–78.
- Porca, E., Jurado, V., Martín-Sánchez, P. M., Hermosin, B., Bastian, F. et al. (2011) Aerobiology: An ecological indicator for early detection and control of fungal outbreaks in caves. *Ecol. Indicators*, **11**, 1594–1598.
- Porca, E., Jurado, V., Zgur-Bertok, D., Saiz-Jiménez, C., and Pasic, L. (2012) Comparative analysis of yellow microbial communities growing on the walls of geographically distinct caves indicates a common core of microorganisms involved in their formation. *FEMS Microbiol. Ecol.*, **81**, 255–266.
- Pore, R. S. (2011) *Prototheca* Krüger. In *The Yeasts, A Taxonomic Study*, 5th ed., ed. by Kurtzman, C. P., Fell, J. W., and Boekhout, T., Elsevier, Amsterdam, pp. 2071–2080.
- Portillo, M. C. and Gonzalez, J. M. (2009a) Sulfate-reducing bacteria are common members of bacterial communities in Altamira Cave (Spain). *Sci. Total Environ.*, **407**, 1114–1122.
- Portillo, M. C. and Gonzalez, J. M. (2009b) Comparing bacterial community fingerprints from white colonizations in Altamira Cave (Spain). *World J. Microbiol. Biotechnol.*, **25**, 1347–1352.
- Portillo, M. C. and Gonzalez, J. M. (2011) Moonmilk deposits originate from specific bacterial communities in Altamira Cave (Spain). *Microbial Ecol.*, **61**, 182–189.
- Portillo, M. C., Gonzalez, J. M., and Saiz-Jiménez, C. (2008) Metabolically active microbial communities of yellow and grey colonizations on the walls of Altamira Cave, Spain. *J. Appl. Microbiol.*, **104**, 681–691.
- Portillo, M. C., Saiz-Jiménez, C., and Gonzalez, J. M. (2009) Molecular characterization of total and metabolically active bacterial communities of “white colonizations” in the Altamira Cave, Spain. *Res. Microbiol.*, **160**, 41–47.
- Riesenman, P. J. and Nicholson, W. L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Appl. Environ. Microbiol.*, **66**, 620–626.
- Roebroeks, W. (2014) Art on the move. *Nature*, **514**, 170–171.
- Ryan, R. P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L. et al. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nature Rev. Microbiol.*, **7**, 514–525.
- Saiz-Jiménez, C. (1995) Microbial melanins in stone monuments. *Sci. Total Environ.*, **167**, 273–286.
- Saiz-Jiménez, C. (ed.) (2003) *Molecular Biology and Cultural Heritage* (Proceedings of the International Congress on Molecular Biology and Cultural Heritage, 4–7 March 2003, Sevilla, Spain), A.A. Balkema Publishers, Lisse, The Netherlands, 287 pp.
- Saiz-Jiménez, C. (2013) Recent research on Lascaux Cave. *Coalition*, **24**, 11–14.
- Saiz-Jiménez, C. (ed.) (2014) *The Conservation of Subterranean Cultural Heritage*, CRC Press (Taylor & Francis Group), London, 328 pp.; doi:10.1201/b17570-1.
- Saiz-Jiménez, C. (2015) The microbiology of show caves, mines, tunnels, and tombs: implications for management and conservation. In *Microbial Life of Cave Systems*, ed. by Annette, S. E., Walter de Gruyter GmbH, Berlin/Boston, pp. 231–262.
- Saiz-Jiménez, C., Cuezva, S., Jurado, V., Fernandez-Cortes, A., Porca, E. et al. (2011) Paleolithic art in peril: Policy and science collide at

- Altamira Cave. *Science*, **334**, 42–43.
- Salvadori, O. and Urzi, C. (2008) Techniques and methods of investigation. In *Plant Biology for Cultural Heritage, Biodeterioration and Conservation*, ed. by Caneva, G., Nugari, M. P., and Salvadori, O., English translation, The Getty Conservation Institute, pp. 347–360.
- Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., and Andersen, B. (2010) *Food and Indoor Fungi*, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, 390 pp.
- Samson, R. A., Yilmaz, N., Houbraken, J., Spierenburg, H., Seifert, K. A. et al. (2011) Phylogeny and nomenclature of the genus *Talaromyces* and taxa accommodated in *Penicillium* subgenus *Biverticillium*. *Stud. Mycol.*, **70**, 159–183.
- Samuels, G. J. (2006) *Trichoderma*: Systematics, the sexual states, and ecology. *Phytopathology*, **96**, 195–206.
- Sanden, G. N. and Weyant, R. S. (2005) *Bordetella* Moreno-López 1952, 178^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. 2 (The *Proteobacteria*), Part C (The *Alpha*-, *Beta*-, *Delta*-, and *Epsilonproteobacteria*), ed. by Brenner, D. J., Krieg, N. R., Staley, J. T., and Garrity, G. M., Springer, New York, pp. 662–671.
- Sandoval-Denis, M., Guarro, J., Cano-Lira, J. F., Sutton, D. A., Wiederhold, N. P. et al. (2016) Phylogeny and taxonomic revision of Microascaceae with emphasis on synnematus fungi. *Stud. Mycol.*, **83**, 193–233.
- Sano, C. (ed.) (2009) International Symposium on the Conservation and Restoration of Cultural Property: Study of Environmental Conditions Surrounding Cultural Properties and Their Protective Measures, National Research Institute for Cultural Properties, Tokyo, Tokyo, 157 pp.
- Sano, C., Mabuchi, H., and Miura, S. (2004) Control of microorganism in Takamatsuzuka Tumulus: Paraformaldehyde fumigation and its concentration in the Tumulus. *Sci. Conserv.*, **43**, 95–105 (in Japanese).
- Sano, C., Mabuchi, H., and Miura, S. (2005) Atmospheric composition and air pollutants in Kitora Tumulus before excavation. *Sci. Conserv.*, **44**, 157–164 (in Japanese).
- Sano, C., Hayakawa, Y., and Miura, S. (2009) Review of the scientific analysis on the painting materials of Takamatsuzuka Tumulus. *Sci. Conserv.*, **48**, 119–131.
- Sano, C., Nishijima, M., Kiyuna, T., Kigawa, R., and Sugiyama, J. (2010) Carboxylic acids productivity of major microorganisms isolated from the stone chamber interior of Takamatsuzuka Tumulus and Kitora Tumulus, Nara Prefecture, Japan. *Sci. Conserv.*, **49**, 209–219 (in Japanese).
- Sato, Y., Kigawa, R., Kiyuna, T., Tazato, T., Nishijima, M. et al. (2013) Culture-independent analysis of the bacterial community in Kitora Tumulus under intermittent UV irradiation. *Sci. Conserv.*, **52**, 1–10 (in Japanese).
- Sato, Y., Kigawa, R., Kiyuna, T., Tazato, T., Nishijima, M. et al. (2015) Pyrosequence analysis for the microbial community in the Kitora Tumulus under intermittent UV irradiation. *Sci. Conserv.*, **54**, 111–120 (in Japanese).
- Satoh, K., Ooe, K., Nagayama, H., and Makimura, K. (2010) *Prototheca cutis* sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin. *Int. J. Syst. Evol. Microbiol.*, **66**, 1692–1704.
- Schabereiter-Gurtner, C., Saiz-Jiménez, J., Piñar, G., Lubitz, W., and Rölleke, S. (2002a) Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Palaeolithic paintings. *Environ. Microbiol.*, **4**, 392–400.
- Schabereiter-Gurtner, C., Saiz-Jiménez, C., Pinar, G., Lubitz, W., and Rölleke, S. (2002b) Altamira cave Paleolithic paintings harbor partly unknown bacterial communities. *FEMS Microbiol. Lett.*, **211**, 7–11.
- Schabereiter-Gurtner, C., Pinar, G., Lubitz, W., Rölleke, S., and Saiz-Jiménez, C. (2003) *Acidobacteria* in Paleolithic painting cave. In *Molecular Biology and Cultural Heritage*, ed. by Saiz-Jiménez, C., A.A. Balkema Publishers, Lisse, The Netherlands, pp. 15–21.
- Schabereiter-Gurtner, C., Saiz-Jiménez, J., Piñar, G., Lubitz, W., and Rölleke, S. (2004) Phylogenetic diversity of bacteria associated with Paleolithic paintings and surrounding rock walls in two Spanish caves (Llonín and La Garma). *FEMS Microbiol. Ecol.*, **47**, 235–247.
- Schroers, H.-J., Samuels, G. J., Zhang, N., Short, D. P. G., Juba, J. et al. (2016) Epitypification of *Fusisporium (Fusarium) solani* and its assignment to a common phylogenetic species in the *Fusarium solani* species complex. *Mycologia*, **108**, 806–819.
- Schumann, P. and Stackebrandt, E. (2012) Genus I. *Promicromonospora* Krasil'nikov, Kalakoutsii and Kirillova 1961, 107^{AL}. In *Bergey's Manual of Systematic Bacteriology*, Vol. 5, Part B, ed. by Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M. E., Suzuki, K. et al., Springer, New York, pp. 995–1002.
- Seifert, K., Morgan-Jones, G., Gams, W., and Kendrick, B. (2011) The Genera of Hyphomycetes (CBS Biodiversity Series 9), CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, 997 pp.
- Shimkets, L. J., Dworkin, M., and Reichenbach, H. (2006) The Myxobacteria. In *The Prokaryotes: A Handbook on the Biology of Bacteria*, 3rd ed., Vol. 7 (*Proteobacteria*: Delta and Epsilon Subclasses. Deeply Rooting Bacteria), ed. by Dworkin, M., Falkow, E., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., Springer, New York, pp. 31–115.
- Sievers, M. and Swings, J. (2005) Genus VIII. *Gluconacetobacter*. Yamada, Hoshino, and Ishikawa 1998b, 32VP (Effective publication: Yamada, Hoshino, and Ishikawa 1997, 1249). In *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. 2, The *Proteobacteria*, Part C, The *Alphaproteobacteria*, the *Betaproteobacteria*, the *Deltaproteobacteria* and the *Epsilonproteobacteria*, ed. by Brenner, D. J., Krieg, N. P., Staley, J. T., and Garrity, G. M., Springer, New York, pp. 72–77.
- Solden, L., Lloyd, K., and Wrighton, K. (2016) The bright side of microbial dark matter: lessons learned from the uncultivated majority. *Curr. Opin. Microbiol.*, **31**, 217–226.
- Stackebrandt, E. and Embley, T. M. (2000) Diversity of uncultured microorganisms in the environment. In *Nonculturable Microorganisms in the Environment*, ed. by Colwell, R. R. and Grimes, D. J., ASM Press, Washington, D.C., pp. 57–75.
- Stark, D., Riley, L. A., Harkness, J., and Marriott, D. (2007) *Bordetella pertussis* from a clinical sample in Australia: isolation and molecular identification. *J. Med. Microbiol.*, **56**, 435–437.
- Sterflinger, K. (2000) Fungi as geologic agents. *Geomicrobiol. J.*, **17**, 97–124.
- Sterflinger, K. (2010) Fungi: Their role in deterioration of cultural heritage. *Fung. Biol. Rev.*, **24**, 47–55.
- Sterflinger, K. and Piñar, G. (2013) Microbial deterioration of cultural heritage and works of art—tilting at windmills? *Appl. Microbiol. Biotechnol.*, **97**, 9637–9646.
- Sugiyama, J., Kiyuna, T., An, K.-D., Nagatsuka, Y., Handa, Y. et al. (2009) Microbiological survey of the stone chambers of Takamatsuzuka and Kitora tumuli, Nara Prefecture, Japan: a milestone in elucidating the cause of biodeterioration of mural paintings. In *International Symposium on the Conservation and Restoration of Cultural Property—Study of Environmental Conditions Surrounding Cultural Properties and Their Protective Measures*, ed. by Sano, C., National Research Institute for Cultural Properties, Tokyo, pp. 51–73.
- Suh, S. O., Nguyen, N. H., and Blackwell, M. (2005) Nine new *Candida* species near *C. membranifaciens* isolated from insects. *Mycol. Res.*, **109**, 1045–1056.
- Summerbell, R. C., Guidan, C., Schroers, H. J., de Hoog, G. S., Starink, M. et al. (2011) *Acremonium* phylogenetic overview and revision of *Gliomastix*, *Sarocladium*, and *Trichothecium*. *Stud. Mycol.*, **68**, 139–162.
- Suzuki, T., Kiuchi, Y., Tanaka, H., Kimura, T., Kiuchi, M. et al. (2012) Physiological activity of microbial volatile organic compounds (MVOCs) as growth regulators in fungi. In *Proceedings of Chemical and Ecological Researches on the Microorganisms for Preserving Cultural Properties in both Japan and Hungary (JSPS International Scientific Exchange: Joint Seminar between Japan and Hungary, July 7 and 8, 2012)*, ed. by Suzuki, T., Nara Women's University, Nara, pp. 83–92.
- Systematics Agenda 2000 (1994) Systematic Agenda 2000: Charting the Biosphere (Technical Report), A Consortium of the American Society of Plant Taxonomists, the Society of Systematic Biologists, and the Willi Hennig Society, in cooperation with the Association of Systematic Collection, New York, 34 pp.
- Takamatsuzuka Tumulus Emergency Conservation Research Commit-

- tee (1972) An interim report by Takamatsuzuka Tumulus Emergency Conservation Research Committee. In National Treasures, the Takamatsuzuka Tumulus Mural Paintings: Conservation and Repair, Agency for Cultural Affairs, Japan, pp. 19–26 (in Japanese).
- Takeuchi, T., Tanaka, H., Ichii, S., and Kimura, T. (2012) Analysis of microbial volatile organic compounds for conservation technology of cultural heritage. In Proceedings of Chemical and Ecological Researches on the Microorganisms for Preserving Cultural Properties in both Japan and Hungary (JSPS International Scientific Exchange: Joint Seminar between Japan and Hungary, July 7 and 8, 2012), ed. by Suzuki, T., Nara Women's University, Nara, pp. 79–83.
- Tazato, N., Nishijima, M., Handa, Y., Kigawa, R., Sano, C. et al. (2012) *Gluconacetobacter tumulicola* sp. nov. and *Gluconacetobacter asukensis* sp. nov., isolated from the stone chamber interior of the Kitora Tumulus, Nara, Japan. *Int. J. Syst. Evol. Microbiol.*, **62**, 2032–2038.
- Tazato, N., Handa, Y., Nishijima, M., Kigawa, R., Sano, C. et al. (2015) Novel environmental species isolated from the plaster wall surface of mural paintings in the Takamatsuzuka tumulus: *Bordetella muralis* sp. nov., *Bordetella tumulicola* sp. nov. and *Bordetella tumbae* sp. nov. *Int. J. Syst. Evol. Microbiol.*, **65**, 4830–4838.
- Urzi, C., Leo, F. D., Bruno, L., and Albertano, P. (2010) Microbial diversity in paleolithic caves: A study case on the phototrophic biofilms of the Cave of Bats (Zuheros, Spain). *Microb. Ecol.*, **60**, 116–129.
- Vandamme, P. A., Peeters, C., Cnockaert, M., Inganäs, E., Falsen, E. et al. (2015). *Bordetella bronchialis* sp. nov., *Bordetella flabilis* sp. nov. and *Bordetella sputigena* sp. nov., isolated from human respiratory specimens, and reclassification of *Achromobacter sediminum* Zhang et al., 2014 as *Verticia sediminum* gen. nov., comb. Nov. *Int. J. Syst. Evol. Microbiol.*, **65**, 3674–3682.
- Vanderwolf, K. J., Malloch, D., McAlpine, D. F., and Forbes, G. J. (2013) A world review of fungi, yeasts, and slime molds in caves. *Int. J. Speleol.*, **42**, 77–96.
- Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S.-B., Klaassen, C. H. W. et al. (2014) Identification and nomenclature of the genus *Penicillium*. *Stud. Mycol.*, **78**, 343–371.
- von Wintzingerode, F., Schattke, A., Siddiqui, R. A., Rösick, U., Göbel, U. B. et al. (2001) *Bordetella petrii* sp. nov., isolated from an anaerobic bioreactor, and emended description of the genus *Bordetella*. *Int. J. Syst. Evol. Microbiol.*, **51**, 1257–1265.
- Weiss, A. (2006) The Genus *Bordetella*. In The Prokaryotes: a Handbook on the Biology of Bacteria, 3rd ed., Vol. 5 (*Proteobacteria: Alpha and Beta Subclasses*), ed. by Dworkin, M., Falkow, E., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., Springer, New York, pp. 648–674.
- Weyant, R. S., Moss, C. W., Weaver, R. E., Hollis, D. G., Jordan, J. G. et al. (1996) Identification of Unusual Pathogenic Gram-negative Aerobic and Facultatively Anaerobic Bacteria, 2nd ed., Baltimore: Williams & Wilkins, Baltimore, 727 pp.
- Whitman, W., Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M. et al. (eds.) (2012) Bergey's Manual of Systematic Bacteriology 2nd ed., Vol. 5, The *Actinobacteria*, Part A and B, Springer, New York, 2083 pp.
- Woese, C. R., Kandler, O., and Wheelis, M. L. (1990) Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA*, **87**, 4576–4579.
- Wolf, A., Fritze, A., Hagemann, M., and Berg, G. (2002) *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *Int. J. Syst. Evol. Microbiol.*, **52**, 1937–1944.
- Woodward, G., Gray, C., and Baird, D. J. (2013) Biomonitoring for the 21st Century: new perspectives in an age of globalisation and emerging environmental threats. *Limnetica*, **32**, 159–174.
- Yamada, Y. (1986) Acetic acid bacteria. In Methods for the Isolation of Microorganisms, ed. by Yamazato, K., Udagawa, S., Kodama, T., and Morichi, T., R&D Planning, Tokyo (in Japanese).
- Yamada, Y., Kawasaki, H., Nagatsuka, Y., Mikata, K., and Seki, T. (1999) The phylogeny of the cactophilic yeasts based on the 18S ribosomal RNA gene sequences: the proposals of *Phaffomyces antillensis* and *Starmera caribaea*, new combinations. *Biosci. Biotechnol. Biochem.*, **63**, 827–832.
- Yamada, Y., Yukphan, P., Thi Lan Vu, H., Muramatsu, Y., Ochaikul, D. et al. (2013) *Komagataeibacter* gen. nov. In List of New Names and New Combinations Previously Effectively, But Not Validly, published, Validation list No. 149. *Int. J. Syst. Evol. Microbiol.*, **63**, 1–5.
- Zengler, K. (ed.) (2008) Access in Uncultured Microorganisms: From the Environment to Organisms and Genome and Back, ASM Press, Washington, D.C., 308 pp.
- Zhang, N., O'Donnell, K., Sutton, D. A., Nalim, F. A., Summerbell, R. C. et al. (2006) Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. *J. Clin. Microbiol.*, **44**, 2186–2190.
- Zhou, J. P., Gu, Y. Q., Zou, C. S., and Mo, M. H. (2007) Phylogenetic diversity of bacteria in an Earth-cave in Guizhou Province, southwest of China. *J. Microbiol.*, **45**, 105–112.
- Zimmermann, J., Gonzalez, J. M., and Saiz-Jiménez, C. (2005) Detection and phylogenetic relationships of highly diverse uncultured Acidobacterial communities in Altamira Cave using 23S rRNA sequence analyses. *Geomicrobiol. J.*, **22**, 379–388.