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*

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Analysis of D1/D2 large-subunit (LSU) rRNA gene sequences predicted that 17 yeast isolates, mainly from viscous gels (biofilms) taken from the stone chamber interior of the Kitora tumulus in Nara, Japan, were placed in the *Yamadazyma* and *Zygoascus* clades. Polyphasic characterization, including morphological, physiological and chemotaxonomic characteristics, multigene sequence divergence and DNA–DNA hybridization, strongly suggested the assignment of one novel species to each of the clades; these are *Yamadazyma kitorensis* f.a., sp. nov., with the type strain JCM 31005^T (ex-type CBS 14158^T=isolate K8617-6-8^T), and *Zygoascus biomembranicola* f.a., sp. nov., with the type strain JCM 31007^T (ex-type CBS 14157^T=isolate K61208-2-11^T). Furthermore, the transfer of five known species of the genus *Candida* as novel combinations to the genera *Yamadazyma* and *Zygoascus* is proposed; these are *Yamadazyma olivae* f.a., comb. nov. (type strain CBS 11171^T=ATCC MYA-4568^T), *Yamadazyma tumulicola* f.a., comb. nov. (type strain JCM 15403^T=ex-type CBS 10917^T=isolate T6517-9-5^T), *Yamadazyma takamatsuzukensis* f.a., comb. nov. (type strain JCM 15410^T=CBS 10916^T=isolate T4922-1-1^T), *Zygoascus polysorbophila* f.a., comb. nov. (type strain NRRL Y-27161^T=CBS 7317^T) and *Zygoascus bituminiphila* f.a., comb. nov. (type strain CBS 8813^T=MUCL 41424^T).

INTRODUCTION

The Kitora tumulus (KT), located in the village of Asuka (Asuka-mura), Nara Prefecture, Japan, was built sometime between the end of the 7th century and the beginning of the 8th century. It was discovered in 1983, designated a special historic site by the government in 2000 and excavated in early 2004. The stone chamber (interior measurements: roughly 1 m wide, 2.4 m deep and 1 m high) is buried within a mound of soil, and its environmental conditions and the characteristics of the murals depicted on thin layers of plaster were very similar to those of the Takamatsuzuka tumulus (TT), which were described previously by Ishizaki & Kigawa (2011) and Nagatsuka *et al.* (2009). The distance between these two tumuli is only 1.2 km.

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Abbreviations: ITS, internal transcribed spacer; KT, Kitora tumulus; LSU, large subunit; ML, maximum-likelihood; MP, maximum-parsimony; MtSm, mitochondrial small-subunit rRNA gene; TT, Takamatsuzuka tumulus.

The GenBank/ EMBL/DDBJ accession numbers for the combined ITS1 region, 5.8S rRNA gene, ITS2 region and D1/D2 LSU rRNA gene, D1/D2 LSU rRNA gene, MtSm gene and *COX2* gene sequences obtained in this study are listed in Table 1.

A supplementary table and two supplementary figures are available with the online Supplementary Material.

Table 1. Origin and GenBank accession numbers for the sequences of the present strains

Isolate/ strain	Culture collection number(s)	Sampling date	Isolation source	Sampling location	GenBank accession no.			
					ITS + D1/D2	D1/D2	MtSm	*COX2*
***Yamadazyma olivae* comb. nov. (group A)**								
K5902-1-4	Died out	2/9/2005	Dark-greenish viscous gels	North wall of stone chamber of KT	–	LC060998	–	–
K5916-5-4-1	–	16/9/2005	White viscous gels	North wall of stone chamber of KT	–	LC060999	–	–
K5916-7-4	JCM 30999	16/9/2005	Dark-greenish viscous gels	Below the paintings of black snake-tortoise (*Genbu*) on north wall in stone chamber of KT	LC060993	–	–	–
K6120-3-4	JCM 31000	20/1/2006	Soil	Stone chamber of KT	–	LC061000	–	–
K6203-4-2	–	3/2/2006	Black mixture of viscous substance and soil	Surface plaster of south area of ceiling wall in stone chamber of KT	–	LC061001	–	–
K6203-5-3	–	3/2/2006	Black viscous gels	Surface plaster of south area of ceiling wall in stone chamber of KT	–	LC061002	–	–
K7511-2	–	11/5/2007	Black sooty moulds	East area of north wall in stone chamber of KT	–	LC061003	–	–
K8617-4-4	JCM 31001	17/6/2008	Black viscous gels	Plaster cracks in central area of ceiling wall in stone chamber of KT	–	LC061004	–	–
K8617-5-6	–	17/6/2008	Piece of plaster (white powdered substance)	Middle east wall in stone chamber of KT	–	LC061005	–	–
K8617-6-7	JCM 31002	17/6/2008	Red viscous gels	Stone wall near relocated area of paintings of the vermillion bird (*Suzaku*) on south wall of stone chamber of KT	–	LC061006	–	–
K8617-7-5	–	17/6/2008	Light-blue viscous gels	South area of floor in stone chamber of KT	–	LC061007	–	–
K8617-8-6	–	17/6/2008	Viscous gels	Black hole in surface plaster of west area of ceiling wall in stone chamber of KT	–	LC061008	–	–
K7706-2-7	JCM 31003	6/7/2007	Viscous gels	East wall of stone chamber of KT	–	LC061009	–	–
***Yamadazyma kitorensis* sp. nov. (group B)**								
K61208-2-10	JCM 31004	8/12/2006	Dried dark-brownish substance (dried viscous gel)	Floor of stone chamber of KT	LC060994	–	–	–
K8617-6-8^T	JCM 31005^T, CBS 14158^T	17/6/2008	Red viscous gels	Stone wall near detached painting of vermillion bird (*Suzaku*) on south wall of stone chamber of KT	LC060995	–	–	–
***Yamadazyma tumulicola* comb. nov.**								
T6517-9-5^T	JCM 15403^T (=CBS 10917^T)	17/5/2006	Viscous gels	Below painting of group of women on east wall 3 of stone chamber of TT	AB365463	–	–	–
***Yamadazyma takamatsuzukensis* comb. nov.**								
T4922-1-1^T	JCM 15410^T (=CBS 10916^T)	22/9/2004	Air	North area of stone chamber of TT	AB365470	–	–	–
***Zygoascus polysorbophila* comb. nov.**								
T6517-9-4	JCM 31006	17/5/2006	Viscous gels (biofilm)	Lower section near rightmost of four women on east wall of stone chamber of TT	LC060996	–	LC091396	LC091398
–	NRRL Y-27161^T (=CBS 7317^T)	NK	Emulsion of white oil and polysorbate	South Africa	–	–	–	–
***Zygoascus biomembranicola* sp. nov.**								

Table 1. cont.

Isolate/ strain	Culture collection number(s)	Sampling date	Isolation source	Sampling location	GenBank accession no.			
					ITS+D1/D2	D1/D2	MtSm	*COX2*
K61208-2-11^T	JCM 31007^T (=CBS 14157^T)	8/12/2006	Dried dark-brownish viscous gel	Floor of stone chamber of KT	LC060997	–	LC091397	LC091399
***Zygoascus bituminiphila* comb. nov.**								
–	CBS 8813^T (=MUCL 41424^T)	NK	Tar	Canada	–	–	–	–

NK, Not known.

All paintings and plaster from KT were detached and relocated to a restoration facility by autumn 2010 because the plaster was loosely attached to the stone at the time of excavation (Kigawa *et al.*, 2005; Ishizaki & Kigawa, 2011). Subsequently, until the end of 2013, the stone chamber was backfilled. Overviews of the serious microbial outbreaks that occurred inside the KT stone chamber over the 10 years from 2004 to 2013, along with the protective measures taken in response, have been provided by Kigawa *et al.* (2009b, 2013, 2015), Sugiyama *et al.* (2009) and Ishizaki & Kigawa (2011).

In the course of our previous microbiological surveys to elucidate the causes of biodeterioration of mural paintings, we isolated numerous bacteria and fungi, including various yeasts, from mouldy spots and viscous gels (biofilms) (Sugiyama *et al.*, 2009). Previously, we described the novel species *Candida tumulicola* and *Candida takamatsuzukensis* (Nagatsuka *et al.*, 2009), assigned to the *Candida membranifaciens* clade (Suh *et al.*, 2005), which were the predominant species in the TT stone chamber interior. In the present study, sequences of the D1/D2 large-subunit (LSU) rRNA gene were determined in 36 of 64 isolates from KT, and 17 of these were assigned to the *C. membranifaciens* clade and were also shown to predominate in KT. Species of the *C. membranifaciens* clade have been suggested to be assigned to the genus *Yamadazyma* (Daniel *et al.*, 2014). On the other hand, two isolates placed in the *Zygoascus* clade (Kurtzman & Robnett, 2007) were also found among the minority yeast isolates from both tumuli. The respective D1/D2 LSU rRNA gene sequence-based phylogenies of our isolates and known species of the *Yamadazyma* and *Zygoascus* clades were supported by concatenated gene sequence-based analyses of D1/D2–internal transcribed spacer (ITS) regions and D1/D2–mitochondrial small-subunit rRNA (MtSm)–cytochrome oxidase II (*COX2*) genes, respectively. On the basis of phenotypic and genotypic characterization, we propose one novel species for each of *Yamadazyma* and *Zygoascus*, and the transfer of three species of *Candida* to *Yamadazyma* and two species of *Candida* to *Zygoascus*. These five species of *Candida* that belong to the mycobiota of mural paintings of KT and TT are herein proposed to be transferred to the corresponding teleomorphic genera.

METHODS

Organisms. The sampling of materials and isolation of microorganisms were performed as described in our previous studies (Kiyuna *et al.*, 2008; Nagatsuka *et al.*, 2009; Sugiyama *et al.*, 2009). Briefly, samples were collected with sterilized cotton swabs from various spots in the stone chamber interiors of TT and KT and maintained in sterilized physiological saline solution, and then streaked directly on potato-dextrose agar (PDA) plates. All yeast isolates and reference type strains were cultivated on yeast extract/malt extract (YM) agar at 25 °C. Voucher isolates, including type and ex-type strains, were deposited in public culture collections (JCM in Tsukuba, Japan, and CBS in Utrecht, Netherlands) as listed in Table 1.

DNA sequencing and phylogenetic analysis. Procedures for DNA isolation and sequencing of the D1/D2 LSU rRNA gene and the ITS

region including the 5.8S rRNA gene were reported previously (Yamada *et al.*, 1999; Nagatsuka *et al.*, 2002, 2005). Amplification and sequencing of MtSm and the *COX2* gene were carried out as reported by Kurtzman & Robnett (2003), using primers MS-1 and MS-2 for MtSm and primers COII-5 and COII-3 for *COX2*. GenBank accession numbers are presented in Table 1. The sequences of the isolates and strains of related species retrieved from GenBank were aligned using MUSCLE (Edgar, 2004). A neighbour-joining (NJ) tree with Kimura's two-parameter model, maximum-likelihood (ML) tree and maximum-parsimony (MP) tree were reconstructed using MEGA6 (Tamura *et al.*, 2013), and a bootstrap test with 1000 replicates was used to assess the reliability of branches (Felsenstein, 1985). We used the model test function of MEGA6 to determine the best substitution model for phylogeny inference by the ML method. The MP analysis was performed using the default settings in MEGA6. Positions with gaps and regions of uncertain nucleotide alignment were excluded from phylogenetic analysis.

Morphological, physiological, biochemical and chemotaxonomic characterization. Physiological and biochemical tests were performed using standard protocols, as described by Kurtzman *et al.* (2011a). The ability of isolates to assimilate isopropanol as a sole carbon source was tested because isopropanol has been used as a disinfectant in the stone chamber interiors since September 2005 (Kigawa *et al.*, 2006; Ishizaki & Kigawa, 2011). Assimilation tests of carbon compounds were performed in liquid medium incubated at 25 °C for up to 4 weeks. Cell morphology was observed using differential interference contrast optics on a light microscope (Olympus BX51) after cultivation on YM agar and in YM broth at 25 °C. Quinone systems were examined using HPLC (Yamada *et al.*, 1973; Kuraishi *et al.*, 1985) with a Waters 996 PDA HPLC system.

DNA G + C content and DNA–DNA hybridization. Nuclear DNA was extracted for the determination of G+C contents and DNA–DNA hybridization, as described previously (Nagatsuka *et al.*, 2002). The DNA base compositions of isolates were determined using an SCL-10A vp HPLC system (Shimadzu) (Tamaoka & Komagata, 1984). DNA–DNA relatedness was determined by fluorometric hybridization using a microdilution plate (Ezaki *et al.*, 1989) at 43 °C in 2× SSC buffer containing 50 % (v/v) formamide on an immunoplate (Nunc). DNA relatedness values (%) are presented as means of triplicate DNA–DNA hybridization determinations.

RESULTS AND DISCUSSION

Isolation

Sixty-four and 78 yeast strains, respectively, were isolated from samples collected from the KT stone chamber interior between June 2004 and June 2008 and from the TT stone chamber between September 2004 and December 2006. Subsequently, we selected 36 KT isolates and 38 TT isolates as representatives based on their sources, their collection sites and their colony characteristics, and the D1/D2 LSU rRNA gene sequences of these selected isolates were determined. Isolates were then grouped according to their D1/D2 LSU rRNA gene sequence divergence (Table S1, available in the online Supplementary Material). Data on the isolation and identification of these yeasts and their ecological implications will be discussed elsewhere.

Sequencing and DNA–DNA relatedness

Group A comprised 13 isolates that were predominant in KT (Tables 1 and S1). All of the D1/D2 LSU rRNA gene sequences of the group-A isolates were identical to that of *Candida olivae* CBS 11171^T (GenBank accession no. FJ715430). The sequence of the ITS region of a representative isolate of group A, K5916-7-4, was identical to that of *C. olivae* CBS 11171^T (FJ715432). From these sequence data, the 13 isolates of group A were assignable to *C. olivae*. *C. olivae*, the dominant species in KT, belongs to the *Yamadazyma* clade (Lachance *et al.*, 2011), as do *C. tumulicola* and *C. takamatsuzukensis*, which were the dominant species in TT. Additionally, two other isolates from KT, K8617-6-8^T and K61208-2-10 of group B, had identical D1/D2 LSU rRNA gene sequences and identical ITS region sequences, and these showed the highest identity to the sequences of *Candida michaelii* CBS 9878^T (GenBank accession no. AY520329), with 15 substitutions and two gaps in the 494 nt sequences, and *Candida germanica* CBS 4105^T (HQ283366), with 91.1 % similarity of the 631 nt sequences (Tables 1 and S1).

The D1/D2 LSU rRNA gene sequences of the KT and TT isolates K61208-2-11^T and T6517-9-4 differed at six positions of the 511 nt sequences (Tables 1 and S1). The D1/D2 LSU rRNA gene and ITS region sequences of isolate T6517-9-4 were identical to those of *Candida polysorbophila* NRRL Y-27161^T (GenBank accession nos DQ438188/DQ911459). Based on these sequence data, isolate T6517-9-4 was assignable to *C. polysorbophila* of the *Zygoascus* clade. The MtSm (549 nt) and *COX2* (635 nt) gene sequences, which have been shown to be effective for species delineation of the *Zygoascus* clade and related clades and genera (Kurtzman & Robnett, 2007), of T6517-9-4 showed 99.2 and 100 % similarity to the sequences of *C. polysorbophila* NRRL Y-27161^T (DQ442717/DQ443045), respectively.

The D1/D2 LSU rRNA gene sequences of K61208-2-11^T had high similarities to those of *Candida bituminiphila* CBS 8813^T (GenBank accession no. AF294910) and *C. polysorbophila* NRRL Y-27161^T (DQ438188) in the *Zygoascus* clade, with two substitutions and five substitutions with a single gap out of 567 nt. Generalizations from D1/D2 LSU rRNA gene sequence analyses (Kurtzman & Robnett, 1998) suggest that isolate K61208-2-11^T belongs to *C. bituminiphila* or a closely related species. Sequences of ITS 1 and 2 in K61208-2-11^T showed 21.4 % difference from those of *C. bituminiphila* CBS 8813^T (AY518583) and 9.2 % difference from those of *C. polysorbophila* NRRL Y-27161^T (DQ911459). Sugita *et al.* (1999) suggested that conspecific strains based on DNA–DNA relatedness have overall nucleotide differences of less than 1 % in ITS 1 and 2. Following their guidelines, isolate K61208-2-11^T could be assigned to a species that is distinct from both *C. bituminiphila* and *C. polysorbophila*. The MtSm sequence similarities of K61208-2-11^T (553 nt) to *C. bituminiphila* NRRL Y-27974^T (DQ911436) and *C. polysorbophila* NRRL Y-27161^T (DQ442717) were 98.2 and 98.7 %, respectively, whereas the *COX2* gene sequence similarities of K61208-2-11^T (622 nt) to *C. bituminiphila* NRRL Y-27974^T (DQ911440) and *C. polysorbophila* NRRL Y-27161^T (DQ443045) were 91.3 and 94.5 %, respectively. The DNA–DNA relatedness of isolate K61208-2-11^T with

Table 2. DNA–DNA relatedness for *Z. biomembranicola* sp. nov. and relatives

Strain	Relative binding (%) with labelled DNA from: 1	2	3
1. *Z. bituminiphila* comb. nov. CBS 8831^T	(100)	31	21
2. *Z. polysorbophila* comb. nov. NRRL Y-27161^T	35	(100)	39
3. *Z. biomembranicola* sp. nov. K61208-2-11^T	42	36	(100)

strains of its two closest neighbours (*C. bituminiphila* and *C. polysorbophila*) was found to be 21–42 % (Table 2). Therefore, considering the recommendation of a threshold value of 70 % DNA–DNA relatedness for the prediction of conspecificity (Kurtzman *et al.*, 2011b), isolate K61208-2-11^T should be assigned to a species distinct from both *C. bituminiphila* CBS 8831^T and *C. polysorbophila* NRRL Y-27161^T.

Phylogeny

The phylogenetic positions of groups A and B in the *Yamadazyma* clade were assigned based on the concatenated sequences of the ITS region and the D1/D2 LSU rRNA gene using ML (Fig. 1). Group A was placed in a phylogenetic position identical to that of *C. olivae*. Both isolates of group B (K8617-6-8^T and K61208-2-10) were in the *Yamadazyma* clade, with 87 % bootstrap support, and were well distinguished from known species. ML (Fig. 1) and MP (data not shown) analyses based on concatenated sequences flanked group B on the clade consisting of the *C. membranifaciens* subclade and the *Candida gorgasii* subclade (with 53 % bootstrap support), whereas NJ analysis based on D1/D2 LSU rRNA gene sequences placed group B basal to a clade comprising the type species *Yamadazyma philogaea* without bootstrap support (Fig. S1). From the preceding analyses, group B represents a novel species belonging to the *Yamadazyma* clade, but more robust datasets will be needed for accurate placement within this clade.

Phylogenetic relationships among isolates T6517-9-4 and K61208-2-11^T and related species were inferred from D1/D2 LSU rRNA gene sequences using ML (Fig. S2), and from concatenated gene sequences of the D1/D2 LSU rRNA, MtSm and *COX2* genes using ML (Fig. 2). Isolate T6517-9-4 was placed in an identical or nearly identical phylogenetic position to *C. polysorbophila*. Isolate K61208-2-11^T was clustered with *C. bituminiphila* with 75 % bootstrap support in the ML tree based on D1/D2 LSU rRNA gene sequences (Fig. S2). It was also clustered with *C. polysorbophila* with 100 % bootstrap support in both the ML (Fig. 2) and MP (data not shown) trees based on the concatenated sequences.

In phylogenetic trees inferred from the D1/D2 LSU rRNA gene sequences, the *Zygoascus* clade was not monophyletic, but was separated into three distinct subclades, one subclade including the type species *Zygoascus hellenicus* and flanked by the *Wickerhamiella*, a second subclade comprising *Zygoascus tannicola* and *Zygoascus ofunaensis* and a third subclade containing *C. bituminiphila*, *C. polysorbophila* and isolate K61208-2-11^T. In phylogenetic trees inferred from the concatenated sequences, the *Zygoascus* clade composed of three subclades appears to be monophyletic with 100 % bootstrap support, and the topologies in the *Zygoascus* clade were identical between the ML (Fig. 2) and MP (data not shown) phylogenies. From the preceding analyses, isolate K61208-2-11^T represents a novel species in the *Zygoascus* clade.

Physiology

The phenotypic characteristics of the group-A isolates K5916-7-4, K6120-3-4, K8617-4-4 and K8617-6-7 were determined and compared with those of *C. olivae* (Nisiotou *et al.*, 2010). The features of the four isolates were similar to those of *C. olivae*, but differences were observed in the ability to assimilate L-sorbose and DL-lactate. Moreover, when the phenotypic characteristics were compared between the group-B isolates and *C. michaelii* (Table 3), the two group-B isolates showed similar features but, in both cases, these features were different from the properties of *C. michaelii*. Specifically, the inability to ferment galactose, maltose and sucrose, the ability to assimilate L-sorbose, D-glucosamine, D-ribose and 2-keto-D-gluconate, the inability to assimilate L-rhamnose, propane-1,2-diol, butane-2,3-diol and D-quinic acid and the ability to grow in 16 % NaCl (w/v)/5 % glucose (w/v) and in vitamin-free medium clearly differentiated the group-B isolates from *C. michaelii*.

In the phenotypic comparison between isolates T6517-9-4 and K61208-2-11^T and *C. polysorbophila* and *C. bituminiphila* (Table 4), the characteristics of T6517-9-4 and *C. polysorbophila* NRRL Y-27161^T were similar, differing only in L-rhamnose and creatinine assimilation. However, isolate K61208-2-11^T clearly differed from *C. bituminiphila* in its ability to assimilate melezitose, D-galacturonic acid monohydrate, citrate, nitrate, ethylamine and creatinine, its ability to grow in the presence of 50 % (w/v) D-glucose and its inability to ferment cellobiose and assimilate D-ribose. In addition, isolate K61208-2-11^T clearly differed from *C. polysorbophila* in its ability to assimilate nitrate and its inability to assimilate D-ribose, sucrose, raffinose, galactitol and D-gluconate.

In TT and KT, ethanol was used occasionally for the removal of colonies of micro-organisms that contaminated the murals as dense colonies that survived even after paraformaldehyde fumigation, as, at high concentrations, it is a strong disinfectant that does not affect the pigments of the mural paintings (Kigawa *et al.*, 2009a). However, ethanol

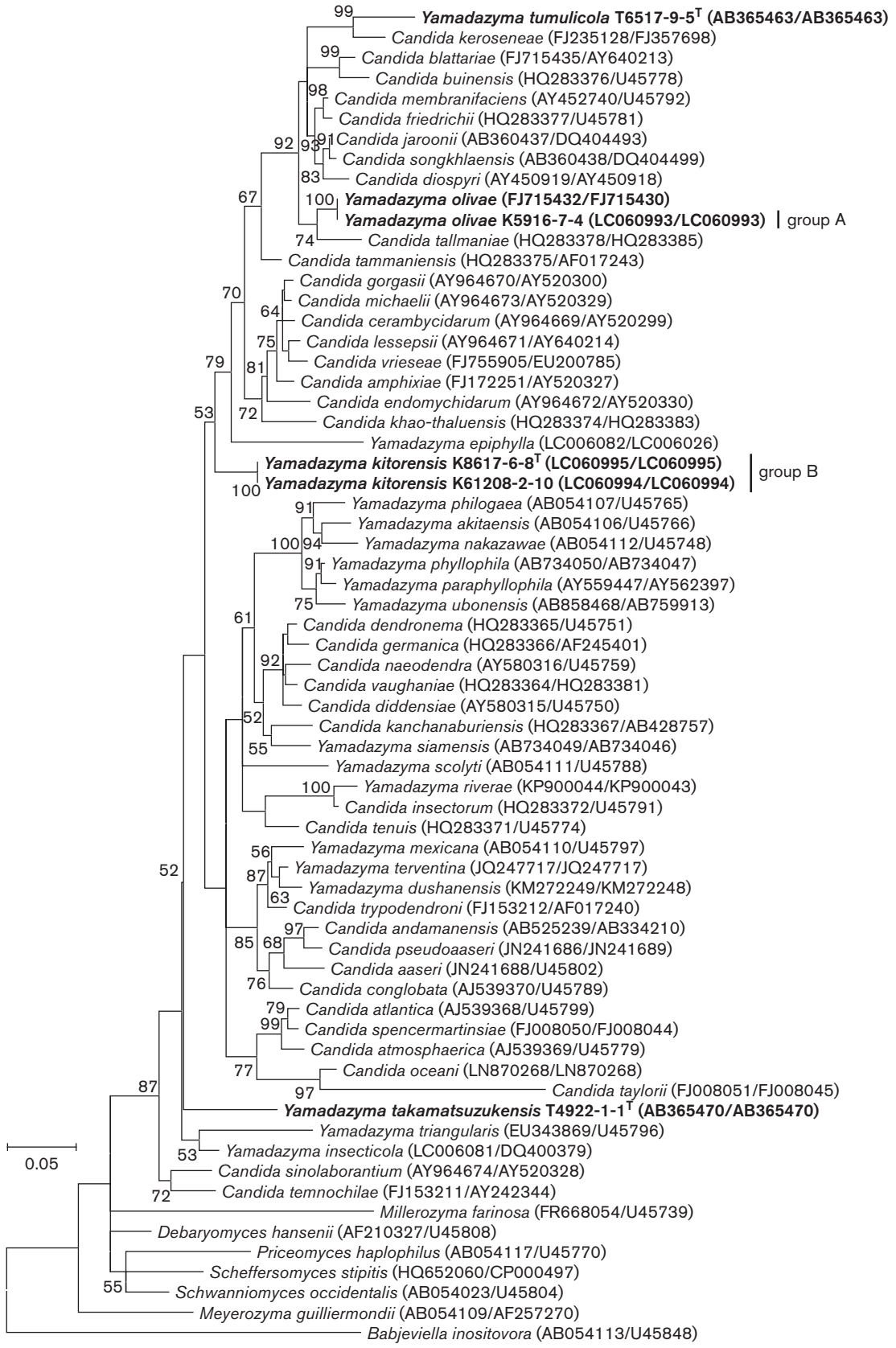

Fig. 1. Phylogenetic positions of *Yamadazyma olivae* comb. nov. (group A), *Yamadazyma kitorensis* sp. nov. (group B), *Yamadazyma tumulicola* comb. nov. and *Yamadazyma takamatsuzukensis* comb. nov. in the *Yamadazyma* clade based on ML

analysis of concatenated sequences of the ITS region and the D1/D2 LSU rRNA gene (871 nt). The ML analysis was conducted using a general time reversible (GTR) model with gamma distribution and invariant sites. *Babjeviella inositovora* was used as an outgroup and all related species are represented by the type strains. Numbers in parentheses are GenBank accession numbers with the ITS region preceding the D1/D2 LSU rRNA gene. Bootstrap values ≥50 % are given. Bar, 5 changes per 100 nucleotide positions.

may have been diluted sufficiently for use as a carbon source by micro-organisms in the stone chamber interiors of TT and KT, the humidity of which was maintained at almost 100 % (Ishizaki & Kigawa, 2011). Some strains of major fungal colonizers, including *C. tumulicola*, *C. takamatsuzukensis* (Nagatsuka *et al.*, 2009; cf. Jones, 2009) and *C. olivae*, which are transferred to *Yamadazyma* as novel combinations in this paper, were found to utilize ethanol (approx. 0.5 % and 1 %, v/v) and not to utilize isopropanol as a single carbon source (Kigawa *et al.*, 2010). From the above results, isopropanol was used as a disinfectant instead of ethanol after September 2005 (Kigawa *et al.*, 2006, 2015). Assimilation tests of the four selected isolates of group A, two isolates of group B, T6517-9-4 and K61208-2-11^T revealed latent or slow ethanol assimilation but no assimilation of isopropanol (Tables 3 and 4), as shown previously for *C. tumulicola* and *C. takamatsuzukensis* (Nagatsuka *et al.*, 2009; cf. Jones, 2009).

Chemotaxonomic characteristics

The DNA G+C contents of T6517-9-4 and K61208-2-11^T were 48.7–49.4 mol% and 46.4–47.2 mol%, respectively.

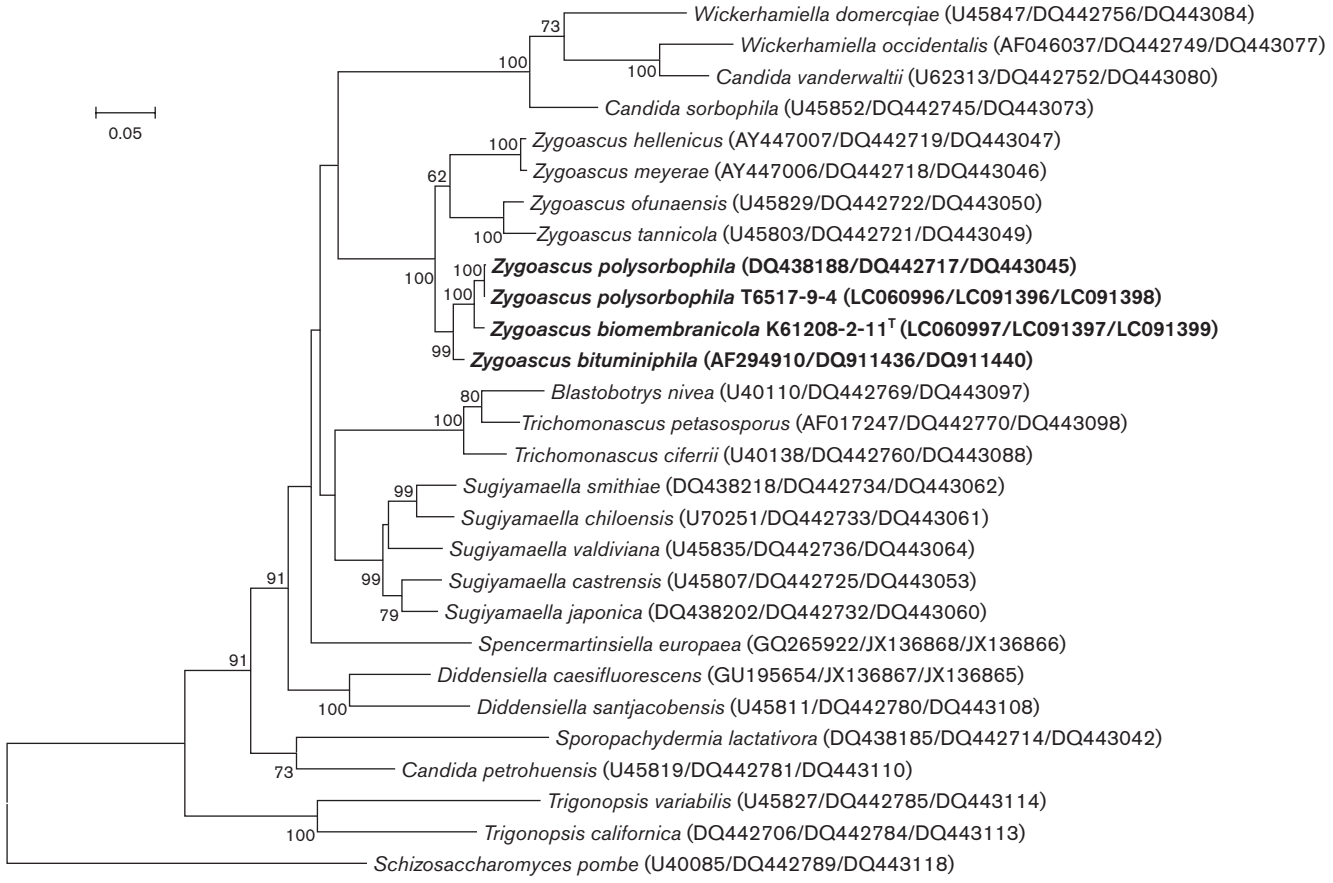

Fig. 2. Phylogenetic positions of *Zygoascus biomembranicola* sp. nov., *Zygoascus polysorbophila* comb. nov. and *Zygoascus bituminiphila* comb. nov. in the *Zygoascus* clade from ML analysis based on concatenated gene sequences of the D1/D2 LSU rRNA, MtSm and *COX2* genes (1221 nt). The ML analysis was conducted using a GTR model with gamma distribution. *Schizosaccharomyces pombe* was used as an outgroup and all related species are represented by type strains. Numbers in parentheses are GenBank accession numbers. Bootstrap values ≥50 % are given. Bar, 5 changes per 100 nucleotide positions.

Table 3. Differential phenotypic characteristics of *Y. kitorensis* sp. nov. and *C. michaelii*

Data for *C. michaelii* are from Suh *et al.* (2005). Methyl α-D-glucoside, lactose, cellobiose and raffinose were not fermented by any strain. Glucose, galactose, D-xylose, L-arabinose, sucrose, trehalose, methyl α-D-glucoside, cellobiose, glycerol, erythritol, ribitol, xylitol, L-arabitol, D-glucitol, D-mannitol, succinate, ethylamine and cadaverine were assimilated by all strains and melibiose, lactose, raffinose, soluble starch, galactitol, inositol, D-glucuronate, methanol, nitrate, nitrite, creatine and creatinine were not. All strains grew at 25 °C in 50 % (w/v) D-glucose and 10 % NaCl/5 % glucose. Growth in media containing 0.01 or 0.1 % cycloheximide was negative. Production of amyloid compounds was negative in all cases. Diazonium blue B and urease reactions were negative in all cases. +, Positive; −, negative; L, delayed positive (latent); S, slow; W, weak; ND, no data.

	Y. kitorensis sp. nov.		*C.*
Characteristic	**K8617-6-8^T**	**K61208-2-10**	***michaelii***
Fermentation of:			
Glucose	W	+	+
Galactose	−	−	+
Maltose	−	−	L
Sucrose	−	−	L
Trehalose	+	+	L
Assimilation of:			
L-Sorbose	+	+	−
D-Glucosamine	L	L	−
D-Ribose	S	S	−
D-Arabinose	L	L	−
L-Rhamnose	−	−	+
Maltose	L	S	+
Salicin	+	S	+
Arbutin	+	S	+
Melezitose	L	L	+
D-Glucono-1,5-lactone	L	L	+
2-Keto-D-gluconate	+	+	−
5-Keto-D-gluconate	−	−	ND
D-Gluconate	S	S	+
D-Galacturonic acid monohydrate	−	−	ND
DL-Lactate	L	L	+
Citrate	+	S	+
Ethanol	L	L	+
Isopropanol	−	−	ND
Propane-1,2-diol	−	−	L
Butane-2,3-diol	−	−	+
D-Quinic acid	−	−	+
N-Acetyl-D-glucosamine	+	+	ND
Hexadecane	−	−	ND
L-Lysine	S	S	+
D-Glucosamine (N)	S	S	W
Growth at/with:			
37 °C	−	−	ND
60 % (w/v) D-glucose	−	−	W
16 % NaCl/5 % glucose	S	S	−
No vitamins	S	S	−

The major ubiquinone system of both isolates was Q-9, which is the same as for *Z. hellenicus*, the type species of the genus *Zygoascus* (Robert & Smith, 2011).

Morphology

Isolates T6517-9-4 and K61208-2-11^T and all isolates of groups A and B were characterized by asexual reproduction via multilateral budding (Fig. 3a, d, e, g), and pseudomycelium-like structures and true hyphae were formed (Fig. 3b, c, e, f, h, i). No sexual states were observed with the formation of ascospores in any isolates, even after mixing within groups A and B and between isolate T6517-9-4 and *C. polysorbophila* NRRL Y-27161^T on YM agar, 2 % malt agar or cornmeal agar after 6 weeks.

Isolates T6517-9-4 and K61208-2-11^T and all isolates of groups A and B except the group-A strains K6120-3-4, K7511-2 and K8617-5-6 were isolated from viscous gel (biofilm) samples, and all isolates, including the group-A strains not isolated from biofilms, formed abundant hyphae and pseudohyphae. Multiple medical studies have documented the close relationships between biofilm development of *Candida albicans* and hyphal formation with extracellular substances (Hawser & Douglas, 1994; Nett *et al.*, 2010). Hyphae and pseudohyphae of these isolates may also have played important roles in biofilm development in TT and KT, leading to the observed contamination.

Identification and taxonomy

The preceding phenotypic and genetic (molecular) evidence indicates that group A and isolate T6517-9-4 are conspecific with *C. olivae* and *C. polysorbophila*, respectively, and that group B and isolate K61208-2-11^T represent novel species of the *Yamadazyma* and *Zygoascus* clades, respectively. From the phylogenetic analyses, the four known species of *Candida* to which KT and TT isolates were assigned, *C. olivae*, *C. polysorbophila*, *C. takamatsuzukensis* and *C. tumulicola*, as well as the closely related *C. bituminiphila*, were shown not to be closely related to the type species *Candida tropicalis* but rather to belong to the *Yamadazyma* or *Zygoascus* clades (Figs 1 and 2). In addition to proposing two novel species of the *Yamadazyma* and *Zygoascus* clades, we also propose the reassignment of the five known species *Candida olivae*, *Candida takamatsuzukensis*, *Candida tumulicola*, *Candida polysorbophila* and *Candida bituminiphila* to the genus *Yamadazyma* or *Zygoascus* as new combinations. Our transfer of these five species to the genera *Yamadazyma* and *Zygoascus* is consistent with the placement suggested by Daniel *et al.* (2014). Reclassifications of other species of *Candida* shown in the trees may be made according to the 'one fungus=one name' principle of the current *International Code of Nomenclature of Algae, Fungi, and Plants (Melbourne Code)* (McNeill *et al.*, 2012; Norvell, 2011).

Table 4. Differential phenotypic characteristics of *Z. biomembranicola* sp. nov., *Z. polysorbophila* comb. nov. and *Z. bituminiphila* comb. nov.

Glucose was fermented by all strains. Lactose and raffinose were not fermented by any of the strains. Glucose, galactose, L-sorbose, D-glucosamine (C), D-xylose, L-arabinose, trehalose, cellobiose, soluble starch, glycerol and D-glucuronate were assimilated by all strains, but methyl α-D-glucoside, melibiose, lactose, erythritol, 2-keto-D-gluconate and methanol were not. All strains grew at 25 °C, but no strain grew at 37 °C. Growth in vitamin-free medium, growth in medium containing 0.01 % cycloheximide, production of amyloid compounds and diazonium blue B and urease reactions were negative in all cases. +, Positive; −, negative; L, delayed positive (latent); S, slow; W, weak; ND, no data; D, delayed positive (after 14 d or more). Data for *Z. polysorbophila* comb. nov. NRRL Y-27161^T were obtained in this study. Data for *Z. bituminiphila* comb. nov. were taken from Robert *et al.* (2001).

Characteristic	*Z. biomembranicola* sp. nov. K61208-2-11^T	*Z. polysorbophila* comb. nov.			*Z. bituminiphila* comb. nov.
		T6517-9-4	NRRL Y-27161^T	Kurtzman (2007)	
Fermentation of:					
Galactose	+	W	S	W	+
Maltose	−	−	−	+	−
Methyl α-D-glucoside	−	−	−	ND	−
Sucrose	−	−	−	W	−
Trehalose	+	S	S	+	+
Cellobiose	−	W	+	ND	+
Assimilation of:					
D-Ribose	−	L	S	+	+
D-Arabinose	−	+	+	+	D/−
L-Rhamnose	+	S	−	−	−
Sucrose	−	+	+	+	D/−
Maltose	+	+	+	+	−/+
Salicin	+	+	S	+	+
Arbutin	+	+	+	ND	+
Raffinose	−	S	+	+	−/+
Melezitose	L	L	L	+	−
Ribitol (adonitol)	S	S	L	+	−/+
Xylitol	+	+	+	ND	−/+
L-Arabitol	S	+	+	ND	−/+
D-Glucitol (sorbitol)	S	L	L	+	+
D-Mannitol	+	S	S	+	+
Galactitol (dulcitol)	−	L	L	+	−
Inositol	+	+	+	+	W
D-Glucono-1,5-lactone	S	+	+	ND	−/+
5-Keto-D-gluconate	L	+	+	+	−/+
D-Gluconate	−	S	S	ND	−/+
D-Galacturonic acid monohydrate	+	+	+	ND	−
DL-Lactate	S	L	S	+	W
Succinate	L	+	+	+	+
Citrate	+	+	+	+	−
Ethanol	L	S	L	+	D/+
Isopropanol	−	−	ND	ND	ND
Propane-1,2-diol	S	S	S	ND	−/+
Butane-2,3-diol	−	−	−	ND	−
D-Quinic acid	−	−	−	ND	ND
N-Acetyl-D-glucosamine	+	+	+	+	ND
Hexadecane	−	−	−	−	ND
Nitrate	S	−	−	−	−
Nitrite	−	−	−	ND	−
Ethylamine	L	L	+	ND	−
L-Lysine	+	+	+	ND	−/+
Cadaverine	+	+	+	+	−/+
Creatine	−	−	−	ND	−
Creatinine	S	S	−	ND	−
D-Glucosamine (N)	+	+	+	ND	−/+

Table 4. cont.

Characteristic	*Z. biomembranicola* sp. nov. K61208-2-11^T	*Z. polysorbophila* comb. nov. T6517-9-4	*Z. polysorbophila* comb. nov. NRRL Y-27161^T	*Z. polysorbophila* comb. nov. Kurtzman (2007)	*Z. bituminiphila* comb. nov.
Growth with:					
0.1 % cycloheximide	+	+	+	ND	S
50 % (w/v) D-glucose	S	L	+	ND	−
60 % (w/v) D-glucose	−	−	−	ND	ND
10 % NaCl/5 % glucose	S	W	S	+	ND
16 % NaCl/5 % glucose	−	−	−	ND	ND

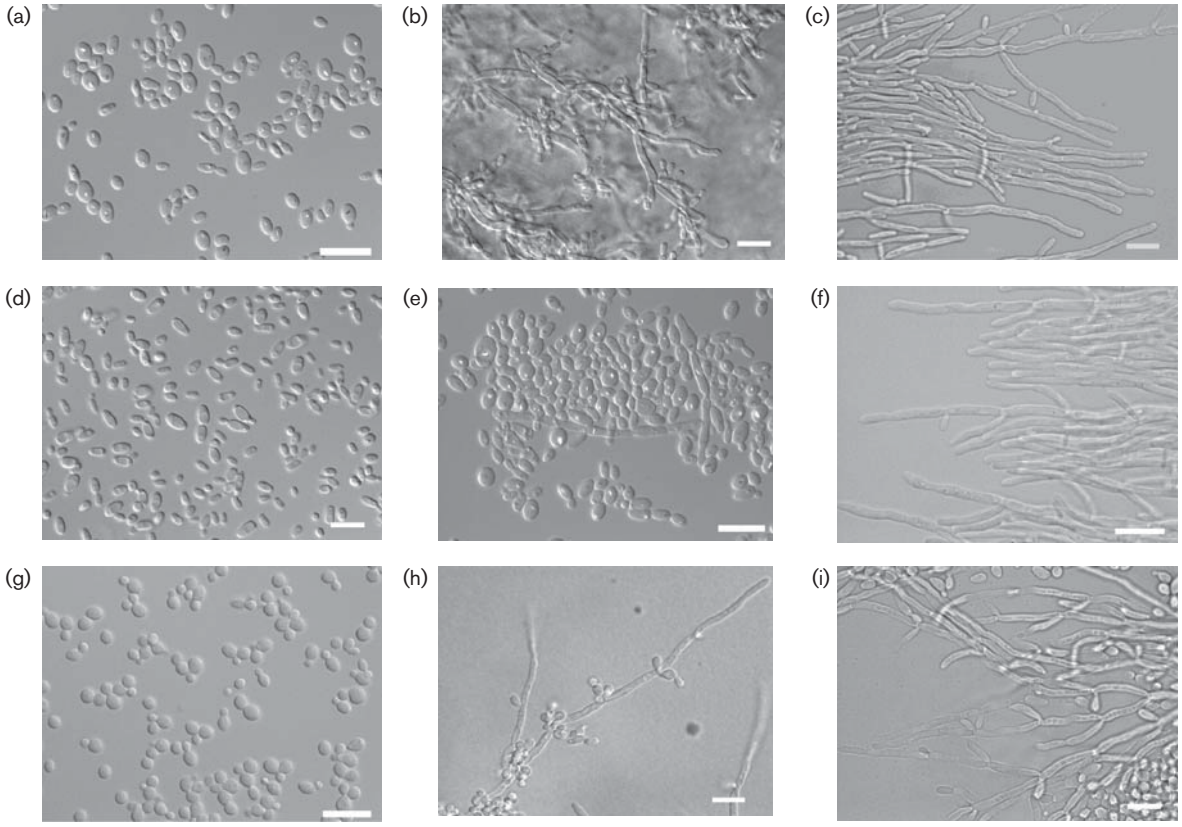

Fig. 3. Micrographs of *Y. olivae* comb. nov. K8617-4-4 (a–c), *Y. kitorensis* sp. nov. K8617-6-8^T (d–f), *Z. biomembranicola* sp. nov. K61208-2-11^T (g, h) and *Z. polysorbophila* comb. nov. T6517-9-4 (i). (a, d, g) Vegetative cells in YM broth for 3 days at 25 °C; (b) pseudohyphae on YM agar for 7 days at 25 °C; (c, i) Dalmau plate culture on PDA for 7 days at 25 °C; (f, h) Dalmau plate culture on cornmeal agar for 7 days at 25 °C; (e) pseudohyphae in YM broth for 3 days at 25 °C. Bars, 10 μm.

Description of *Yamadazyma kitorensis* Nagatsuka, Ninomiya, Kiyuna, Kigawa & Sugiyama sp. nov.

Yamadazyma kitorensis (ki.to.ren′sis. N.L. fem. adj. *kitorensis* referring to the Kitora tumulus in Asuka village, Nara Prefecture, Japan, the collection site of the type strain).

Growth in YM broth

After 3 days at 25 °C, cells are ovoid to ellipsoidal (4.0–8.8 × 6.4–12.0 μm) and appear singly or in pairs. Asexual reproduction is by multilateral budding (Fig. 3d). Simple to elaborate pseudohyphae are present (Fig. 3e). Sediment and thick pellicles are formed in fermentation medium.

Growth on YM agar

After 10 days of incubation at 25 °C, streak cultures are glistening, white–cream in colour and convex and have entire, filamentous margins.

Dalmau plate culture on YM agar

Septate hyphae are observed after 6 days of incubation at 25 °C (Fig. 3f).

Sexual reproduction

No sexual states of individual or combined strains are observed on YM agar after 1 month at 25 °C.

Physiological and biochemical characteristics

See Table 3.

Typification

The type strain, JCM 31005^T (ex-type CBS 14158^T = isolate K8617-6-8^T), was isolated from red viscous gels on a stone wall near the detached painting of a vermillion bird (*Suzaku*) on the south wall inside the KT stone chamber in Asuka-mura, Nara Prefecture, Japan, on 17 June 2008 by one of the authors (T. K.).

Identifier

MycoBank no. MB814344.

Isolates examined

One isolate, K61208-2-10, was examined in addition to the type strain identified as *Y. kitorensis* (for strain data, see Table 1).

Description of *Zygoascus biomembranicola* Nagatsuka, Ninomiya, Kiyuna, Kigawa & Sugiyama sp. nov.

Zygoascus biomembranicola (bi.o.mem.bra.ni′co.la. Gr. n. *bios* life; L. fem. n. *membrana* film; L. suffix. *-cola* from L. n. *incola* inhabitant, dweller; N.L. n. *biomembranicola* biofilm dweller).

Growth in YM broth

After 3 days at 25 °C, cells are spherical to ovoid (4.8–6.4 × 5.6–8.8 μm) and appear singly or in pairs. Asexual reproduction is by multilateral budding (Fig. 3g). Sediment and pellicles are formed in fermentation medium.

Aerobic growth on YM agar or PDA

After 14 days of incubation at 25 °C, streak cultures are cream-coloured with slightly pink centres, are smooth and glistening and have entire margins and are pulvinate.

Dalmau plate culture on cornmeal agar

After 7 days of incubation at 25 °C, true hyphae with clusters of blastoconidia at septa (Fig. 3h) are present.

Sexual reproduction

Sexual states on YM, 2 % malt or cornmeal agar remain unknown after 1 month at 25 °C.

Physiological and biochemical characteristics

See Table 4.

Chemotaxonomic characteristics

The major ubiquinone system is Q-9 and the nuclear DNA G+C content is 46.7 mol%, as determined using HPLC.

Typification

The type strain, JCM 31007^T (ex-type CBS 14157^T=isolate K61208-2-11^T), was isolated from a dried dark-brownish viscous gel on the floor of the KT stone chamber interior in Asuka village, Nara Prefecture, Japan, on 8 December 2006 by one of the authors (T. K.).

Identifier

MycoBank no. MB814345.

Isolate examined

Only one isolate, K61208-2-11^T, was examined (for strain data, see Table 1).

Description of *Yamadazyma olivae* (Nisiotou, Panagou & Nychas) Nagatsuka, Kiyuna & Sugiyama comb. nov.

Basionym

Candida olivae Nisiotou, Panagou & Nychas, *Int J Syst Evol Microbiol* **60**, 1219 (2010); MycoBank no. MB514467.

Type strain

CBS 11171^T (=ATCC MYA-4568^T).

MycoBank no

MB814339.

Isolates examined

Thirteen KT isolates were examined (for strain data, see Table 1).

Description of *Yamadazyma tumulicola* (Nagatsuka, Kiyuna, Kigawa & Sugiyama) Nagatsuka, Kiyuna, Kigawa & Sugiyama comb. nov.

Basionym

Candida tumulicola Nagatsuka, Kiyuna, Kigawa & Sugiyama, *Int J Syst Evol Microbiol* **59**, 186 (2009); MycoBank no. MB508970.

Type strain

JCM 15403^T (=CBS 10917^T=NBRC 104392^T=isolate T6517-9-5^T); for strain data, see Table 1.

MycoBank no

MB814340.

Description of *Yamadazyma takamatsuzukensis* (Nagatsuka, Kiyuna, Kigawa & Sugiyama) Nagatsuka, Kiyuna, Kigawa & Sugiyama comb. nov.

Basionym

Candida takamatsuzukensis Nagatsuka, Kiyuna, Kigawa & Sugiyama, *Int J Syst Evol Microbiol* **59**, 186 (2009); MycoBank no. MB508971.

Type strain

JCM 15410^T (=CBS 10916^T=NBRC 104391^T=isolate T4922-1-1^T); for strain data, see Table 1.

MycoBank no

MB814341.

Description of *Zygoascus bituminiphila* (V. Robert, B. Bonjean, Karutz, Paschold, W. Peeters & Wubbolts) Nagatsuka, Kiyuna & Sugiyama comb. nov.

Basionym

Candida bituminiphila V. Robert, B. Bonjean, Karutz, Paschold, W. Peeters & Wubbolts, *Int J Syst Evol Microbiol* **51**, 2171 (2001); MycoBank no. MB484846.

Type strain

CBS 8813^T (=MUCL 41424^T); for strain data, see Table 1.

MycoBank no

MB814342.

Description of *Zygoascus polysorbophila* (Kurtzman) Nagatsuka, Kiyuna & Sugiyama comb. nov.

Basionym

Candida polysorbophila Kurtzman, *Antonie van Leeuwenhoek* **92**, 221 (2007); MycoBank no. MB510412.

Type strain

NRRL Y-27161^T (=CBS 7317^T).

MycoBank no

MB814343.

Isolate examined

One isolate, T6517-9-4, was examined (for strain data, see Table 1).

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