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ORIGINAL PAPER

The identity of *Penicillium* sp. 1, a major contaminant of the stone chambers in the Takamatsuzuka and Kitora Tumuli in Japan, is *Penicillium paneum*

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Abstract *Penicillium* appeared as the major dweller in the Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT) stone chambers, both located in the village of Asuka, Nara Prefecture, in relation to the biodeterioration of the 1,300-year-old mural paintings, plaster walls and ceilings. Of 662 *Penicillium* isolates from 373 samples of the TT (sampling period, May 2004–2007) and the KT (sampling period, June 2004–Sep 2007), 181 were phenotypically assigned as *Penicillium* sp. 1 which shared similar phenotypic characteristics of sect. *Roqueforti* in *Penicillium* subg. *Penicillium*. Fifteen representative isolates of *Penicillium* sp. 1, 13 from TT and 2 from KT, were selected for molecular phylogenetic analysis. The 28S rDNA D1/D2, ITS, β-tubulin, and *lys2* gene sequence-based phylogenies clearly demonstrated that the three known species *P. roqueforti*, *P. carneum* and *P. paneum* in sect. *Roqueforti*, and all TT and KT isolates grouped together. In addition to this, TT and KT isolates formed a monophyletic group with the ex-holotype strain CBS 101032 of *P. paneum* Frisvad with very strong bootstrap supports. So far, *P. paneum* has been isolated only from mouldy rye breads, other foods, and baled grass silage. Therefore, this is the first report of *P. paneum* isolation from samples relating to the biodeteriorated cultural properties such as mural paintings on plaster walls.

Keywords Biodeterioration · *Penicillium* sect. *Roqueforti* · Molecular phylogeny · Murals · Takamatsuzuka and Kitora Tumuli

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Introduction

Penicillium, the phialidic, anamorphic, species-rich genus of the *Trichocomaceae* in the *Eurotiomycetes*, inhabits a variety of substrates and environments.

Members of this genus are important in human life; they commonly appear as soil, storage, indoor and airborne fungi or as food contaminants (e.g., Pitt 1979; Domsch et al. 2007; CBS databases [http://www.cbs.knaw.nl/fungi/BioloMICS.aspx]). Fungal biodeterioration is well known to affect cultural properties such as catacombs (Saarela et al. 2004) and murals (Berner et al. 1997; Dhawan et al. 1993; Guglielminetti et al. 1994) in Europe. In addition, the roles of fungi in the deterioration of murals, as well as their decay mechanism, have been reviewed by Nugari et al. (1993) and Garg et al. (1995); those studies listed, at the species level and including *Penicillium* spp., fungi that have been isolated from deteriorated murals.

On the other hand, in Japan, microbiological surveys related to the conservation of stone chamber interiors and murals were conducted for the Ozuka and Chibusan Tumuli in Fukuoka Prefecture (Emoto and Emoto 1974), the Torazuka Tumulus in Ibaraki Prefecture (Emoto et al. 1983; Arai 1984, 1990a), and the Takamatsuzuka (Arai 1984, 1987, 1990b; Kigawa et al. 2004, 2006b) and Kitora (Kigawa et al. 2005, 2006a) Tumuli, both in Nara Prefecture. Anamorphic fungi such as species of *Cladosporium, Doratomyces, Fusarium, Penicillium*, and *Trichoderma* were detected from samples taken from these tumuli (cf. Tables 1 and 3 in Kiyuna et al. 2008). In most cases, however, identification of *Penicillium* isolates in these previous studies was limited to the generic level.

The Takamatsuzuka Tumulus (TT) and the Kitora Tumulus (KT), which are thought to have been built in late 7th century, are well known in Japan as invaluable cultural heritage sites because of their beautiful mural paintings, which were drawn directly onto thin plaster in the small stone chamber interior. The mural paintings of the TT were discovered in 1972, whereas the KT was discovered in 1983 and excavated in early 2004. The Japanese government designated the TT and KT as Special Historic Sites in 1973 and 2000, respectively.

The TT is located in the village of Asuka, Nara Prefecture, Japan. It is a circular burial mound measuring approximately 20 m in diameter. The stone chamber was constructed of cut slabs of volcanic tuff; the interior area is approximately 2.7 m deep, 1.0 m wide, and 1.1 m tall (for the schematic diagram, see Fig. 1 in Kiyuna et al. 2008). The colorful paintings adorn the inner walls and a ceiling of the stone chamber with various colors, including red, green, yellow, blue, and gold. The figures comprise star constellations (*Seishuku*) on the ceiling, and the sun and the moon (*Nichi-Getsu*), the four heavenly guardian gods (*Shishin*), and groups of men and women on the four walls. The group of women on the west wall is called the "Asuka beauties" (*Asuka bijin*) (see Fig. 2 in Kiyuna et al. 2008). All the paintings were designated as National Treasures by the government in 1974. In accordance with specialists' advice, the Japanese Agency for Cultural Affairs decided in 1973 to preserve the murals on site and they were never opened to the public. It was thought that the beauty and stability of the plaster paintings could be preserved if conditions inside the stone chamber would be kept as before, naturally buried state; high humidity (100% RH) and cool temperature (14–20°C) could be established. In addition, the stone chamber was kept in darkness except for regular inspections by the staff involved in its conservation.

The KT is also located 1.2 km south of the TT in the same village of Asuka. Its circular tomb is approximately 14 m diameter; the stone chamber interior is approximately 2.4 m deep and 1 m wide and tall; a diagram of the stone chamber and related facilities is not illustrated here, but the KT adjacent small room corresponds to the adjacent space of the TT. Similar colorful murals are drawn on the thin plaster wall within the stone chamber, and a star chart on the ceiling is in the same style as in the TT. The interior environmental conditions are quite similar to those of the TT, and no conspicuous fungal colonies were seen for some time after the excavation. In 2004, in the course of the excavation, species of *Acremonium, Fusarium, Penicillium*, and *Trichoderma* appeared as the main contaminants (Kigawa et al. 2005, 2006a, 2007). In the case of the KT, the paintings on the plaster wall had become partly detached from their support. Considering such situation, it was decided to relocate these paintings to a controlled environment in July 2004. By the end of 2008, all the paintings except for possible ones hidden by thin wall layer, were relocated.

We have detected and reported the haplotypes of *Fusarium* and *Trichoderma*, the two predominant fungal colonizers, using the 28S rDNA D1/D2 region (28S) and EF-1α (=tef1: protein-coding gene

translation-elongation factor 1-alpha) gene sequence-based analyses (Kiyuna et al. 2008). We have also proposed two new species of *Candida* assignable to the *C. membranifaciens* clade: *C. tumulicola* and *C. takamatsuzukensis*, isolated mainly from biofilm samples from the stone chamber interior of the Takamatsuzuka Tumulus (Nagatsuka et al. 2009). In the previous papers (Sugiyama et al. 2008, 2009; Kiyuna et al. 2008), we have mentioned that *Penicillium* isolates, which comprised one of the predominant fungal groups, were obtained from samples in the stone chamber interiors and adjacent spaces or rooms of both tumuli. Of 1,780 fungal isolates, 662 were assignable to *Penicillium*. And 181 of those isolates have been phenotypically assigned as *Penicillium* sp. 1.

In the present study, we reveal phenotypically and genotypically the identity of *Penicillium* sp. 1 isolates as the major *Penicillium* colonizers in the stone chamber environments in the Takamatsuzuka and Kitora Tumuli.

Materials and methods

Sample sources, isolation, and culturing

A total of 224 samples, including mold spots, viscous gels (biofilms), and mixtures of plaster fragments and soil, were collected from the stone chamber interior, spaces between the stone walls, and stone chamber exterior of the Takamatsuzuka Tumulus (TT) between May 2004 and May 2007. As well, a total of 149 samples were collected from the stone chamber interior and exterior of the Kitora Tumulus (KT) between June 2004 and September 2007. Isolation methods have been mentioned in Sugiyama et al. (2008, 2009) and Kiyuna et al. (2008). The isolates have been maintained on potato dextrose agar (PDA; Nihon Pharmaceutical, Tokyo, Japan). For strain data originally identified as *Penicillium* sp. 1 from the Takamatsuzuka and Kitora Tumuli, see Table 1 and supplementary Table S1. The 11 selected isolates are deposited with the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Wako, Saitama Prefecture, Japan, as JCM 15985–15995 (Table 1). The remaining *P. paneum* isolates from both tumuli are maintained at the Biology Laboratory of the National Research Institute of Cultural Properties, Tokyo as lyophilised cultures (Table 1, supplementary Table S1).

Cultural and morphological observations

A total of 50 isolates, comprising 31 and 19 isolates from TT and KT, respectively, were used in the cultural and morphological observations. These isolates were selected from the standpoint of sampling sites and dates as well from the standpoint of kinds of substrates. The detailed data on isolates are shown in supplementary Table S1. All isolates were grown using the media and growth conditions adopted by Pitt (1979, 2000) and Frisvad and Samson (2004). The isolates were incubated on Czapek yeast autolysate (CYA) agar at 25°C, malt extract agar (MEA) at 25°C, 25% glycerol nitrate (G25N) agar at 25°C, yeast extract sucrose (YES) agar at 25°C, and creatine sucrose (CREA) agar at 25°C all for 7 days in darkness, respectively. Growth rates were measured on CYA inoculated with conidial suspension and incubated at 5, 10, 15, 20, 25, 30, 37 and 40°C all for 7 days in the dark. Furthermore, growth rates were determined in the moist chamber, about 100% RH. The colony colors of the isolates on all media were determined by using Kornerup and Wanscher's color standard (1978). Microscopic slides were prepared from portions of the colonies grown on MEA plate cultures and were mounted in lactophenol mounting medium without dye (Bills and Foster 2004). Microscopic examinations were made using a fluorescence microscope BX51 (Olympus, Tokyo, Japan) with Normarski interphase contrast at up to $\times$1,000 magnification. All micrographs were taken with a Coolpix P5000 digital camera (Nikon, Tokyo, Japan).

Ubiquinone determination, a chemotaxonomic characteristic

Mycelia grown on Difco^{TM} Potato Dextrose Broth (Becton–Dickinson, MD, USA) were used for the extraction of ubiquinones. The isolates and other authentic strains were cultured at 25°C for 3 days on a rotary shaker (Taitec, Saitama, Japan) at 125 revolutions per min^{-1}. Extraction, purification, and determination of ubiquinones by high-performance liquid chromatography were carried out as described previously (Kuraishi et al. 1985, 1991). When one

Table 1 Strain data of TT and KT isolates, with major ubiquinone system data and the GenBank accession numbers, for 28S, ITS, *β*-tubulin, and *lys2* gene sequences determined in this study

Isolate[a]	JCM no.	Isolation source	Sampling date	GenBank accession no.				Ubiquinone system[b]
				28S	ITS	*β*-tubulin	*lys2*	
T4519-5-5	15985	Mouldy spots on the floor of the stone chamber of Takamatsuzuka Tumulus	19 May 2004	AB479290	AB479322	AB479337	AB479366	
T4906-11-8	15986	Surface of an insect body (Isopoda) from the wall in the stone chamber of Takamatsuzuka Tumulus	6 Sep 2004	AB479291	AB479323	AB479338	AB479367	
T5916-1-1		Viscous gels below the forefoot of the painting of the white tiger on the west wall 2 in the stone chamber of Takamatsuzuka Tumulus	16 Sep 2005	AB479292	AB479324	AB479339	AB479368	
T5916-3-1		Viscous gels below the paintings of the group of women on the west wall 3 in the stone chamber of Takamatsuzuka Tumulus	16 Sep 2005	AB479293	AB479325	AB479340	AB479369	
T5916-6-1	15987	Viscous gels below the paintings of the group of women on the east wall 3 in the stone chamber of Takamatsuzuka Tumulus	16 Sep 2005	AB479294	AB479326	AB479341	AB479370	Q-9
T6517-1-2	15988	Black spots on the paintings of the group of women on the west wall 3 in the stone chamber of Takamatsuzuka Tumulus	17 May 2006	AB479295	AB479327	AB479342	AB479371	
T7214-8-1	15989	Black spots on western area of the adjacent space of Takamatsuzuka Tumulus (during excavation)	14 Feb 2007	AB479296	AB479328	AB479343	AB479372	Q-9
T7425-3-1		Black substances in small pits on the top surface of west wall stone 3 of Takamatsuzuka Tumulus (during relocation of the stone chamber)	25 Apr 2007	AB479297	AB479329	AB479344	AB479373	
T7425-4-1	15990	Black substances from the top surface of east wall stone 3 of Takamatsuzuka Tumulus (during relocation of the stone chamber)	25 Apr 2007	AB479298	AB479330	AB479345	AB479374	Q-9
T7510-5-1	15991	Pieces of side plaster from the northern side of west wall stone 2 of Takamatsuzuka Tumulus (during relocation of the stone chamber)	10 May 2007	AB479299	AB479331	AB479346	AB479375	
T7521-8A-4	15992	Plastic cover over the thieving hole (stone chamber interior side) of Takamatsuzuka Tumulus	21 May 2007	AB479300	AB479332	AB479347	AB479376	
T7528-21-1	15993	Southern area of the ceiling plaster of ceiling stone 2 of Takamatsuzuka Tumulus (during relocation)	28 May 2007	AB479301	AB479333	AB479348	AB479377	
T7530-4-1		Black viscous gels and pieces of side plaster between east wall stone 1 and ceiling stone 1 of Takamatsuzuka Tumulus (during relocation)	30 May 2007	AB479302	AB479334	AB479349	AB479378	
K5916-7-1	15994	Dark-greenish viscous gels below the paintings of the tortoise and snake on the north wall in the stone chamber of Kitora Tumulus	16 Sep 2005	AB479303	AB479335	AB479350	AB479379	
K6203-2-1	15995	Black viscous substance in a hole on the east wall in the stone chamber of Kitora Tumulus	3 Feb 2006	AB479304	AB479336	AB479351	AB479380	

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

[b] The major ubiquinone system was determined in this study

type of ubiquinone molecule constituted more than 90% of the types found in a particular strain, it was determined to be the major ubiquinone type (Kuraishi et al. 1985, 1991).

DNA extraction, PCR amplification, and sequencing

The isolates used for the DNA sequencing are listed in Table 1. Their genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The four gene regions sequenced were the 28S rDNA D1/D2 region (28S), ITS-5.8S rDNA (ITS), part of the β-tubulin, and part of the *lys2* (aminoadipate reductase) gene. The primers used included NL1 and NL4 (O'Donnell 1993) for 28S, ITS5 and ITS4 (White et al. 1990) for ITS, Bt2a and Bt2b (Glass and Donaldson 1995) for β-tubulin, and lys2F and lys2R (An et al. 2002) for *lys2*. Polymerase chain reactions (PCR) were performed using puRe*Taq* Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK). Thermal cycling was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Initial denaturation at 95°C for 5 min was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s (28S, ITS)/58°C for 30 s (β-tubulin)/56°C for 1 min (*lys2*), 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, Hilden, Germany). The PCR products of the *lys2* gene were cloned using a PCR Cloning Kit (Qiagen, Hilden, Germany). Two to three white clones of each the PCR products were sampled. Sequencing of the PCR products and the cloned plasmids was performed using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, CA, USA). Reactions were incubated in a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) and the products cleaned using a DyeEx^{TM} 2.0 Spin Kit (Qiagen, Hilden, Germany). Sequences were determined using electrophoresis in an ABI3130xl DNA sequencer (Applied Biosystems, CA, USA). The sequences determined in this study were deposited in GenBank/EMBL/DDBJ, and their accession numbers are given in Table 1. Other sequences downloaded from GenBank (Table 2) are shown in the respective molecular phylogenetic trees.

28S and ITS molecular phylogenetic analyses

The sequences were assembled using ChromasPro 1.42 (Technelysium Pty Ltd., Tewantin QLD, Australia). Multiple alignments were performed using CLUSTAL W version 1.83 (Thompson et al. 1994), and then the final alignments were manually adjusted. Ambiguous positions and alignment gaps were excluded from the analysis. The alignments and trees were deposited in TreeBASE, accession number SN4551. The neighbor-joining (NJ) tree based on the respective gene sequences was constructed using the multiple alignments in MEGA ver3.1 (Kumar et al. 2004), with 1,000 bootstrap replicates (Felsenstein 1985).

Molecular phylogenetic analyses of β-tubulin and *lys2* genes

The β-tubulin and *lys2* gene analysis for *Penicillium*, the phylogenetic tree by NJ, and the maximum likelihood (ML) and Bayesian (Bayes) analyses were performed. The alignments and trees were deposited in TreeBASE, accession number SN4551. NJ trees based on the respective gene sequences were constructed using the multiple alignments in MEGA ver3.1 (Kumar et al. 2004), with 1,000 bootstrap replicates (Felsenstein 1985). ML trees based on the respective gene sequences were also constructed using the multiple alignments in PHYLIP version 3.6 (Felsenstein 2002), with 100 bootstrap replicates. The Bayesian approach to phylogenetic reconstruction (Rannala and Yang 1996) was implemented using MrBayes v3.1.1-p1 (Huelsenbeck and Ronquist 2001). The model of DNA substitution was calculated using Modeltest 3.0 (Posada and Crandall 1998). The results were obtained by the K80 model and gamma correction (G) rate variation among sites for β-tubulin, and the general time-reversible (GTR) model and G rate variation among sites for *lys2*. 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 1,500,000 generations for β-tubulin and 1,000,000 generations for *lys2*. Searches were conducted with four chains (three cold, one hot) with trees sampled every 100 generations. The average standard deviations of split frequencies were 0.008

Table 2 Strain data used from molecular phylogenetic analyses with the major ubiquinone system and GenBank accession numbers for 28S, ITS, β-tubulin, and *lys2* gene sequences

Taxon	Strain no.	Nomenclatural status	Source (substrate) and origin	GenBank accession no.[a]				Ubiquinone system
				28S	ITS	β-tubulin	*lys2*	
Penicillium albocoremium	IBT 10682	Ex-holotype	Salami, Denmark	–	AJ004819	–	–	–
P. allii	DAR 74613		Antarctica, ornithogenic soils in penguin colony areas, Windmill Islands	–	AF218787	–	–	–
P. allii	IBT 3056		Food, Great Britain	–	AJ005484	–	–	–
P. atramentosum	NRRL 795	Ex-neotype	French Camembert cheese, USA	AF033483	–	–	–	Q-9
P. aurantiogriseum	CBS 324.89	Ex-neotype		**AB479286**	**AB479318**	AY674296	**AB479365**	–
P. brevicompactum	CBS 257.29	Ex-neotype		–	–	AY674437	–	Q-9
P. brevicompactum	JCM 22849	Ex-neotype		**AB479275**	**AB479306**	–	–	Q-9
P. camemberti	CBS 299.48	Ex-neotype	French Camembert cheese, USA	**AB479282**	**AB479314**	AY674368	**AB479361**	Q-9[b]
P. carneum	CBS 112297	Ex-holotype	Mouldy rye bread, Denmark	**AB479279**	**AB479310**	AY674386	**AB479358**	Q-9[b]
P. chrysogenum	CBS 306.48	Ex-neotype	Cheese, USA	–	–	AY495981	–	Q-9
P. chrysogenum	JCM 22826	Ex-neotype	Cheese, USA	**AB479274**	**AB479305**	–	**AB479353**	Q-9
P. commune	CBS 311.48	Ex-isotype	Cheese, USA	AY213616	–	–	–	–
P. concentricum	NRRL 2034			–	DQ339561	–	–	–
P. coprophilum	NRRL 13627		*Sorghum*, Africa	AF033469	–	–	–	–
P. cordubense	NRRL 13072			AF527055	–	–	–	–
P. digitatum	CBS 136.65		Fruits of *Citrus medica limonium* ? (*Rutaceae*), Netherlands	**AB479276**	**AB479307**	AY674404	**AB479355**	–
P. echinulatum	CBS 317.48	Ex-isotype	Culture contaminant, Canada	–	–	AY674341	–	Q-9
P. echinulatum	JCM 22836	Ex-isotype	Culture contaminant, Canada	**AB479284**	**AB479316**	–	**AB479363**	Q-9
P. expansum	CBS 325.48	Ex-neotype	Fruits of *Malus sylvestris* (*Rosaceae*), USA	**AB479278**	**AB479309**	AY674400	**AB479357**	–
P. gladioli	NRRL 939	Ex-neotype	Gladiolus corm, Africa	AF033480	–	–	–	–
P. glandicola	CBS 498.75	Ex-epitype	Mouldy wine cork, Portugal	**AB479277**	**AB479308**	AY674415	**AB479356**	–
P. griseofulvum	NRRL 2300	Ex-neotype		AF033468	–	–	–	Q-9
P. griseofulvum	NRRL 3523			–	DQ339549	–	–	–
P. hirsutum	JCM 22835	Ex-neotype	Aphid, green fly, Netherlands	**AB479283**	**AB479315**	–	**AB479362**	Q-9
P. hirsutum	CBS 135.41	Ex-neotype	Aphid, green fly, Netherlands	–	–	AY674328	–	Q-9
P. inflatum	CBS 682.70	Ex-holotype	Root surface of *Picea abies*, Denmark	–	–	–	**AB480887**	–
P. madriti	NRRL 3452	Ex-holotype	Garden soil, Spain	AF033482	AF033482	–	–	–

Table 2 continued

Taxon	Strain no.	Nomenclatural status	Source (substrate) and origin	GenBank accession no.[a]				Ubiquinone system
				28S	ITS	β-tubulin	*lys2*	
P. olsonii	CBS 232.60	Ex-neotype	Root of *Picea abies*, Austria	–	–	AY674445	–	–
P. paneum	CBS 101032	Ex-holotype	Mouldy rye bread, Denmark	**AB479273**	**AB479312**	AY674387	**AB479321**	Q-9[b]
P. paneum	CBS 465.95		Mouldy baker's yeast, Denmark	**AB479280**	**AB479311**	AY674388	**AB479359**	Q-9[b]
P. polonicum	NRRL 995	Ex-holotype	Soil, Poland	AF033475	AF033475	–	–	–
P. roqueforti	CBS 221.30	Ex-neotype	French Roquefort cheese, USA	–	–	AY674383	–	Q-9[b]
P. roqueforti	JCM 22842	Ex-neotype	French Roquefort cheese, USA	**AB479281**	**AB479313**	–	**AB479360**	Q-9[b]
P. sclerotigenum	NRRL 3461	Ex-neotype	Plant tuber, Sweet potato, Japan	AF033470	–	–	–	–
P. tricolor	ATCC 10413			–	AY373935	–	–	–
P. venetum	IBT 5464		Flower bulb, Denmark	–	AJ005485	–	–	–
P. verrucosum	CBS 603.74	Ex-neotype		**AB479285**	**AB479317**	AY674323	**AB479364**	Q-9
P. viridicatum	NRRL 961			AF033478	AF033478	–	–	Q-9
Eupenicillium baarnense	NRRL 2086	Ex-neotype	Soil, Netherlands	AF033481	–	–	–	–
E. catenatum	CBS 431.69		Soil, USA	–	–	AY674433	–	–
E. crustaceum	CBS 581.67		Soil, Pakistan	**AB479289**	**AB479321**	–	–	–
E. crustaceum	CBS 244.32	Ex-neotype	Soil, Egypt	**AB479287**	**AB479319**	–	–	–
E. molle	CBS 456.72	Ex-holotype	Soil, Pakistan	**AB479288**	**AB479320**	–	–	Q-9
E. tularense	NRRL 5273	Ex-holotype	Soil under *Pinus ponderosa* & *Quercus* sp., USA	AF033487	AF033487	–	–	Q-9

IBT Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark, *ATCC* American Type Culture Collection, Manassas, Virginia, USA, *NRRL* Agricultural Research Service (ARS) Culture Collection, Northern Utilization Regional Research Center, Peoria, Illinois, USA, *NBRC* NITE Biological Resource Center, Kisarazu, Chiba, Japan, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, *JCM* Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan, *DAR* Department of Agriculture, Biological and Chemical Research Institute, Rydalmere, NSW, Australia

[a] Bold accession numbers indicate sequences determined in this study, whereas other sequences were obtained from GenBank

[b] The major ubiquinone system were determined in this study, whereas the remaining ubiquinone data were cited from Kuraishi et al. (1991)

– No data

and 0.006, respectively, 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.

Results and discussion

Cultural and morphological characterization of *Penicillium* sp. 1 isolates

Cultural and morphological characterization of *Penicillium* sp. 1 was based on 50 representative isolates from the TT and KT stone chamber interiors and exteriors (for details, see Table 1 and supplementary Table S1).

Colonies on CYA at 25°C for 7 days grew rapidly and were 30–40 mm in diameter, radially sulcate, velutinous, predominantly greyish turquoise (25CD-6) or greyish green (28B2-3), and had a white (25A1-2) margin; the reverse was pale yellow. No growth was observed at 37°C for 7 days. Colonies on MEA at 25°C for 7 days grew rapidly, and were 43–50 mm in diameter, velutinous, and predominantly pale green (26A-3) to greenish white (26A-2), with a greenish white (25A-2) margin; the reverse was pale yellow. Colonies on YES at 25°C for 7 days grew rapidly, and were 40–43 mm in diameter, radially sulcate, velutinous, and predominantly greyish white (25A-2) to pale green (25A-3); the reverse was pale yellow. Colonies on G25N at 25°C for 7 days were 26–30 mm in diameter, velutinous, and predominantly greyish green (25B-5), with a white (25A-1) margin; the reverse was pale yellow. Colonies on CREA at 25°C for 7 days attained a diameter of 18–20 mm and were velutinous, predominantly pale green (25A-3). The optimum temperature for growth on CYA, in the four selected *Penicillium* sp. 1 isolates (T4519-5-5, T5916-6-1, T6517-1-2 and K5916-6-1), was 20–25°C; the colonies attained ca. 50–60 mm diameter after 7 days. The growth rates were similar to the moist chamber, about 100% RH. These results suggested that TT and KT isolates were mesophilic.

Conidiophores arose from the subsurface and submerged hypha, and stripes reached lengths of 100–200 (–250) μm, characteristically with very rough and tuberculate walls. The penicillus was typically terverticillate, occasionally quaterverticillate, with appressed elements; the rami were 20–25 (–30) μm long with tuberculate walls; the metulae were 12–15 μm long; and the phialides were ampulliform, 7.5–8.5 (–10) μm long, with short collula. Conidia were spherical, (3–) 3.5–4 (–4.5) μm in diameter, with smooth or finely rough walls, pale green to dark green, borne in long chains, and closely packed. No sclerotium-like structures were observed.

The above-mentioned cultural and morphological characterization of *Penicillium* sp. 1 isolates differed from those of *P. roqueforti* and *P. carneum,* both in *Penicillium* subg. *Penicillium* sect. *Roqueforti* by a pale yellow colony reverse on CYA and YES. The phenotypic characteristics of *Penicillium* sp. 1 isolates agreed well with those of the ex-holotype (CBS 101032) of *P. paneum* Frisvad, the third species in the same section and its previous descriptions (Boysen et al. 1996; Frisvad and Samson 2004), except for producing finely rough conidia. As a whole, the results of our morphological observations were in concordance with those of O'Brien et al. (2008). Further studies are needed to clarify whether or not the finely rough conidial formation is a common characteristic of *P. paneum*.

Chemotaxonomic characterization of *Penicillium* sp. 1 isolates

Three selected *Penicillium* sp. 1 isolates (T5916-6-1, T7214-8-1, and T7425-4-1) and the ex-holotype strain CBS 101032 and CBS 465.95 of *P. paneum*, which were determined in this study, had Q-9 as the major ubiquinone system, a chemotaxonomic characteristic (Table 1). It agreed with that of the remaining two species within the same section *Roqueforti*, *P. roqueforti* and *P. carneum*. As a result, three known species comprising *P. roqueforti, P. carneum* and *P. paneum* in the same section *Roqueforti*, have been characterized by the same ubiquinone system Q-9 (Table 2; cf. Kuraishi et al. 1991).

Molecular phylogenetic position of *Penicillium* sp. 1 isolates

Here we show evidence from our molecular phylogenetic analyses of four different gene sequences (28S, ITS, *β*-tubulin, and *lys2*; Figs 1, 2, supplementary

Figs. S1 and S2) that the selected 15 isolates of *Penicillium* sp. 1, i.e., 13 from the TT and 2 from the KT (Table 1), are assignable to *Penicillium paneum* in sect. *Roqueforti* subg. *Penicillium*.

Using *P. brevicompactum* JCM 22849 (*Penicillium* subg. *Penicillium* sect. *Coronata*) as an outgroup taxon, the NJ tree (supplementary Fig. S1) was inferred from 594 bp of 28S sequences of 44 isolates of *Penicillium* and relatives. In the 28S phylogeny, all the TT and KT isolates were placed in a cluster comprised only of *P. paneum*, including the ex-holotype strain CBS 101032 (isolated from mouldy rye bread in Denmark) of *P. paneum*. However, the bootstrap value showed that the reliability of the phylogeny was not sufficient to endorse a taxonomic position at a species level because of a comparatively low confidence level (80%).

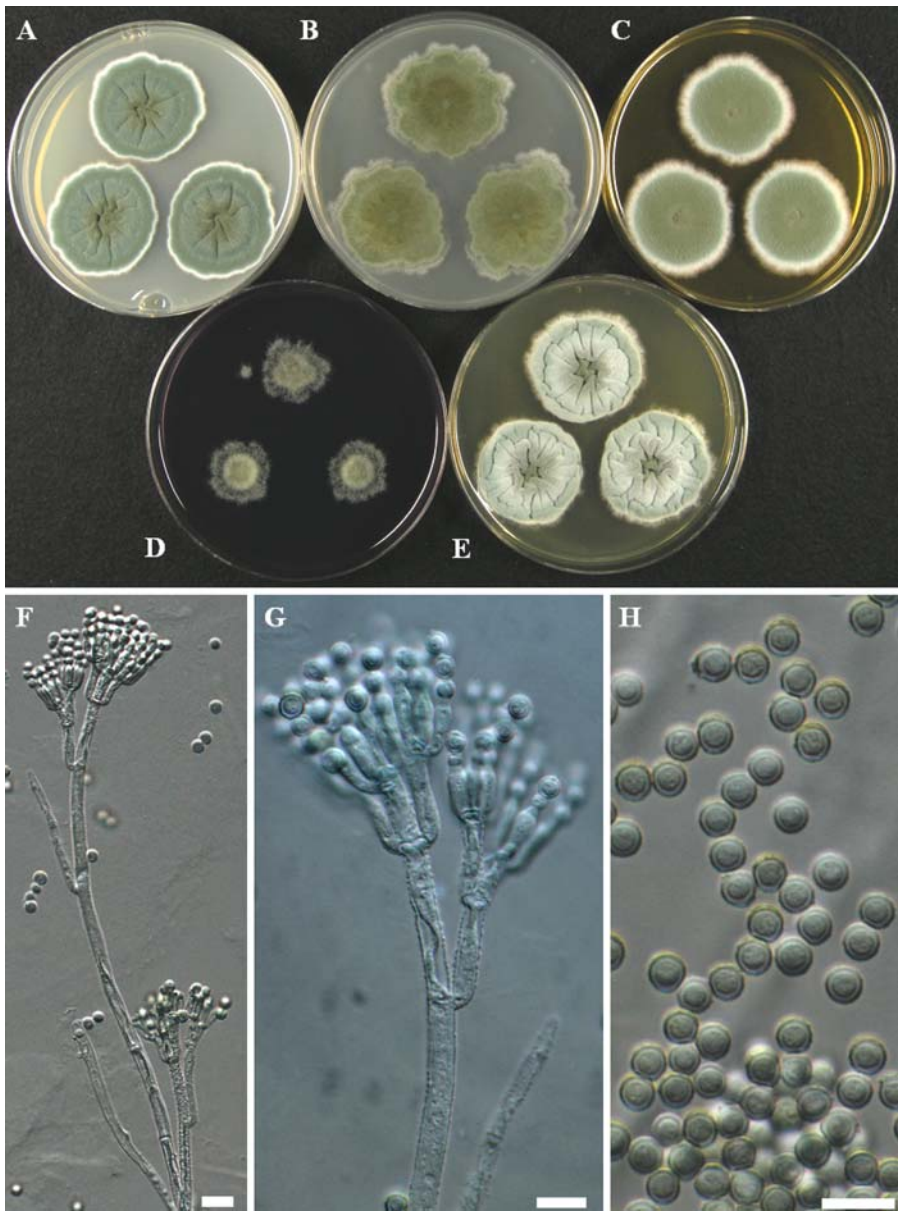

Fig. 1 Cultural and morphological characteristics of *Penicillium* sp. 1 (isolate T5916-6-1). Colonies on CYA (**a**), MEA (**b**), G25N (**c**), CREA (**d**), and YES (**e**) at 25°C, 7 days. Conidiophores and penicilli (**f**, **g**), and conidia (**h**). *Bars* 10 μm

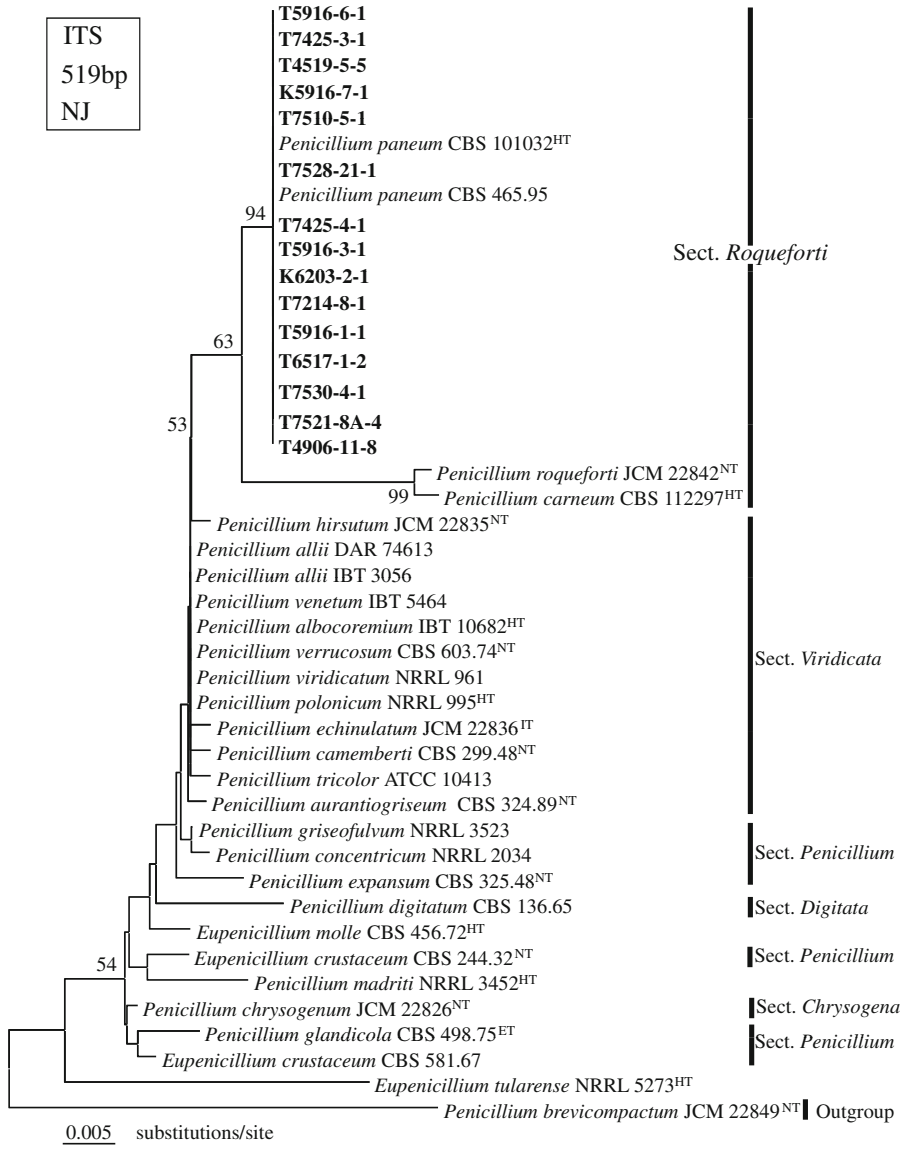

Fig. 2 Phylogenetic relationships among 15 TT and TK isolates and 28 known *Penicillium* isolates for which the sequences were obtained from GenBank, based on NJ analysis of ITS sequence data of 519 aligned nucleotide sites using MEGA ver3.1. *Numbers* on the branch nodes represent bootstrap support values (%) based on 1,000 replications; bootstrap values greater than 50% are indicated. *T* of the respective *isolate numbers* indicates isolates from TT, whereas *K* indicates those from KT. *Right vertical bars* indicate the section(s) and clade(s) defined by Samson et al. (2004). Strains with a *superscript* indicate the strains derived from; *HT*, Holotype; *NT*, Neotype; *IT*, Isotype; and *ET*, Epitype

Using the same outgroup taxon as the 28S phylogeny, the NJ tree (Fig. 2) was inferred from 519 bp of ITS sequences of 43 isolates of *Penicillium* and relatives. In the ITS phylogeny, all the TT and KT isolates were placed in the cluster containing only *P. paneum*, including its ex-holotype strain CBS 101032. The bootstrap support showed a comparatively high value, 94%. Incidentally, ITS is now expected to be the primary target gene among possible barcodes for fungi (e.g., All Fungi Barcoding [http://www.allfungi.com/index.php]). An assessment of the DNA barcode for *Penicillium* subg. *Penicillium* was done by Seifert et al. (2007) using ITS, CO1 (mitochondrial gene cytochrome oxidase I; also known as Cox1), and β-tubulin gene sequences. CO1 provided barcodes for *Penicillium* subgen. *Penicillium*, superior to the resolution of ITS, but inferior to that of the β-tubulin gene. Our case study of the barcode for TT and KT fungal isolates is now in progress. The results will appear elsewhere in the near future.

Using *Eupenicillium catenatum* CBS 431.69 as an outgroup, the NJ, ML, and Bayesian trees (Fig. 3) were inferred from 254 bp of β-tubulin sequences of 31 isolates of *Penicillium* and relatives. In the β-tubulin phylogeny, all the TT and KT isolates were placed in a cluster containing only *P. paneum*,

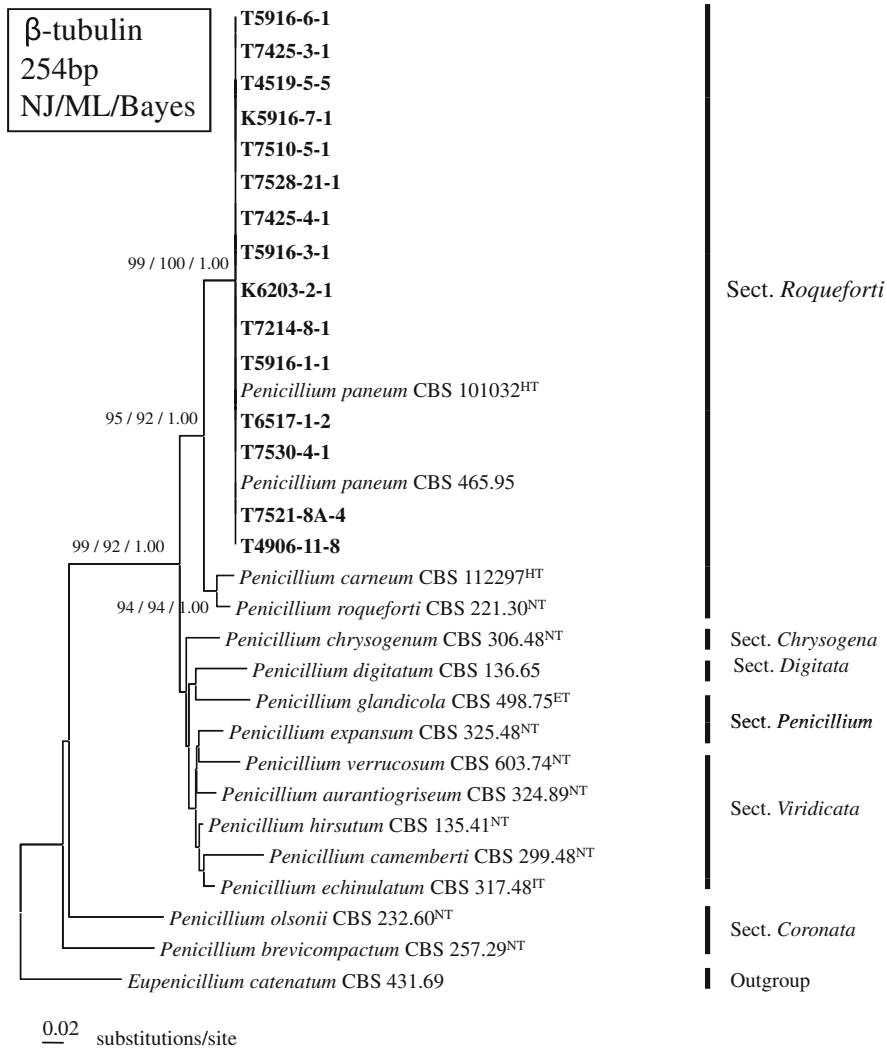

Fig. 3 Phylogenetic relationships among 15 TT and TK isolates and 16 known *Penicillium* isolates for which the sequences were obtained from GenBank based on NJ, ML, and Bayesian analyses of *β*-tubulin gene sequence data of 254 aligned nucleotide sites, using MEGA ver3.1., PHYLIP version 3.6., and MrBayes. *Numbers* on branches indicate Bayesian posterior probability and bootstrap support values (%) based on 1,000 replications; bootstrap values greater than 50% are indicated. For other abbreviations, see Fig. 2

including its ex-holotype strain CBS 101032, with strong statistical supports. The bootstrap values were 99 and 100%, respectively, and the posterior probability was 1.00. The respective three species of sect. *Roqueforti* were well-supported by the bootstrapping, as was the topology of the species phylogeny demonstrated by Boysen et al. (1996). Very recently, O'Brien et al. (2008) adopted *β*-tubulin and acetyl CoA synthetase sequences to identify their isolates in this section. The *β*-tubulin sequence-based phylogeny of TT and KT *Penicillium* sp. 1 isolates were identical with that of *P. paneum* isolated from baled grass silage by O'Brien et al. (2008).

In fungi, aminoadipate reductase converts 2-aminoadipate to 2-aminoadipate 6-semialdehyde. An et al. (2002) have already shown that the *lys2* gene (encoding of aminoadipate reductase) fragment is useful for the phylogenetic analyses of ascomycetes. However, the prokaryotes that biosynthesize lysine through the aminoadipate pathway have no *lys2* gene; they synthesize lysine from the aminoadipate using a different pathway (An et al. 2002, 2003; Nishida and Nishiyama 2000). The *lys2* gene is fungal-specific (An et al. 2002).

Using *P. brevicompactum* JCM 22849 as an outgroup taxon, the NJ, ML, and Bayesian trees (not shown herein; see supplementary Fig. S2) were inferred from 384 bp of the *lys2* sequences of 30 isolates of *Penicillium* and relatives. In the *lys2* phylogeny, all the TT and KT isolates were placed in a cluster comprised only of *P. paneum*, including its ex-holotype strain CBS 101032. The bootstrap supports

were 99 and 96%, respectively, whereas the posterior probability was 0.70. No sequence variation was observed in the 28S, ITS, and β-tubulin gene sequences from the *Penicillium* sp. 1 isolates. In the *lys2* gene, however, T6517-1-2 differs isolates T7425-4-1, T7510-5-1, T7528-21-1, T4906-11-8 and K6203-2-1 by one or two nucleotides. Consequently, the *lys2* gene may suggest a potential to distinguish populations of *P. paneum* in genealogical studies. Further analyses are needed to test the availability for fungi.

Our molecular phylogenies inferred from the 28S, ITS, β-tubulin, and *lys2* gene sequences clearly indicate that the 15 selected *Penicillium* sp.1 isolates of TT and KT, as well as the ex-holotype and another strain of *P. paneum*, formed a monophyletic cluster with high bootstrap supports and posterior probability.

In our previous paper, we detected several haplotypes of *Fusarium* and *Trichoderma* isolates from the TT and KT stone chamber interiors and exteriors (Kiyuna et al. 2008). However, our molecular phylogenies suggest that there are no haplotypes within *Penicillium* sp. 1 (i.e., *P. paneum*). As shown in Table 1 and supplementary Table S1, 50 representative strains of *Penicillium* sp. 1, which were isolated from a variety of substrates such as mouldy spots and viscous gels (biofilms) on plaster walls, soil, air, and Isopoda's body surface collected in different periods, are thought to be the same species, *P. paneum*.

On the other hand, the most abundant phylotype *P. namyslowskii* (=*Geosmithia namyslowskii*), a common soil colonizer, has been reported from the Chamber of Felines in the prehistoric painted cave of Lascaux in France (Bastian and Alabouvette 2009). *P. paneum* (this paper) and *P. namyslowskii* (Peterson 2000) shows a difference of 46 nucleotides in the ITS region. In addition to this, the former is characterized by the Q-9 system, whereas the latter has Q-8 (23%) + 9 (77%) as the major ubiquinone system (Ogawa et al. 1997). Therefore, there is a big difference between the two.

So far *P. paneum* has been isolated from mouldy rye breads, other foods (Frisvad and Samson 2004), and baled grass silage (O'Brien et al. 2006, 2008). The present report is thus the first account of *P. paneum* isolated from samples relating to the biodeterioration of cultural properties such as mural paintings. This novel finding will help elucidate the cause of biodeterioration of the TT and KT murals. Very recently an important role of arthropods in the dispersion of fungal spores and development has been expressed by Bastian et al. (2009) in the prehistoric painted cave of Lascaux. In the near future, we will discuss on roles of *P. paneum* in the biodeterioration of mural paintings and plaster walls, and also on the invasion route to the TT and KT stone chamber interiors.

In conclusion, the phenotypic and genotypic characteristics of *Penicillium* sp. 1 isolates from TT and KT samples, agreed well with those of the authentic strains (including the ex-holotype) and related references of *P. paneum* Frisvad (Boysen et al. 1996; Frisvad and Samson 2004; O'Brien et al. 2008).

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