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FULL PAPER

Bristle-like fungal colonizers on the stone walls of the Kitora and Takamatsuzuka Tumuli are identified as *Kendrickiella phycomyces*

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Abstract Using an integrated analysis of phenotypic and genotypic characterizations, a total of 18 isolates of "bristle-like" fungal colonizers of the Kitora and Takamatsuzuka Tumuli, which had been provisionally identified as *Phialocephala phycomyces*, were here determined to be *Kendrickiella phycomyces* (Auersw.) K. Jacobs & M. J. Wingf. The 18 isolates consisted of 10 from stone surfaces or viscous gels (biofilms) of the stone chamber interior and adjacent small room, and air in the adjacent small room of the Kitora Tumulus, and 8 from viscous gels on the stone surfaces of the stone chamber interior, plant roots, and soil in the adjacent space or stone wall interspaces (interstices) in the stone chamber of the Takamatsuzuka Tumulus. Plaster and stone walls in both tumuli were recorded as novel substrates of this fungus. Our 18S sequence-based phylogeny indicated that *K. phycomyces* and species of the leotiomycetous anamorph genera *Chaetomella*, *Pilidium*, *Sphaerographium*, and *Synchaetomella* formed a monophyletic lineage distant from the core taxa of the Leotiomycetes (Pezizomycotina, Ascomycota). The relationship between the physicochemical characteristics of these isolates on GYC agar plates, i.e., soluble brownish pigments and dissolution of calcium carbonate ($CaCO_3$), and the biodeterioration of the plaster and plaster walls of both tumuli, are briefly discussed.

Keywords Biodeterioration · $CaCO_3$ solubilization · Cultural properties · *Kendrickiella phycomyces* · Molecular systematics

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Introduction

Since May 2004, we have been conducting a series of microbiological surveys to elucidate the cause of the biodeterioration of the mural paintings at the Kitora and Takamatsuzuka Tumuli (hereafter KT and TT, respectively) in Asuka-mura (the village of Asuka), Nara, Japan. These 1,300-year-old wall paintings were drawn directly on thin plaster in the small chamber interiors of both tumuli, and their historical background and the history of their conservation have been described in previous papers (e.g., Kiyuna et al. 2008, 2011; Sugiyama et al. 2008, 2009; Kigawa et al. 2009; Ishizaki and Kigawa 2011). We identified the predominant colonizers that were isolated from numerous samples of moldy spots and viscous gels (biofilms) from KT and TT, using integrated phenotypic and genotypic approaches. The results have been published in four papers: species of the genera *Fusarium* and *Trichoderma* (Kiyuna et al. 2008), two novel species of *Candida*

(*Candida tumulicola* Nagatsuka et al. and *Candida takamatsuzukensis* Nagatsuka et al.), mainly from biofilms (Nagatsuka et al. 2009), *Penicillium paneum* Frisvad as the major *Penicillium* dweller in both TT and KT (An et al. 2009), and species of *Acremonium* sect. *Gliomastix*, including the new species *Acremonium tumulicola* Kiyuna et al. and the new combination *Acremonium felinum* (Marchal) Kiyuna et al. (Kiyuna et al. 2011).

During a microbiological inspection and survey of the KT in September 2004, we encountered "bristle-like" fungal colonizers that had a niche on the stone wall (tuff stone) of the small room adjacent to the KT (Kigawa et al. 2007, 2008; Sugiyama et al. 2008, 2009). Based on a brief morphological and molecular (i.e., 28S rDNA D1/D2 gene sequences) characterization, we (Sugiyama et al. 2009) provisionally identified these colonizers, viz., only three (K4910-1, K5906-1-1, and K5225-12-1 in Table 1) of the KT isolates, as *Phialocephala phycomyces* (Auersw.) W. B. Kendr. The reference sequence of *Phialocephala phycomyces* CMW 2556 (AF269216; 28S ribosomal RNA gene, partial sequence) from GenBank was poor, and therefore the sequence comparison in our previous

Table 1 Strain data of Kitora and Takamatsuzuka isolates, with the GenBank accession numbers for rDNA sequences determined in this study

Isolate no.	JCM no.	Source[a]	Sampling date	GenBank accession no.		
				18S	28S	ITS
K4910-1	18027	Stone surface of the stone ceiling in the adjacent small room of KT	10 Sept 2004	AB671437	AB671441	AB671474
K5225-12-1		Air from the north area in the adjacent small room of KT	25 Feb 2005		AB671442	AB671475
K5906-1-1	18028	Brownish bristle-like structures below the area of the west wall in the stone chamber of KT	6 Sept 2005	AB671438	AB671443	AB671476
K5906-2-1		Brownish bristle-like structures below the area of the west wall in the stone chamber of KT	6 Sept 2005		AB671444	AB671477
K7706-1-2		Viscous gels (substances) on the east wall in the stone chamber of KT	6 July 2007		AB671445	AB671478
K7724-1-2		Beige viscous gels (substances) on the floor in the stone chamber of KT	24 July 2007		AB671446	AB671479
K61208-2-2		Brownish substances on the floor in the stone chamber of KT	8 Dec 2006		AB671447	AB671480
K8617-6-13	18029	Reddish viscous gels on the stone wall near the relocated area of the paintings of the vermillion bird (*Suzaku*) on the south wall in the stone chamber of KT	17 June 2008		AB671448	AB671481
K101008-6-1	18030	Blackish and brownish viscous gels and bristle-like structures on the southern area of the west walls in the stone chamber of KT	8 Oct 2010		AB671449	AB671482
TBK-5		Stone surface of the stone ceiling in the adjacent small room of KT	30 Aug 2004		AB671450	AB671483
T61114-1-1	18031	Black substances on the sealed stone in the southwest first stratum above the adjacent space of TT	14 Nov 2006		AB671451	AB671484
T611xx-2-1		Soil (treated by polysiloxane resin) in the adjacent space of TT	Nov 2006		AB671452	AB671485
T61213-12-3[b]		Viscous gels in upper area of the north wall in the stone chamber of TT	13 Dec 2006		AB671453	AB671486
T61213-21-3		Soil on the western stone wall of TT	13 Dec 2006		AB671454	AB671487
T61213-27-1		Plant roots and soil in the hole beside the stone wall of the TT	13 Dec 2006		AB671455	AB671488
T7302-19-3		Brownish soil and plaster mixtures on the surface of the ceiling in stone wall 2 of TT	2 Mar 2007		AB671456	AB671489
T7320-1-2		Soil on the surface tomb passage of TT	20 Mar 2007		AB671457	AB671490
T7608-1-7	18032	Blackish particle plasters and soil between west stone wall 1 and the south stone wall of TT	8 June 2007		AB671458	AB671491

[a] *KT* and *TT* indicate the Kitora and Takamatsuzuka Tumuli, respectively

[b] Living isolate and specimen were not preserved

molecular phylogenetic analysis was insufficient. At that time we did not take the systematic treatment of *Kendrickiella phycomyces* by Jacobs et al. (2001) into account and provisionally adopted the name *Phialocephala phycomyces*. In our previous treatment, the distinction between *Phialocephala* and *Kendrickiella* remained unresolved (for details, see p. 62 in Sugiyama et al. 2009). In Index Fungorum http://www.indexfungorum.org/Names/Names.asp, *K. phycomyces* is now treated as anamorphic Eurotiales, which obviously is erroneous.

Between the first discovery in 2004 and October 2010, we isolated 18 strains from "bristle-like" fungal colonizers of both tumuli; viz., 10 isolates from the stone surfaces or viscous gels of the stone chamber interior and the adjacent small room, and air in the adjacent small room of the KT, and 8 from viscous gels on the stone surfaces of the stone chamber interior, plant roots, and soil in the adjacent space or stone wall interspaces (interstices) in the stone chamber interior of TT (for details, see Table 1). By integrating the phenotypic (cultural and morphological characters) and genotypic (18S rRNA, 28S rRNA D1/D2, and ITS genes) characterizations, we could reliably identify these isolates. During the cultural characterization, we also found that the isolates dissolved calcium carbonate ($CaCO_3$) in glucose–yeast extract–calcium carbonate (GYC) agar plates (De Ley et al. 1984). This finding is relevant in explaining the biodeterioration of the stone and plaster walls of the tumuli in light of Karbowska-Berent's review (2003) and a paper by Pangallo et al. (2009).

The principal objective of this study was to carry out a reliable identification of their molecular systematic/phylogenetic positions, with the detection of their physicochemical characteristics on GYC agar plates, toward the ultimate goal of elucidating the cause of the biodeterioration of the stone chamber interiors of both tumuli.

Materials and methods

Sampling, isolating, and culturing

In our survey of mycobiota related to biodeterioration of the mural paintings of the KT and TT, which has been ongoing since 2004, we recognized dark mononematous conidiophores bearing a spore ball (a slimy mass of conidia) at the tip in the 10 and 8 samples collected at KT and TT, respectively, between August 2004 and October 2010. The methods used for the collection, isolation, and preservation of the isolates were noted in our previous papers (Kiyuna et al. 2008; Sugiyama et al. 2008, 2009). Detailed data on the isolates from both tumuli and accession numbers of DNA sequences in GenBank are listed in Table 1. Six isolates are deposited as vouchers with the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Wako, Saitama Prefecture, Japan, as JCM 18027–18032 (Table 1). The remaining living isolates from both tumuli are maintained at the Biology Laboratory of the National Research Institute of Cultural Properties, Tokyo, as lyophilized vouchers (Table 1).

Cultural and morphological observations

All isolates were grown using mainly the media and growth conditions adopted by Jacobs et al. (2001), i.e., incubation in Petri dishes with 2 % malt extract agar (MEA; 20 g Bacto Malt Extract, 20 g Wako Agar Powder, and 1,000 ml distilled water) at 20 °C, and on potato dextrose agar (PDA; Nihon Pharmaceutical, Tokyo, Japan) at 20 °C, for 20 days in darkness. In addition, all isolates were inoculated on GYC agar plates to investigate their ability to solubilize $CaCO_3$. GYC agar is usually used for isolation of acetic acid bacteria (De Ley et al. 1984); the composition was based on the formula in Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) medium no. 105 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium105.pdf). The colony colors of the isolates on all media were determined by using the Kornerup and Wanscher color standard (1978). Microscopic slides were prepared from portions of the colonies grown on MEA plate cultures, mounted in lactophenol (with or without cotton blue) or lactic acid without dye (Gams et al. 1987; Bills and Foster 2004). Microscopic examinations were made using a BX51 microscope (Olympus, Tokyo, Japan) with Nomarski interference contrast at up to 1,000× magnification. All micrographs were taken with a Coolpix 5000 digital camera (Nikon, Tokyo, Japan).

Phylogenetic analyses

DNA extraction, PCR amplification, and sequencing

The isolates and reference strains from the various culture collections used for DNA sequencing are listed in Tables 1 and 2. Their genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The three gene regions sequenced were the nuclear 18S rDNA region (hereafter 18S), 28S rDNA D1/D2 region (28S), and internal transcribed spacer-5.8S rDNA region (ITS). The primers used for the polymerase chain reactions (PCR) are listed in Table 3. PCR was performed using puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). Thermal cycling was performed using a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA). An initial denaturation at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at

Table 2 Strain data and rDNA sequences determined in this study for comparison

Species[a]	Strain no.[b,c]	Source[d]	Location[d]	GenBank accession no.		
				18S	28S	ITS
Phialocephala phycomyces	MUCL 4271[e]	Carpophore of basidiomycete, polypore	Guyane, France	AB671439	AB671459	AB671492
Phialocephala phycomyces	MUCL 38565[e]	Contaminant in a culture of *Ganoderma* (rain forest)	Ecuador, USA	AB671440	AB671460	AB671493
Scopularia phycomyces	CBS 145.41	Wood pulp	Sweden		AB671461	AB671494
Myxocephala albida	CBS 962.87^{IT}	*Picea abies* (Pinaceae), root surface	Freudenstadt, Germany		AB671462	AB671495
Phialocephala bactrospora	NBRC 8770	–	–		AB671463	AB671496
Phialocephala bactrospora	NBRC 8852	–	–		AB671464	AB671497
Phialocephala dimorphospora	CBS 300.62	Slime in pulp mill	New Brunswick, Canada		AB671465	AB671498
Phialocephala fortinii	CBS 443.86^{IT}	*Pinus sylvestris* (Pinaceae), root	Suonenjoki, Finland		AB671466	AB671499
Phialocephala fusca	CBS 300.85	*Acer* (Aceraceae), decayed wood	Quebec, Canada		AB671467	AB671500
Phialocephala humicola	CBS 420.73^{IT}	Soil	New Jersey, USA		AB671468	AB671501
Phialocephala humicola	NBRC 31686	Paddy field soil	–		AB671469	AB671502
Phialocephala humicola	NBRC 100578	Decayed tree	Hachijo Is., Tokyo, Japan		AB671470	AB671503
Phialocephala trigonospora	CBS 100161^{T}	Gallery of *Orthotomicus laricis* from *Pinus sylvestris*	Hessen, Germany		AB671471	AB671504
Phialocephala virens	CBS 452.92^{T}	Root or butt rot on standing *Tsuga* or *Picea*	Alaska, USA		AB671472	AB671505
Phialocephala xalapensis	CBS 218.86^{IT}	Rotten twig	Karnataka, India		AB671473	AB671506

[a] Species names are noted as registered in each Culture Collection

[b] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MUCL, Mycothèque de l'Université catholique de Louvain, Belgium; NBRC, NITE Biological Resource Center, Kisarazu, Japan

[c] *T* ex-type strain, *IT* ex-isotype strain

[d] –, source and location are unknown

[e] MUCL 4271 (=DAOM 63734) and MUCL 38565 (=CMW 2556) were strains examined by Kendrick (1964) and Jacobs et al. (2001), respectively

55 °C for 30 s, extension at 72 °C for 1 min, and then a final extension at 72 °C for 10 min. The amplified DNA fragments were purified with a QIAquick PCR Purification Kit (Qiagen). Direct sequencing for the PCR products was performed using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems), and the tubes were incubated in a GeneAmp PCR System 9600 (Applied Biosystems). The completed reactions were cleaned using a DyeExTM 2.0 Spin Kit (Qiagen). Sequences were determined using electrophoresis in an ABI3130xl DNA sequencer (Applied Biosystems). The sequences determined in this study were deposited in GenBank/EMBL/DDBJ. Their accession numbers are given in Tables 1 and 2. Other known sequences downloaded for comparison for the respective molecular phylogenetic analyses from GenBank are shown in Figs. 1, 2, and 3.

Molecular phylogenetic analyses

The sequences were assembled using ChromasPro 1.42 (Technelysium, Tewantin, QLD, Australia). Three subsets of the segment were also made into data sets: 18S, ITS, and 28S. Multiple alignments were performed using CLUSTAL W version 1.83 (Thompson et al. 1994); the final alignments were manually adjusted. Ambiguous positions and alignment gaps were excluded from the analysis. The

Table 3 Polymerase chain reaction (PCR) primer sequences used in this study

Primer name	Sequence (5′–3′)	Target gene	References
NS1	GTAGTCATATGCTTGTCTC	18S	White et al. (1990)
NS2	GGCTGCTGGCACCAGACTTGC	18S	White et al. (1990)
NS3	GCAAGTCTGGTGCCAGCAGCC	18S	White et al. (1990)
NS4	CTTCCGTCAATTCCTTTAAG	18S	White et al. (1990)
NS5	AACTTAAAGGAATTGACGGAAG	18S	White et al. (1990)
NS6R	TGGACCTGGTGAGTTTCC	18S	This study
NS7F	GGAGTGATTTGTCTGCTT	18S	This study
NS8	TCCGCAGGTTCACCTACGGA	18S	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	ITS	White et al. (1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG	ITS	White et al. (1990)
NL1	GCATATCAATAAGCGGAGGAAAAG	28S	O'Donnell (1993)
NL4	GGTCCGTGTTTCAAGACGG	28S	O'Donnell (1993)

alignments and molecular phylogenetic trees are deposited in TreeBASE as S12231. The neighbor-joining (NJ) tree was constructed using the multiple alignments in MEGA ver. 5 (Tamura et al. 2011), with 1,000 bootstrap replicates (Felsenstein 1985). The phylogenetic reconstruction approach using Bayesian trees based on 18S, ITS, and 28S sequences (Rannala and Yang 1996) was implemented using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). The model of DNA substitution was calculated using Modeltest2.2 (Nylander 2004). The results were obtained by the GTR + I + G model for 18S and 28S and the SYM + G model for ITS. Bayesian metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses (Mau et al. 1999) were performed with MrBayes for phylogenetic estimation inferred from the respective gene sequences. MrBayes was run for 4,500,000 generations for 18S, 1,000,000 generations for ITS, and 7,500,000 generations for 28S. Searches were conducted with four chains (three cold, one hot), with trees sampled every 100 generations. The average standard deviations of split frequencies were 0.007 for 18S and 28S, and 0.005 for ITS at the end of the run. The confidence levels of nodes were measured by posterior probabilities obtained from the majority rule consensus after deletion of the trees during burn-in. The higher-level phylogenetic classification of the fungi in this article was fundamentally based on Hibbett et al. (2007) and Schoch et al. (2009a,b).

Results and discussion

Cultural and morphological characterization

The 18 isolates treated as bristle-like fungal colonizers from the KT and TT stone chamber interior and exterior, as enumerated in Table 1, were identified as the anamorph species *Kendrickiella phycomyces* (Auersw.) K. Jacobs & M.J. Wingf. based on cultural and morphological characteristics.

The anamorph genus *Kendrickiella* was proposed by Jacobs et al. (2001) to accommodate only *Phialocephala phycomyces* of the *Leptographium* complex, which produces conidia from phialides at the apex of mononematous reddish-brown conidiophores and has poorly developed collarettes on the phialides, characteristics that distinguish them from the other species of *Phialocephala* (Jacobs et al. 2001); the specific epithet '*phycomycoides*' used by Jacobs et al. (2001) has been corrected to '*phycomyces*' in the Index of Fungi (Anonymous 2003). *Kendrickiella* currently consists of only a single species, *K. phycomyces* (formerly known as *Phialocephala phycomyces*). This species was previously recorded from fungi (culture of *Ganoderma* and other polypores), plant substrates (an oak barrel in a cellar, wood, wood pulp), and soil (Kendrick 1964; Matsushima 1980; Jacobs et al. 2001; Grünig et al. 2002). Changes in the taxonomic classification of *K. phycomyces* and *P. phycomyces* were documented by Kendrick (1964) and Jacobs et al. (2001) in detail.

Very recently, *Kendrickiella*, which consists of only the single species *K. phycomyces* (≡*Phialocephala phycomyces*), was compiled by Seifert et al. (2011) in *The Genera of Hyphomycetes* as an accepted anamorphic genus in addition to *Leptographium* and *Phialocephala*. The major differences among the three genera are summarized in Table 4, which incorporates our new data.

The phenotypic characteristics of our isolates from both tumuli and two authentic strains from MUCL (MUCL 4271 and 38565, both labeled as *Phialocephala phycomyces*; for the strain data, see Table 2) agreed well with the characteristics provided by Kendrick (1964) and Jacobs et al. (2001). However, in our observations, the structures at the base of conidiophores of all available isolates grown on MEA and the natural substratum consisted of some rhizoid-like and swollen cells. This feature was distinct from that

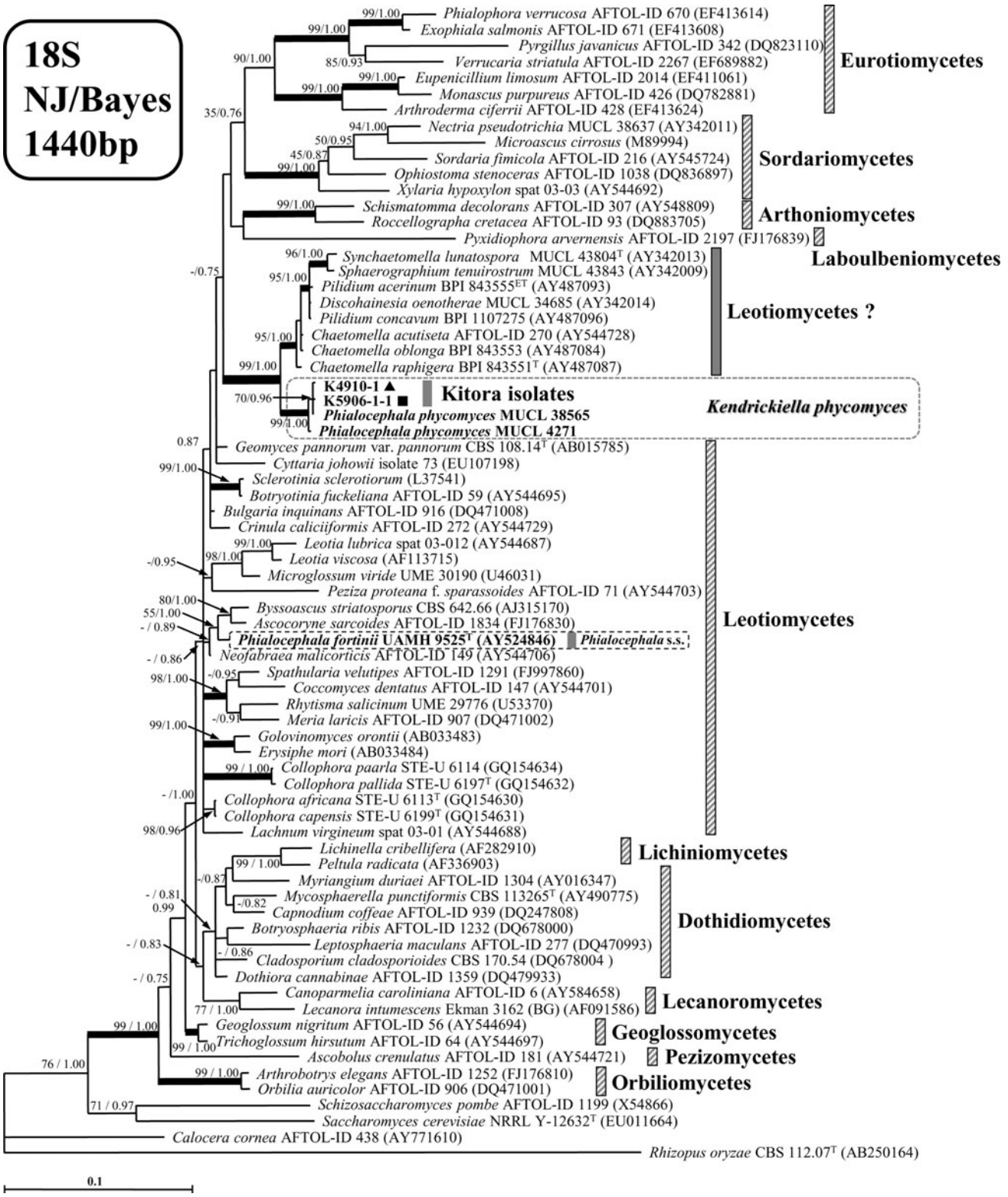

Fig. 1 Phylogenetic relationships of two Kitora Tumuli (KT) isolates on Bayesian analysis of the 18S rDNA region sequence data of 1,440 aligned nucleotide sites using MEGA ver. 5. *Numbers on the branch nodes* represent neighbor-joining (*NJ*) bootstrap support values (≥50 %) based on 1,000 replications and Bayesian posterior probabilities. Branches significantly supported by bootstrap values (≥99 %) and Bayesian posterior probabilities (1.00) are shown by *thick lines*. *K* indicates isolates from the KT. *Filled square* indicates the isolates from the stone chamber interiors, and *filled triangle* indicates isolates from the adjacent small room of KT. The superscripts ^{T} and ^{ET} indicate ex-type and ex-epitype strains, respectively. *Right vertical bars* indicate the taxonomic rank class based on Hibbett et al. (2007) and Schoch et al. (2009a,b)

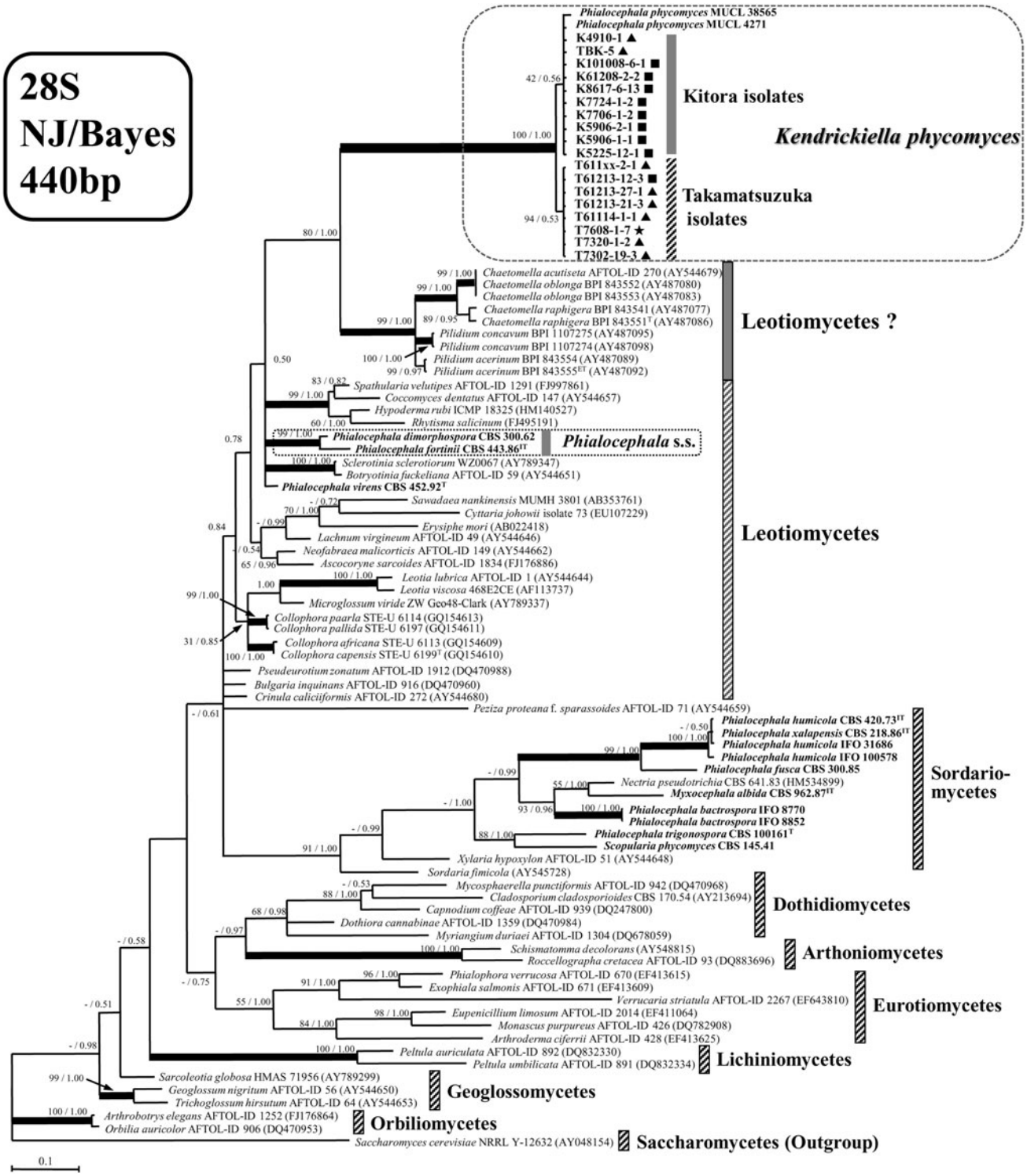

Fig. 2 Phylogenetic relationships among ten Kitora Tumuli (KT) and eight Takamatsuzuka Tumulus (TT) isolates based on Bayesian analysis of the 28S rDNA D1/D2 region sequence data of 440 aligned nucleotide sites, using MEGA ver. 5. *Star* indicates the isolate from between the stones in the wall of the TT. The superscript IT indicates ex-isotype strain. Further details are as in Fig. 1

described by Kendrick (1964) and Jacobs et al. (2001). Therefore, we added our condensed results to the full description, and partial modification, of *Kendrickiella phycomyces* in the following "Systematics" section. This is the first case in which *K. phycomyces* was isolated from biodeteriorated stone and plaster walls or murals in tumuli.

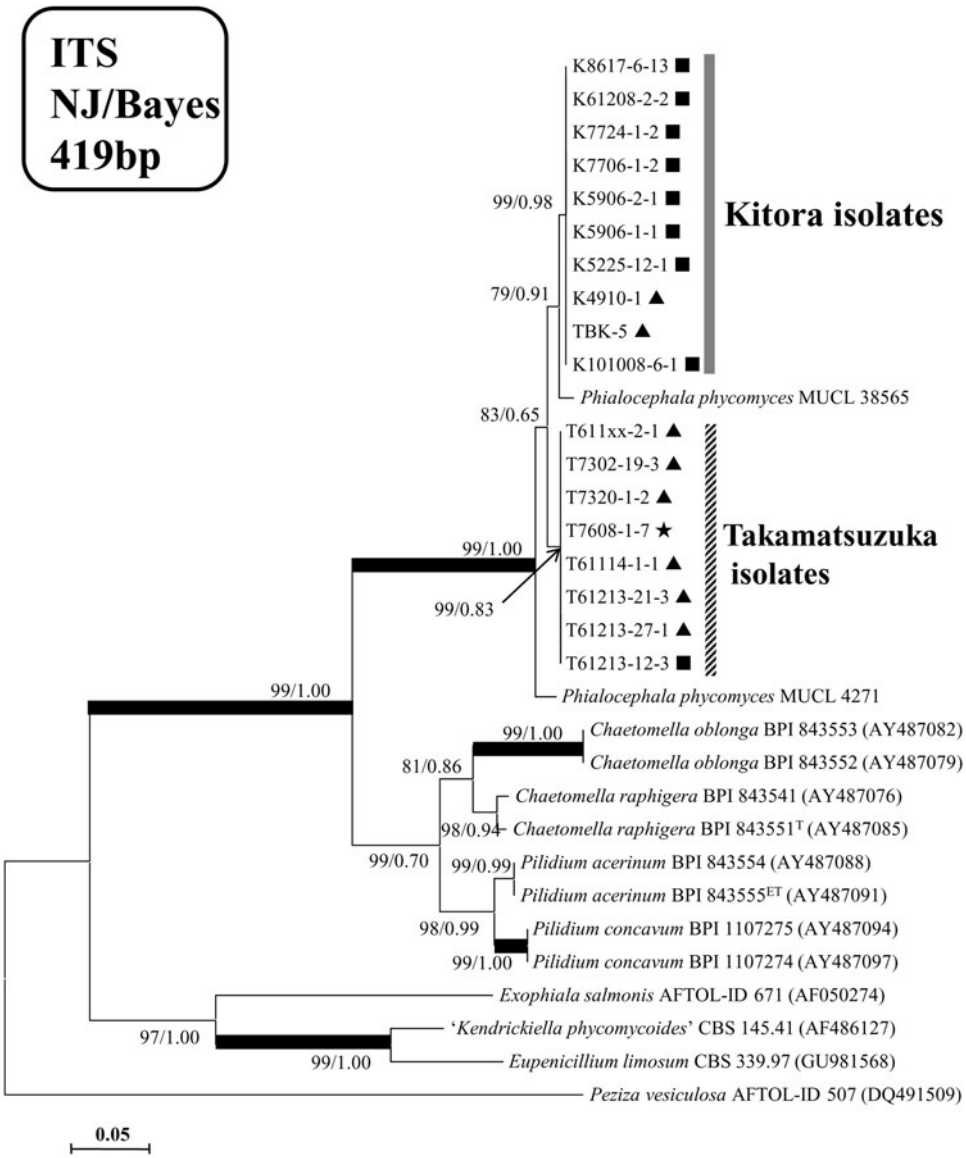

Fig. 3 Phylogenetic relationships among ten Kitora Tumulus (KT) and eight Takamatsuzuka Tumulus (TT) isolates based on Bayesian analysis of the ITS–5.8S rDNA region sequence data of 414 aligned nucleotide sites, using MEGA ver. 5. Further details are as in Figs. 1 and 2

Furthermore, this fungus constitutes a new addition to the mycobiota (or fungal inventory) of Japan.

Molecular analyses and phylogeny

The 18S, 28S, and ITS datasets contained 18 isolates (Table 1) and two authentic reference strains that had been labeled as *Phialocephala phycomyces*, which were obtained from the Mycothèque de l'Université catholique de Louvain (MUCL) in Belgium (Table 2). The phylogenetic trees generated are shown in Figs. 1, 2, and 3.

No base changes for the 18S sequences were detected between the two KT isolates. The 18S Bayesian tree, using the mucoralean fungus *Rhizopus oryzae* Went & Prins. Geerl. (Mucoromycotina) and the dacrymycetalean fungus *Calocera cornea* (Batsch) Fr. (Basidiomycota) as outgroup taxa, suggested that the two KT isolates, two reference strains (MUCL 4271 and 38565), and species of the leotiomycetous anamorph genera *Chaetomella*, *Pilidium*, *Sphaerographium*, and *Synchaetomella* were grouped together with very high bootstrap support (99 %) and high Bayesian posterior probability (1.00) (Fig. 1). Rossman et al. (2004) and Decock et al. (2005) reported that the latter four genera formed a monophyletic lineage, with 95 % bootstrap confidence, that was basal to the core taxa of the Leotiomycetes. Rossman et al. (2004) assigned it as "a distinct lineage within the ascomycetes that is allied with the Leotiomycetes." For the higher-level phylogenetic classification of the genus *Kendrickiella*, therefore, further comprehensive studies are required.

The 28S and ITS datasets (Tables 1, 2) comprise sequences from the 18 KT and TT isolates and the two

Table 4 Comparison of selected characteristics between *Kendrickiella*, *Phialocephala*, and *Leptographium*

Character/item	*Kendrickiella*[a] K. Jacobs & M.J. Wingf. 2001	*Phialocephala* W.B. Kendr. 1961	*Leptographium* Lagerb. & Melin 1927
Conidiogenous cell	Phialides (intercalary in axis), hyaline	Phialides (sub)hyaline, collarettes	Sympodial or percurrent, (sub)hyaline
Base of conidiophores	**Rhizoids (present), swollen**	Rhizoids (present or absent)	Rhizoids (present or absent)
Substrate	Wood, fungi (cultures of *Ganoderma* and other polypores), soil, **stone, plaster wall, viscous gels (biofilms)**	Wood (often associated with bark beetles), litter, soil	Wood, roots (conifers), often associated with bark beetles
Distribution	Africa, Europe, South America, **Japan**	Cosmopolitan	Cosmopolitan
Systematics (Order to Class)	**Incertae sedis**[b]	Helotiales	Ophiostomatales
		Leotiomycetidae	Sordariomycetidae
		Leotiomycetes	Sordariomycetes
Reference source	Kendrick (1964)	Kendrick (1961)	Jacobs and Wingfield (2001)
	Jacobs et al. (2001)	Seifert et al. (2011)	Seifert et al. (2011)
	Seifert et al. (2011)		

[a] Data obtained in this study are indicated in bold

[b] The systematic assignment of *Kendrickiella* was treated as anamorphic Eurotiales by both Jacobs et al. (2001) and the current Index Fungorum http://www.indexfungorum.org/Names/Names.asp, but this is not mentioned in Seifert et al. (2011). In our molecular phylogenetic analyses, *Kendrickiella* took a position near the Leotiomycetes; however, its molecular phylogenetic relationship to the core members in the Leotiomycetes remains unresolved. In this study, therefore, the systematic position of *Kendrickiella* is treated as incertae sedis in the Pezizomycotina, Ascomycota; for details, see text

authentic strains labeled as *P. phycomyces* (MUCL 4271 and 38565). Using the ascomycetous yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen NRRL Y-12632 (Saccharomycetes, Saccharomycotina, Ascomycota) as an outgroup taxon, the Bayesian tree (Fig. 2) was inferred from 440 bp of the 28S sequences dataset. As demonstrated in Fig. 2, 18 of the KT and TT isolates and the 2 *P. phycomyces* strains (MUCL 4271 and 38565) formed a monophyletic lineage with very high bootstrap support (100 %) and Bayesian posterior probability (1.00). No base changes were seen among the 28S gene sequences from either the ten KT or the eight TT isolates, but between the KT and TT isolates three nucleotides differed. Two reference strains, MUCL 4271 and 38565, coincided with the KT isolates. A comparatively low genetic diversity was detected within this lineage. Jacobs et al. (2001) reported that the 28S-based phylogenetic position of *Kendrickiella phycomyces* CMW 2556 (=MUCL 38565) was basal to the Eurotiales–Onygenales clade. However, their sequence comparisons were based on only a 321-bp sequence (accession no.: AF269216). In this study, we determined a 546-bp sequence of the 28S rDNA D1/D2 region of MUCL 38565. In our molecular phylogeny of 18S and 28S (Figs. 1, 2), *P. phycomyces* did not group with any taxon samples from the two orders Eurotiales or Onygenales.

Using the cup fungus *Peziza vesiculosa* Bull. AFTOL-ID 507 (Pezizomycetes, Pezizomycotina) as an outgroup taxon, a Bayesian tree was inferred from 414 bp of the ITS sequences dataset. As shown in Fig. 3, our 18 isolates and the two *P. phycomyces* strains (MUCL 4271 and 38565) again formed a monophyletic lineage with high bootstrap support (99 %) and Bayesian posterior probability (1.00). No base changes were seen among the ITS gene sequences from either the 10 KT or the 8 TT isolates, but 13 nucleotides differed between the KT and TT isolates. One reference strain, MUCL 38655, was clustered with the KT isolates, whereas another reference strain, MUCL 4271, was basal to both subclades (i.e., the Kitora isolates plus MUCL 38565 and Takamatsuzuka isolates); thus yielding four haplotypes. Grünig et al. (2002) reported that the molecular phylogenetic position of *K. phycomyces* CBS 145.41 (preserved as *Scopularia phycomyces* (Auersw.) Goid.) was within the Eurotiales in their ITS1–5.8S–ITS2 tree. In our ITS tree (Fig. 3), the GenBank sequence of *Scopularia phycomyces* (registered as *K. phycomycoides*) CBS 145.41 (AF486127), and *Eupenicillium limosum* S. Ueda CBS 339.97 (GU981568) as a representative of the Eurotiales clustered together with 99 % bootstrap and 1.00 Bayesian posterior probability. In our observation, *Scopularia phycomyces* CBS 145.41, deposited by E. Rennerfelt in 1941, did not sporulate in culture, and the cultural characteristics differed between CBS 145.41 and our *K. phycomyces* isolates. In addition, the ITS sequence (AB671494) of CBS 145.41 determined in our study (not shown in Fig. 3) differed from that (AF486127) of GenBank registered by Grünig et al. (2002). Consequently, the identity of and the phylogenetic placement of '*Scopularia phycomyces*' CBS 145.41 (AF486127) listed in table 1 in Grünig et al. (2002) is questionable. Further

study is needed to reveal the "true" identity of *Scopularia phycomyces* CBS 145.41.

Several previous molecular phylogenies have clearly shown that the taxa included in *Phialocephala* are polyphyletic (e.g., Jacobs et al. 2001, 2003; Grünig et al. 2002). Our integrated analysis of phenotypic (mainly morphological; see the description in the following "Systematics" section) and genotypic (18S, 28S, and ITS sequence comparisons already mentioned) characteristics revealed that the 18 KT and TT isolates and two authentic strains (MUCL 4271 and 38565) labeled as *P. phycomyces* are conspecific. The correct name for the lineage comprising these isolates from both tumuli and the two reference strains is *Kendrickiella phycomyces*, as defined in Jacobs et al. (2001), the type species of *Kendrickiella*, rather than *Phialocephala*, typified by *P. dimorphospora* W.B. Kendr. (1961), which was phylogenetically placed within the core of the Leotiomycetes (Fig. 2). A full redescription of *K. phycomyces* with synonymy is given in the "Systematics" section following.

$CaCO_3$ solubilization on GYC agar plates

We recognized novel characteristics of organic acid production (Sano et al. 2010; Sugiyama et al. 2010) and the abilities of $CaCO_3$ solubilization in our isolates of *K. phycomyces*. Because most of the isolates were found on stone, the fungus may be involved in deterioration of tumulus stone walls. In both tumuli, mural paintings were applied on plaster walls that contained mainly $CaCO_3$. Thus, the ability of fungi to produce organic acids and to solubilize $CaCO_3$ were important aspects relevant to the biodeterioration.

Our isolates and reference strains of *K. phycomyces* were inoculated on GYC agar plates, and the cultural characteristics of $CaCO_3$ solubilization were observed. KT isolates showed stronger solubilization and recalcification of calcium carbonate (e.g., strain K5906-1-1) than TT isolates (e.g., strain T61114-1-1) (Fig. 4). These differences of solubilization activity were correlated with differences of haplotype in the molecular phylogenetic relationships of KT and TT isolates.

Pangallo et al. (2009) used $CaCO_3$ glucose agar to assess solubilization activity in their search for microbial communities responsible for the biodegradation of indoor artwork and air environments. In several studies, $CaCO_3$-containing agar plates were also used to test for solubilization by fungi (Sterflinger 2000). In our surveys we observed novel characteristics of organic acid production (e.g., citric acid and formic acid) (Sano et al. 2010, personal communication; Sugiyama et al. 2010) and abilities of $CaCO_3$ solubilization. GYC agar is useful for determining whether individual fungi have the ability to solubilize $CaCO_3$ and thereby contribute to the biodeterioration of plaster murals.

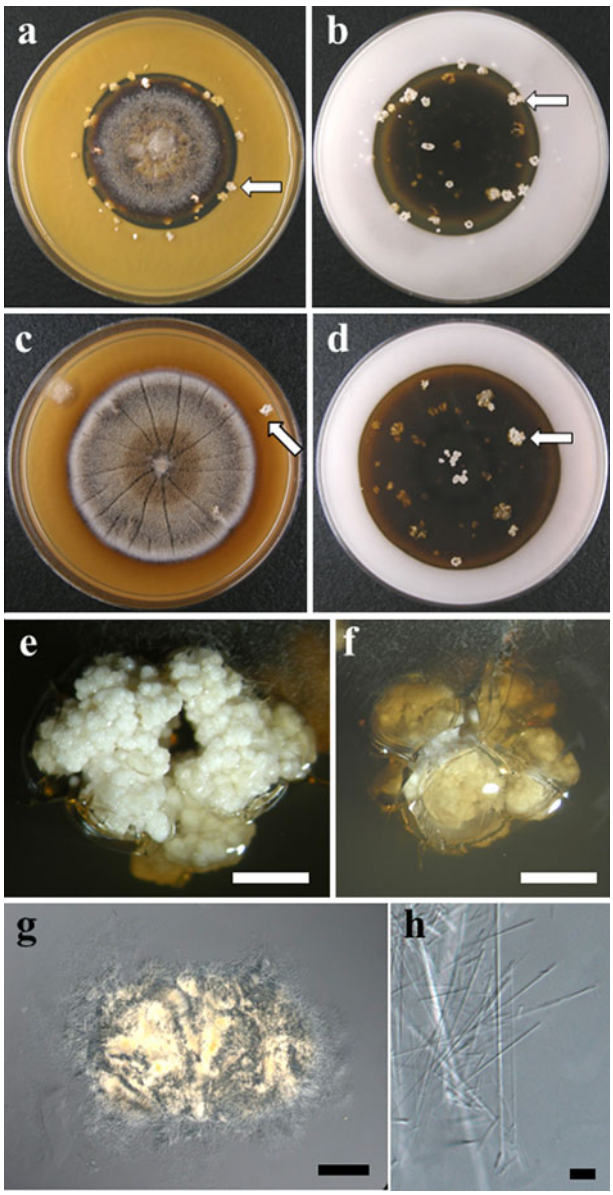

Fig. 4 Cultural characteristics of GYC (glucose–yeast extract–calcium carbonate) agar plates of Kitora Tumulus (KT) and Takamatsuzuka Tumulus (TT) isolates, and materials of recalcification of calcium carbonate at 25 °C, 4 weeks. **a**, **b** Surface and reverse of a GYC agar plate of K5906-1-1. **c**, **d** Surface and reverse of a GYC agar plate of T61114-1-1. **e**, **f** Recalcification of calcium carbonate; magnified figures from the portions shown by the *arrow* in **a** under the stereomicroscope, on GYC agar of K5906-1-1. **g**, **h** Needle-like crystals formed on GYC agar of K5906-1-1 that were seen in slide preparations made directly from the portion of the recalcification structures shown in **e**. *Bars* **e**, **f** 20 μm; **g**, **h** 5 μm. *Arrows* indicate white recrystallized materials formed on and in the agar plates

On GYC agar plates, we detected recrystallization of calcium carbonate by all isolates (Fig. 4a–f). White recrystallized material was formed on the surface of agar plates and embedded in the agar. These crystals exhibited a

needle-like structure under the light microscope (Fig. 4g–h). Further studies are needed to elucidate the chemical characteristics of these crystals in detail. Similar phenomena have been reported in bacteria and some fungi (Pangallo et al. 2009; Banks et al. 2010). Furthermore, white powder-like excretions were observed on the surface of the east wall of the stone chamber interior of the KT on 3 July 2009. This material showed the same needle-like crystals as the aggregations in our culture plates (Kiyuna et al., unpublished data). This $CaCO_3$ biomineralization is obviously related to biodeterioration of the plaster murals.

Origin and mechanisms of invasion into the stone chamber

In the KT, *K. phycomyces* was first observed on the surface of the stone ceiling in the adjacent small room in August of 2004 (Table 1). After that, this fungus was continuously observed in the stone chamber (Table 1). Conidial masses produced on the apex of conidiophores in *K. phycomyces* are considered suitable for dispersal by small animals (e.g., arthropods), just like species of the ophiostomatoid anamorph genus *Leptographium* (Jacobs and Wingfield 2001). Therefore, a likely route of invasion of *K. phycomyces* into the stone chamber is thought to be via arthropods (mites or collembolans). On the other hand, in TT this fungus was observed on plant roots in a hole beside the stone wall above the adjacent space (Fig. 5i–m; e.g., isolate no. T61213-27-1). Thus, another possible route of invasion of *K. phycomyces* into the stone chamber is thought to be via plant roots.

Systematics

Kendrickiella phycomyces (Auersw.) K. Jacobs & M.J. Wingf., Can. J. Bot. 79:113, 2001. Figs. 5, 6

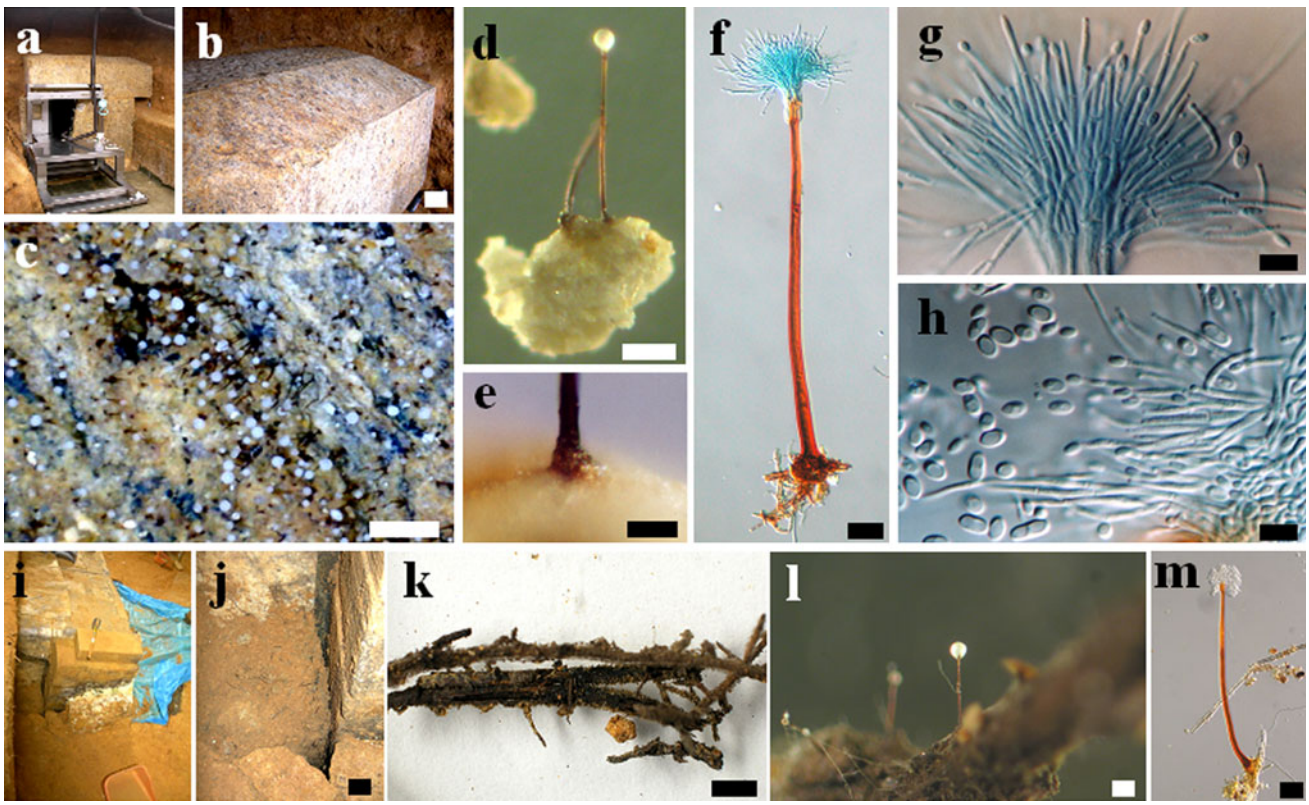

Fig. 5 Bristle-like structures on the stone wall in the Kitora Tumulus (KT) (**a**–**h**) and in roots collected from the adjacent space of the Takamatsuzuka Tumulus (TT) (**i**–**m**). **a**, **b** Entrance of the stone chamber and stone wall in the adjacent small room of the KT. **c** Bristle-like structures on the stone wall in the adjacent small room of the KT (photograph taken on 10 Sept 2004 by the Agency for Cultural Affairs, Japan; sample no. K4910). **d**, **e** Conidiophores and the base of a conidiophore digging into the stone substrate. This structure has a significant impact on the biodeterioration of stone substrates. **f**–**h** Conidiophores and conidia in slide preparations made directly from the part of the stone wall indicated in **c**. Conidiogenous cells are stained by cotton blue in **f**–**h**. Upper area of the adjacent space of TT (**i**) and connecting side space (**j**) into the adjacent space of TT (photograph taken on 13 Dec 2006). This side space was thought to be an invasion route. **k** Roots collected from the side space upper areas of the adjacent space of the TT in **j** (photograph taken on 13 Dec 2006; sample no. T61213-27). **l** A conidiophore on the root. **m** A conidiophore in a slide preparation made directly from the part of the root indicated in **l**. *Bars* **b**, **j** 30 cm; **c** 10 cm; **d**, **l** 100 μm; **e**, **f**, **m** 10 μm; **g**, **h** 5 μm; **k** 1 cm

≡*Hantzschia phycomyces* Auersw., Hedwigia 2:60, 1862 (basionym).
≡*Graphium phycomyces* (Auersw.) Sacc., Syll. Fung. 4:614, 1886.
≡*Leptographium phycomyces* (Auersw.) Grosmann, Hedwigia 71:193, 1932.
≡*Scopularia phycomyces* (Auersw.) Goid., Boll. Staz. Patol. Veg. Roma 16:1, 1936.
≡*Phialocephala phycomyces* (Auersw.) W. B. Kendr., Can. J. Bot. 42:1292, 1964.

Colonies in 12 days at 25 °C in darkness on MEA reaching 26–28 mm diameter, cream-buff (19A5) (Fig. 6a), on PDA 26–30 mm diameter, also cream-buff (Fig. 6b). Colony margin smooth. Hyphae submerged or in aerial mycelium, hyaline, smooth, not constricted at the septa, 2–3(–4) µm wide. Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (150–)200–450(–550) µm tall (Figs. 5f, 6d), with some rhizoid-like and swollen structures at the base (Figs. 5e; 6d,f). Stipes reddish brown, smooth, cylindrical, simple, 1–4-septate, (150–)200–350(–450) µm long (from first basal septum to the base of the primary branches), 5–7 (–9) µm wide below the primary branches, apical cell not swollen, 7.5–12.5(–15.5) µm wide at the base (Figs. 5f, 6d). Conidiogenous apparatus (50–)60–75(–90) µm long, excluding the conidial mass, with three or four series of cylindrical branches; 2–4 primary branches in whirls, reddish brown, smooth, cylindrical, aseptate, (12–)15–17 (–20) × 5–6(–8) µm, secondary branches light reddish brown to hyaline, aseptate, (7–)8–10(–12) × 3–4 µm; tertiary branches hyaline, aseptate, 5–10 × 2–2.5 µm, quaternary branches aseptate, hyaline, 5–7 × 1.5–2 µm (Figs. 5g, 6e). Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering slightly at the apex, (5–)8–10(–20) × 1–1.5 mm (Figs. 5g, 6e). Conidium development phialidic with poorly developed collarettes (Figs. 5g, 6e). Conidia aseptate, ellipsoidal, (3–)4–5(–7) × (1.5–)2–3 µm, accumulating in cream-colored slimy droplets at the apex of the conidiogenous apparatus (Figs. 5h; 6c,g).

Gene sequence data: The GenBank accession numbers for nuclear 18S, 28S, and ITS regions are listed in Tables 1 and 2.

Isolates examined: Ten isolates from the stone walls and other substrates of the stone chamber interior and exterior of KT, from 2004 to 2010; eight isolates from the adjacent space, the stone chamber interior and between stone walls of the TT, from 2006 to 2007 (Table 1). Two authentic MUCL strains labeled as *Phialocephala phycomyces*: MUCL 4271, derived from DAOM 63734, isolated as a

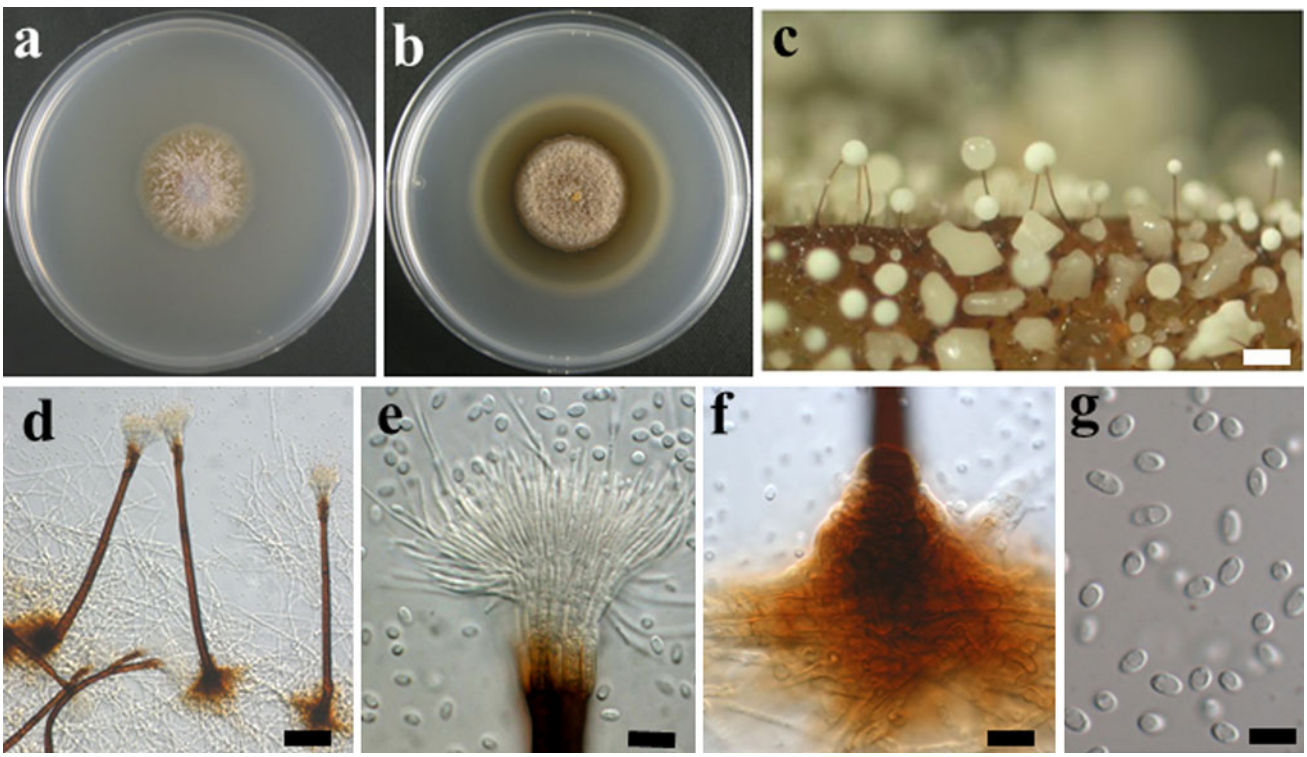

Fig. 6 *Kendrickiella phycomyces* (K4910-1). **a** Colony on malt extract agar (MEA) at 20 °C, 20 days. **b** Colony on potato dextrose agar (PDA) at 20 °C, 20 days. **c** Conidiophores on PDA. **d** Conidiophores. **e** Conidiogenous apparatus. **f** Basal structure of the conidiophore. **g** Conidia. *Bars* **c** 200 µm; **d** 50 µm; **e**, **f** 10 µm; **g** 5 µm

contaminant in culture derived from a polypore (Kendrick 1964), and MUCL 38565, derived from CMW 2556 examined in the description of *K. phycomyces* as a single living isolate by Jacobs et al. (2001).

The phenotypic characteristics, except for the basal structures of conidiophores, of our isolates from both tumuli and the two reference strains from MUCL agreed well with the characteristics of *Phialocephala phycomyces* and *Kendrickiella phycomycoides* [sic] provided by Kendrick (1964) and Jacobs et al. (2001), respectively. We also observed some rhizoids and swollen cells grown on MEA and on the natural substratum at the base of the conidiophores. This observation deviates from the descriptions by Kendrick (1964) and Jacobs et al. (2001). These structures might have been an adaptation to the habitat, i.e., the stone substrates observed in KT.

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