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Two new yeast species Cystobasidium psychroaquaticum f.a. sp. nov. and Cystobasidium rietchieii f.a. sp. nov. isolated from natural environments, and the transfer of members of Rhodotorula minuta clade to the genus Cystobasidium.

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Abstract:	Yeasts inhabit diverse habitats worldwide, including sphagnum swamp ecosystems, which have been studied intensively, yielding several novel yeast species. Here we describe two new basidiomycetous yeasts related to R. minuta (Cystobasidiales). Multi-gene analyses resolved the phylogenetic relationships between the members of the R. minuta clade and the mycoparasite Cystobasidium fimetarium. Based on these results, we propose the transfer of nine species belonging to the R. minuta clade into the genus Cystobasidium. As a result, the clinical relevant species Rhodotorula minuta will be renamed Cystobasidium minutum. This proposal follows ongoing reassessments of the anamorphic genus Rhodotorula reducing the polyphyly of this genus. This change in the taxonomy of yeast fungi will help to delimit the aforementioned group from the species comprising Sporidiobolales that includes the generic type species R. glutinis. Our proposal will also help to distinguish most common red yeasts from clinical samples such as members of Sporidiobolales and Cystobasidiales. We emend the diagnosis of the genus Cystobasidium by including additional characteristics known for related yeast species. The two novel species are described here as Cystobasidium

	psychroaquaticum f.a. sp. nov. (K-833T = KBP 3881T = VKPM Y-3653T = CBS 11769T = MUCL 52875T = DSM 27713T) and Cystobasidium rietchiei f.a. sp. nov. (K-780T = KBP 4220T = VKPM Y-3658T = CBS 12324T = MUCL 53589T = DSM 27155T). The new species were registered in MycoBank under MB 809336 and MB 809337, respectively.

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Dear Iain Sutcliffe,

Herewith, I would like to submit to Antonie van Leeuwenhoek the manuscript studying phylogenetic relationships between yeasts of the *Rhodotorula minuta* clade and the mycoparasite *Cystobasidium*. In the paper, we describe two new species of in this group. Furthermore, based on multi-gene analyses we propose the transfer of nine species belonging to the *R. minuta* clade into the genus *Cystobasidium*. As a result, the clinical relevant species *Rhodotorula minuta* will be renamed *Cystobasidium minutum*. This proposal follows ongoing reassessments of the anamorphic genus *Rhodotorula* reducing the polyphyly of this genus. This change in the taxonomy of yeast fungi will help to delimit the aforementioned group from the species comprising Sporidiobolales that includes the generic type species *R. glutinis*. Our proposal will also help to distinguish most common red yeasts from clinical samples such as members of Sporidiobolales and Cystobasidiales. We emend the diagnosis of the genus *Cystobasidium* by including additional characteristics known for related yeast species.

Following the requirements, the type culture was deposited in several recognized culture collections: CBS (The Netherlands), BCCM/MUCL (Belgium), VKM and VKPM (both in Russia). All taxa and new combinations were registered in MycoBank, and the partial sequences of 18S, 26S rRNA genes, the complete sequence of the ITS-region, and partial sequences of *TEF-1* gene were deposited in GenBank.

Sincerely yours,

Andrey Yurkov
Curator for Fungi and Yeasts

Two new yeast species *Cystobasidium psychroaquaticum f.a.* sp. nov. and *Cystobasidium rietchieii f.a.* sp. nov. isolated from natural environments, and the transfer of members of *Rhodotorula minuta* clade to the genus *Cystobasidium*.

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Running title: Cystobasidium yeasts

Keywords: yeasts, fungi, basidiomycetes, Pucciniomycotina, new species, *Rhodotorula*, *Cystobasidium*

Summary: Yeasts inhabit diverse habitats worldwide, including sphagnum swamp ecosystems, which have been studied intensively, yielding several novel yeast species. Here we describe two new basidiomycetous yeasts related to *R. minuta* (Cystobasidiales). Multi-gene analyses resolved the phylogenetic relationships between the members of the *R. minuta* clade and the mycoparasite *Cystobasidium fimetarium*. Based on these results, we propose the transfer of nine species belonging to the *R. minuta* clade into the genus *Cystobasidium*. As a result, the clinical relevant species *Rhodotorula minuta* will be renamed *Cystobasidium minutum*. This proposal follows ongoing reassessments of the anamorphic genus *Rhodotorula* reducing the polyphyly of this genus. This change in the taxonomy of yeast fungi will help to delimit the aforementioned group from the species comprising Sporidiobolales that includes the generic type species *R. glutinis*. Our proposal will also help to distinguish most common red yeasts from clinical samples such as members of Sporidiobolales and Cystobasidiales. We emend the diagnosis of the genus *Cystobasidium* by including additional characteristics known for related yeast species. The two novel species are described here as *Cystobasidium psychroaquaticum f.a.* sp. nov. (K-833^T = KBP 3881^T = VKPM Y-3653^T = CBS 11769^T = MUCL 52875^T = DSM 27713^T) and *Cystobasidium rietchiei f.a.* sp. nov. (K-780^T = KBP 4220^T = VKPM Y-3658^T = CBS 12324^T = MUCL 53589^T = DSM 27155^T). The new species were registered in MycoBank under MB 809336 and MB 809337, respectively.

Introduction

Many species of basidiomycetous yeasts are known only in their asexual phase and are classified in heterogeneous anamorphic genera such as *Rhodotorula*

Harrison or *Cryptococcus* Vuillemin. However, phylogenetic analyses have repeatedly demonstrated the polyphyletic nature of these two genera (Fell et al. 2000; Scrorzetti et al. 2002; Aime et al. 2006; Bauer et al. 2006; Hibbet et al. 2006). The anamorphic basidiomycetous genus *Rhodotorula* comprises species that are currently distributed in two subphyla, Pucciniomycotina and Ustilagomycotina. Most of the known species are placed in the Microbotryomycetes: either in Sporidiobolales that includes the generic type species *R. glutinis*, in Microbotryales that includes the species *R. hordea*, in Kriegeriales that includes several white-colored species, or in the unclassified mitosporic Microbotryomycetes (Sampaio et al. 2003; Bauer et al. 2006). Another large group of *Rhodotorula* is distantly related to Microbotryomycetes and it places phylogenetically in the family Cystobasidiomycetes, in orders Cystobasidiales and Erythrobasidiales (Aime et al. 2006). The *Rhodotorula* species in the order Cystobasidiales present the second largest group after Sporidiobolales and contains *R. minuta* and allied taxa such as the mycoparasites *Occultifur* Oberwinkler, *Naohidea* Oberwinkler and *Cystobasidium* (Lagerheim) Neuhoff as their closest teleomorphic relatives (Sampaio et al., 2003; Aime et al. 2006). Several attempts have been made to reduce polyphyly and heterogeneity of the genus *Rhodotorula* that resulted in the reclassification of *Rhodotorula*-like yeasts that are unrelated to Sporidiobolales. This group includes the type species of *Rhodotorula*, *R. glutinis*. Thereby, several new genera have emerged to accommodate these anamorphic species, e.g. *Glaciozyma*, *Leucosporidiella*, *Farysizyma*, *Microbotryozyma* and *Meredithblackwellia* (Sampaio et al. 2003; Inacio et al. 2008; Turchetti et al. 2011; Suh et al. 2012; Toome et al. 2013). Recent changes in the International Code of Nomenclature for algae, fungi and plants (ICBN, Melbourne Code) terminated the use of dual nomenclature for fungi and ensures that only one name is used for anamorphic and teleomorphic stages of the same pleomorphic fungus (Hawksworth et al. 2011; McNeill & Turland 2012). This opens the possibility to follow evolutionary relationships and reduce the excessive complexity of fungal taxonomy by classifying asexual yeast stages of respective parasitic fungi in teleomorphic genera.

In the course of the study that focused on assessing the yeast diversity in *Sphagnum* moss ecosystems (Kachalkin et al. 2008; Kachalkin 2010; Kachalkin &

Yurkov 2012), two yet unknown yeast species related to the *R. minuta* clade (Cystobasidiales) were isolated. Other conspecific strains originating from diverse yeast surveys were included in this study. Phylogenetic analysis of the nearly entire nuclear ribosomal DNA cistron (18S, 5.8S and 26/28S rDNA genes) and partial sequences of the gene encoding the alpha subunit of the elongation factor-1 complex (*TEF1*) suggested that the two yeast species belong to the Cystobasidiales clade and are closely related to the mycoparasite *Cystobasidium fimetarium*. Taking into consideration the polyphyletic nature of the genus *Rhodotorula* and relationships of the studied group with *C. fimetarium*, we propose the transfer of the members of the *R. minuta* clade to the genus *Cystobasidium*.

Material and methods.

Four strains of *Rhodotorula spp.*, K-833 (= KBP3881 = VKPM Y-3653 = CBS 11769 = MUCL 52875 = DSM 27713), K-653 (= KBP4209 = VKPM Y-3654), K-915 (= KBP4210 = VKPM Y-3655) and K-780 (= KBP4220 = VKPM Y-3658 = CBS 12324 = MUCL 53589 = DSM 27155) were isolated from vascular bog plants, roots and leaves of Leatherleaf (*Chamaedaphne calyculata* (L.) Moensch) and cranberry (*Oxycoccus palustris* Pers.) leaves during September 2008 - January 2009 in Russia (Pushkino, Moscow region). Strain MUCL 30688 (= CBS 8018) was obtained from seawater collected at the cost of Sweden by B. Norkrans (Norkrans 1966). Other strains were isolated from glacial ice in Norway (Svalbard archipelago, Spitsbergen, Kongsfjorden glacier near Ny-Ålesund), EXF-3800 and EXF-3973 (Butinar et al. 2007); in Argentina (Cerro Otto, Bariloche, Patagonia) CRUB 1888 (leaves of Southern Beech *Nothofagus pumilio*); in Antarctica, CRUB 1786, CRUB 1787 and CRUB 1788 (from seawater collected in Bellingshausen sea); in Bulgaria, CBS 12423 (from a springtail, Sminthuridae feeding on a fungus collected in Natural park Vitosha).

Additionally, DNA isolated from *C. fimetarium* RB2079 (= DB1489) was used to produce the nucleotide sequences for phylogenetic analyses. This culture, RB2079 was isolated from the fruiting body of a coprophilus ascomycetous fungus *Lasiobolus* *equinus*, collected in Marburg, Germany by K.H. Rexer and maintained by R. Bauer. The yeast *Rhodotorula* sp. MB27 (= DSM 28479) was isolated as epiphyte from false oat-grass (*Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl; Poaceae) collected in October 2010 in Bochum, Germany by Marie Buchholz, and maintained by R. Prior and D. Begerow. It has been found to be the closest outgroup to the *R. minuta - Occultifur* clade.

Physiological and biochemical characteristics

Mating experiments were performed using Potato-Dextrose and Corn Meal agars (Yarrow 1998; Kurtzman et al. 2011) as well as Malt extract-Yeast extract-Soytone agar (MYP; (Sampaio et al. 2003). Phenotypic characterization of isolates was carried out according to Yarrow (1998) and Kurtzman et al. (2011) using both solid and liquid media. Additional assimilation tests using aldaric acids and aromatic compounds were performed as described by Fonseca (1992) and Sampaio (1999), respectively.

Molecular characterization

Yeast cultures were identified using nucleotide sequences of the three regions from the nuclear ribosomal DNA cistron (rDNA), the small subunit (18S or SSU), the internal transcribed spacer (ITS) region and the D1/D2 domains of the large subunit (26S/28S or LSU). Methods used for the DNA extractions, PCR amplifications, purification and sequencing of the SSU, LSU and the ITS regions were performed as described before (Glushakova et al. 2010; Yurkov et al. 2012). Sequencing of the fragment of the gene encoding translation elongation factor 1 alpha (*TEF1*) was performed with primers 983F, EF-df, EF-gr, and 1953R as described before by Matheny et al. (2006) and Rehner & Buckley (2005). Based on available sequences of the gene *TEF1* a few novel primers were designed to increase the PCR amplification success for the given group of fungi (Table 1). The following primers showed to be the most suitable for the studied group of yeasts, 983F, TEF-1150F as forward, and CBEF-04r and CBEF-a08r as reverse primers.

The assembly and editing of sequence data were performed using Sequencher 4.10 (Gene Codes Corp., USA). Alignments were made using the MAFFT algorithm (Katoh et al. 2002). Maximum likelihood analysis was performed with RaxML (version 7.2.8) using raxmlGUI (Silvestro & Michalak, 2012) and the GTRCAT option with 100 or 1000 rounds of bootstrap replicates (Stamatakis et al. 2008). Neighbor Joining analysis was performed using PAUP* (Wilgenbusch & Swofford 2003).

Priors for the Bayesian analyses were determined using MrModeltest 2.2 (Nylander et al. 2004) and the analyses were performed using MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001). Four incrementally heated simultaneous Markov chains were run over 2,000,000 generations using random starting trees and default starting parameters of the respective DNA substitution model (Huelsenbeck & Ronquist 2001). Trees were sampled every 100th generation resulting in an overall sampling of 20,001 trees. From these the first 5,000 trees were discarded and the remaining were used to compute a 50% majority rule consensus tree to obtain estimates for posterior probabilities.

Nucleotide sequences were deposited in GenBank under the accession numbers given on the phylogenetic trees (Fig. 1 - 3). Additional sequences were retrieved from GenBank (www.ncbi.nih.gov) and MycoID (www.mycobank.org) databases. Accession numbers and strain numbers are indicated on the phylogenetic trees.

Results and discussion

Previous studies, which used sequences of the D1/D2 domains of the LSU reported the polyphyletic nature of the genus *Rhodotorula* (e.g. Fell et al., 1995; Fell et al. 2000; Aime et al. 2006; Libkind et al. 2010; Sampaio 2011). These studies also revealed the phylogenetic relationship among some *Rhodotorula* species and the mycoparasites *Cystobasidium fimetarium* and *Occultifur externus*. Analysis of the D1/D2 domains of the surveyed yeasts suggested that they represented two hitherto undescribed species that are phylogenetically related to the *R. minuta* clade in Cystobasidiales. Specifically, strain K-833 = CBS 11769 showed seven and eight substitutions to *Rhodotorula sp.* CBS 10222 (EU002841) and “Antarctic yeast” CBS 8913 (AY040648), respectively. The nearest match (7 substitutions) among currently described species for strain K-780 = CBS 12324 was obtained with the type strain of *Rhodotorula laryngis* CBS 2221 (AF189937). Results of the phylogenetic analysis were consistent with earlier studies and showed that the two novel species, together with the mycoparasites *C. fimetarium* and *O. externus*, formed a monophyletic group that has a high statistical support with neighbor-joining (NJ, 100%), maximum likelihood (ML, 100%) and Bayesian (BI, 1.0) analyses (Fig. 1). The overall topologies of the trees obtained with NJ, BI and ML algorithms were consistent except for the position of *Cyrenella elegans*, which appeared as an outgroup taxon to Cystobasidiales in NJ, but not in ML and BI analyses (Fig. 1; grey dashed line).

In the light of the well-documented polyphyletic nature of the genus *Rhodotorula*, we faced the problem of the appropriate classification of the two novel species. Due to the fact that the type species of the genus *Rhodotorula* is placed in the Sporidiobolales lineage, the use of this generic name for phylogenetically unrelated yeasts such as members of the Cystobasidiomycetes will further increase the taxonomic complexity within *Rhodotorula*. Furthermore, recent changes in the fungal nomenclature have stopped the practice of using different names for naming sexual and asexual stages of the same fungus. This now opens the possibility to rename *Rhodotorula* species that are currently classified in the order Cystobasidiales and use names of teleomorphic genera like *Cystobasidium or Occultifur* or a new generic name for both taxa. Therefore, our study was aimed to resolve the phylogenetic relationships among yeasts that belong to the *R. minuta* clade and the two mycoparasites using multi-gene phylogenetic analyses. We sequenced the SSU, ITS and *TEF1* loci of *C. fimetarium* RB2079 and the undescribed yeast isolate *Rhodotorula* sp. MB27, which appears to be the closest outgroup to the *R. minuta* - *Occultifur* clade (Fig. 1).

Combined phylogenetic analyses of the rDNA cistron performed on 16 taxa confirmed the monophyly of the *R. minuta* clade with good support (93%) under ML analysis (Fig. 1). Due to the high variability of nucleotide sequences of the two spacers (ITS1 and ITS2) of the species belonging to the *R. minuta* clade, only a few terminal branches were additionally supported (> 75%) in this analysis. This result is congruent with what was previously reported by Nagahama et al. (2006) who found also low support for the studied clade in the ITS rDNA analysis. Adding nucleotide sequences of *TEF1* decreased support for the *R. minuta* clade but provided better resolution of inner nodes (Fig. 2). Specifically, *Rhodotorula* spp. and the *C. fimetarium* clade as well as the entire *R. minuta* clade including *O. externus* received moderate support (ML) being 80% and 76%, respectively (Fig. 2). Interestingly, this analysis revealed two well supported groups: (1) *C. fimetarium*, *R. minuta*, *R.*

slooffiae, and *R. calyptogenae*; and (2) *R. pinicola*, *R. laryngis*, *R. benthica*, *R. pallida* and the two novel yeast species. The use of *TEF1* slightly increased the support for *Rhodotorula* spp. and the *C. fimetarium* clade. However, when only the coding sequence was considered, the overall support for the *R. minuta* clade, including *O. externus*, was high (ML, 100%) whereas internal nodes were not resolved (data not shown). This observation is also in agreement with earlier study by Nagahama et al. (2006), who reported similar support values for the *R. minuta* clade (ML, 76%).

Our study confirmed close relationships among the yeasts that belong to the *R. minuta* clade and the mycoparasite *C. fimetarium*. Herewith, we describe two new yeast species in this group and propose the transfer of *Rhodotorula* spp. from the *R. minuta* clade to *Cystobasidium*. In spite of the observation of two phylogenetically well supported groups (Fig. 2), we faced the choice of delimiting the genus *Cystobasidium* either to the clade consisting of *C. fimetarium*, *R. minuta*, *R. slooffiae*, and *R. calyptogenae*, or the entire *R. minuta* clade. We are in favor of the transfer of the entire *R. minuta* clade to the genus *Cystobasidium* given this is the most conservative option, which will reduce taxonomic complexity of the Cystobasidiales lineage. This proposal enables stable classification of species known from asexual states only and circumscribes the genus *Cystobasidium* to a larger monophyletic group until sufficient knowledge regarding phylogenetic relationships of the type species of the genus *Cystobasidium* (*C. fimetarium*) with other species of this genus will be obtained (see also discussion below). Our proposal follows ongoing reassessment of the anamorphic genera *Cryptococcus* and *Rhodotorula* (e.g. Inacio et al. 2008; Wang & Bai 2008; Wang et al. 2011; Wuzkowski et al. 2011; Turchetti et al. 2011; Valente et al. 2012; Toome et al. 2013). The proposed taxonomical changes have the intention to unify the classification scheme of the mitosporic and meiosporic taxa of dimorphic basidiomycetes and to restrict the genera to phylogenetically related groups of species. Sexual structures have not been reported for any of these *Rhodotorula* species, and *C. fimetarium* does not produce sexual structures in laboratory conditions (Sampaio & Oberwinkler 2011a; this study). In contrast, *O. externus*, which occupies a position basal to the analyzed clade, produces a sexual state in culture. Furthermore, several traits are distinctive for these two teleomorphic species, e.g. the formation of basidiocarps, the presence of probasidia, and the mode of basidium germination and conidiogenesis (Sampaio & Oberwinkler 2011b). Therefore, we restrict our proposal to the transfer of *Rhodotorula* species but not *O. externus* to the genus *Cystobasidium*.

Morphological characters alone have limited power in resolving evolutionary relationships among yeast-like fungi and currently available sequence data suggest that the members of the order Platygloeales, where *Cystobasidium* was assigned to (e.g. Bandoni 1995; Diederich 1996), are placed in distantly related phylogenetic groups, namely classes Microbotryomycetes, Cystobasidimycetes (both Pucciniomycotina) and Tremellomycetes (Agaricomycotina). We are not aware of molecular data for other species of *Cystobasidium* but we expect that the genus *Cystobasidium* in its current circumscription, including also the species known only from light microscopy *C. hypogymniicola* and *C. usneicula* (Diederich 1996), might not be monophyletic, like it has been already demonstrated for some other lichenicolous heterobasidiomycetes listed by Diederich (1996), such as *Tremella* and *Syzygospora* (Millanes et al. 2011).

The proposed reclassification of the *R. minuta* clade species in *Cystobasidium* reduces the polyphyly and heterogeneity of *Rhodotorula*, shows the evolutionary link of the transferred species to *C. fimetarium* as the type species of the genus, and encourage further studies to add biological knowledge to this group of fungi. In our opinion, this reclassification is essential to reduce the taxonomic complexity and enhance stability of the genus *Rhodotorula* also in view of the ongoing description of novel anamorphic species related to *Cystobasidium*, which otherwise were to be classified in the genus *Rhodotorula*. These include the recently described *Rhodotorula oligophaga* (Satoh et al. 2013) and a few potential novel taxa originating from deep-sea environments; i.e. *Rhodotorula “nymphaeae”*, *R. “cassiicola”*, *R. “samaneae”* (Nagahama, 2006) and several CBS strains (CBS 8913, CBS 8923, CBS 9086, CBS 10222) from various environments. Last but not the least, our proposal will help to distinguish most common ‘red yeasts’ reported from clinical samples, species from Sporidiobolales lineage (referred to as *R. mucilaginosa* and *R. glutinis*) and members of Cystobasidiales (*Cystobasidium* spp.) presently mostly named as *R. minuta* (Libkind & Sampaio 2010).

We emend the diagnosis of the genus *Cystobasidium* to include available characteristics determined for its asexual counterparts. Some of these characteristics were summarized previously by Sampaio and Oberwinkler (2011), and Sampaio (2011). The authors favor the use of the expression *forma asexualis* (*f.a.*) in the description of anamorphic species of the genus *Cystobasidium* and this decision follows the current practice of reclassification of asexual yeast taxa (see e.g. Lachance 2012; Groenewald & Smith 2013; Daniel et al. 2013; Selbmann et al., 2014).

Emendation of *Cystobasidium* (Lagerheim) Neuhoff (1924) Bot. Arch. 8: 272 (1924) emend. Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout and Begerow.

Yeast cells are ovoid to elongate, and budding is predominately polar. Cultures are often pigmented and pink to orange in color. Synthesis of mycosporines may be present (Libkind et al. 2005). Main carotenoid pigments are torulene and torularhodin (Buzzini et al. 2007; Yurkov et al. 2008). Physiological characteristics, typical for the yeasts of the Cystobasidiomycetes lineage have been reported before (Bauer et al. 2006; Libkind et al. 2010; Sampaio et al. 2011a). Assimilation of *myo*-inositol is variable (Table 2). Nitrate is not assimilated, starch-like compounds (PSC) are not produced, and D-glucuronate is utilized. Fermentative abilities are absent. The diazonium blue B reaction and production of urease is positive. Discrimination growth tests of the species belonging to the order Cystobasidiales are summarized in Table 2.

The genus *Cystobasidium* comprises mycoparasitic species. Asexual yeast states of species belonging to the genus *Cystobasidium* are repeatedly found in the phylloplane in temperate to cold regions (Fonseca & Inacio 2006; Yurkov et al., 2008; Glushakova & Chernov 2010; Kachalkin & Yurkov 2012), soil-related substrates (de Azeredo et al. 1998; Golubtsova et al. 2007; Connell et al. 2008), aquatic environments (Sláviková & Vadkertiová 1997; Libkind et al. 2003; de Garcia et al. 2007; Kachalkin 2014), and deep-sea sediments (Nagahama 2006). Furthermore, *R. minuta* has been reported among the most common red-colored yeasts, after *R. mucilaginosa* and *R. glutinis*, from clinical specimens and are considered as emergent pathogens (Tuon & Costa 2008; Libkind & Sampaio 2010; Zhou et al.

2014). It has to be also emphasized that identification of clinical isolates may not be conform with the currently used approaches and, thus, different morphologically indistinguishable species of the genus *Cystobasidium* can be mistaken for *R. minuta* in these reports (Libkind & Sampaio 2010).

Novel combinations

Cystobasidium minutum (Saito) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: MB 809340.

Basionym: *Torula minuta* Saito (Saito. Journal of Japanese Botany 1:1-54,1922); MB 246433. Synonym: *Rhodotorula minuta* (Saito) F.C. Harrison; MB 271309.

Cystobasidium slooffiae (Novák & Vörös-Felkai) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: MB 809341.

Basionym: *Rhodotorula slooffiae* Novák & Vörös-Felkai (Novák, Vörös-Felkai. Acta Microbiologica Academiae Scientiarum Hungaricae 9: 261-263, 1962); MB 456473.

Cystobasidium benthicum (Nagahama, Hamamoto, Nakase & Horikoshi) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a.comb. nov.* MycoBank No.: MB 809342.

Basionym: *Rhodotorula benthica* Nagahama, Hamamoto, Nakase & Horikoshi (Nagahama, Hamamoto, Nakase, Horikoshi. International Journal of Systematic and Evolutionary Microbiology 53: 897-903, 2003); MB 489309.

Cystobasidium pinicola (Bai, Guo & Zhao) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: MB 809344.

Basionym: *Rhodotorula pinicola* Bai, Guo & Zhao (Zhao, Bai, Guo, Jia. FEMS Yeast Research 2: 159-163, 2002); MB 373538.

Cystobasidium laryngis (Reiersöl) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: 809345.

Basionym: *Rhodotorula laryngis* Reiersöl (Reiersöl. Antonie van Leeuwenhoek. 21: 286-288, 1955); MB 338576.

Cystobasidium calyptogenae (Nagahama, Hamamoto, Nakase & Horikoshi) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: 809346.

Basionym: *Rhodotorula calyptogenae* Nagahama, Hamamoto, Nakase & Horikoshi (Nagahama, Hamamoto, Nakase, Horikoshi. International Journal of Systematic and Evolutionary Microbiology 53: 897-903, 2003); MB 489308.

Cystobasidium lysinophilum (Nagahama, Hamamoto, Nakase & Horikoshi) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: 809347.

Basionym: *Rhodotorula lysinophila* Nagahama, Hamamoto, Nakase & Horikoshi (Nagahama, Hamamoto, Nakase, Horikoshi. International Journal of Systematic and Evolutionary Microbiology 53: 897-903, 2003); MB 489310.

Cystobasidium pallidum (Lodder) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: 809348.

Basionym: *Rhodotorula pallida* Lodder (Diddens, Lodder. Verhandelingen Koninklijke Nederlandse Akademie van Wetenschappen Afdeling Natuurkunde 32: 1-256, 1934); MB 273323.

Cystobasidium oligophagum (Satoh & Makimura) Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow *f.a. comb. nov.* MycoBank No.: MB 809613.

Basionym: *Rhodotorula oligophaga* Satoh and Makimura (Satoh, Maeda, Umeda, Sugamata, Makimura. Antonie van Leeuwenhoek 104: 83-93, 2013); MB 802689.

Description of *Cystobasidium psychroaquaticum* Yurkov, Kachalkin, Daniel, Groenewald, Libkind, de Garcia, Zalar, Gouliamova, Boekhout & Begerow. *f.a.* sp. nov. (MB 809336).

Etymology: ***psychroaquaticum*** refers to the psychrophilic aquatic source of most of currently know isolates, except for CRUB 1888 and CBS 12423.

Streak culture after one week on GYP agar at 20–22°C, salmon-orange to pinkish, shiny, mucilaginous, smooth, with entire margins. Yeast cells after four days on GYP agar ovoid to elongate, 4–5 x 6–9 µm, proliferating by polar budding (Fig. 3). Ballistospores and pseudohyphae not produced.

Sugars not fermented. Assimilation of carbon compounds: D-glucose, sucrose, trehalose, melezitose, D-xylose, L-arabinose, glycerol, ribitol, D-mannitol, salicin, D-glucuronate, succinic acid, 2-ketogluconic acid, 5-ketogluconic acid, arbutin, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, ferulic acid, vanillic acid, and veratric acid. No growth on D-galactose, maltose, lactose, melibiose, raffinose, inulin, starch, L-rhamnose, glucosamine, erythritol, dulcitol (galactitol), methyl-a-D-glucoside, citric acid, *myo*-inositol, protocatechuic acid, gallic acid, gentisic acid, salicylic acid, L-tartaric acid, and saccharic acid. Variable tests: L-sorbose, cellobiose, D-arabinose, D-ribose, ethanol, D-sorbitol (D-glucitol), DL-lactic acid, and on mucic acid. Potassium nitrate, sodium nitrite, ethylamine, L-lysine, cadaverine, creatine, creatinine, glucosamine, and imidazole are not assimilated. Growth is absent on 50% (w/w) glucose-yeast extract-agar and 5% glucose medium with 10% NaCl (w/v). Maximal growth temperature: 25ºC.

Molecular characteristics (type strain): nucleotide sequences of SSU rRNA, ITS–LSU (D1/D2 domains) rRNA, and *TEF1* deposited in NCBI/EMBL (GenBank) under the accession numbers LM644062, FN868153, and LM644068, respectively.

Deposits: holotype, strain K-833^T (= KBP3881^T), isolated from leatherleaf (*Chamaedaphne calyculata*, Ericaceaea) in Pushkino, Moscow region, Russia, and ex-type cultures are deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 11769^T), the Russian National Collection of Industrial Microorganisms, Moscow, Russia (VKPM Y-3653^T), the Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL), Louvain-la-Neuve, Belgium (MUCL 52875^T), and the German Collection of Microorganisms and Cell Cultures, Brunswick, Germany (DSM 27713^T).

Strains studied: K-833, MUCL 30688, EXF-3800, EXF-3973, CRUB 1888, CRUB1786, CRUB1787, CRUB1788 and CBS 12423.

Description of *Cystobasidium ritchiei* Yurkov, Kachalkin, Daniel, Groenewald, Boekhout & Begerow. *f.a.* sp. nov. (MB 809337).

Etymology: ***ritchiei*** refers to the late computer scientist Dennis MacAlistair Ritchie for creating the *C* programming language, which has been widely used for writing computer tools we use every day in molecular phylogeny and microbial ecology.

Streak culture after one week on GYP agar at 20–22°C, red, shiny, mucilaginous, smooth, with entire margins. Yeast cells after four days on GPY agar ovoid to elongate, 2.5–4 x 5–7 µm, proliferating by polar budding (Fig. 4). Ballistospores and pseudohyphae not produced.

Sugars not fermented. Assimilation of carbon compounds: D-glucose, L-sorbose (weak), sucrose, cellobiose, trehalose (weak), melezitose, D-xylose, L-arabinose, D-arabinose, D-ribose, glucosamine (weak), ethanol (weak), glycerol, ribitol, D-mannitol, D-sorbitol (D-glucitol), salicin, DL-lactic acid (weak), succinic acid, D-glucuronate, 2-ketogluconic acid, 5-ketogluconic acid, arbutin, gallic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, ferulic acid, vanillic acid, veratric acid, and mucic acid (weak). No growth on D-galactose, maltose, lactose, melibiose, raffinose, inulin, starch, L-rhamnose, erythritol, dulcitol (galactitol), methyl-a-D-glucoside, citric acid, *myo*-inositol, protocatechuic acid, gentisic acid, salicylic acid, L-tartaric acid, and saccharic acid. Potassium nitrate, sodium nitrite, ethylamine, L-lysine, cadaverine, creatine, creatinine, glucosamine, and imidazole are not assimilated. Growth absent on 50% (w/w) glucose-yeast extract-agar and 5% glucose medium with 10% NaCl (w/v). Maximal growth temperature: 25ºC.

Molecular characteristics (type strain): nucleotide sequences of SSU rRNA, ITS–LSU (D1/D2 domains) rRNA, and *TEF1* deposited in NCBI/EMBL (GenBank) under the accession numbers LM644063, LM644066, and LM644069, respectively.

Deposits: holotype, strain K-780^T (= KBP 4220^T), isolated from leatherleaf (*Chamaedaphne calyculata*, Ericaceae) in Pushkino, Moscow region, Russia, and ex-type cultures are deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 12324^T), the Russian National Collection of Industrial Microorganisms, Moscow, Russia (VKPM Y-3658^T), the Mycothèque de l'Université catholique de Louvain (BCCM/MUCL), Louvain-la-Neuve, Belgium (MUCL 52875^T), and the German Collection of Microorganisms and Cell Cultures, Brunswick, Germany (DSM 27155^T).

Strains studied: K-780.

Acknowledgments

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Figure captions:

Fig. 1 – Phylogenetic relationships of yeasts and related taxa from the Cystobasidiomycetes lineage obtained by Neighbor-Joining analysis of LSU (D1/D2 domains) rRNA (backbone tree) and detailed placement of the *Rhodotorula minuta* clade, *Cystobasidium fimetarium* and *Occultifur externus* obtained with Maximum Likelihood analysis of SSU rRNA, LSU (D1/D2 domains) rRNA, and the ITS region. The numbers given on branches are frequencies (>75%) with which a given branch appeared in 100 bootstrap replications. The scale bars indicate the numbers of expected substitutions accumulated per site. Branches given as dashed lines were collapsed. Position of *Cyrenella elegans* (grey dashed line) was inconsistent between different phylogenies. Maximum Likelihood tree is rooted with *Sakaguchia dacryoidea* (DQ832205, DQ832206, DQ832207) and *Erythrobasidium hasegawianum* (AF189899, D12803, AF444522).

Fig. 2 – Maximum likelihood analysis of an alignment of the SSU rRNA, the ITS region, the LSU (D1/D2 domains) rRNA and *TEF1* for the *Rhodotorula minuta* clade, *Cystobasidium fimetarium* and *Occultifur externus*. The numbers given on branches are frequencies (>50%) with which a given branch appeared in 1000 bootstrap replications. The scale indicates the number of expected substitutions accumulated per site.

Fig. 3 - Phase contrast micrograph of *Cystobasidium psychroaquaticum* K-833^T. Vegetative cells reproducing by budding after 7 days on GPY agar at room temperature, bar = 10 µm.

Fig. 4 - Phase contrast micrograph of *Cystobasidium ritchiei* K-780^T. Vegetative cells reproducing by budding after 7 days on GPY agar at room temperature, bar = 10 µm.

Figure 1

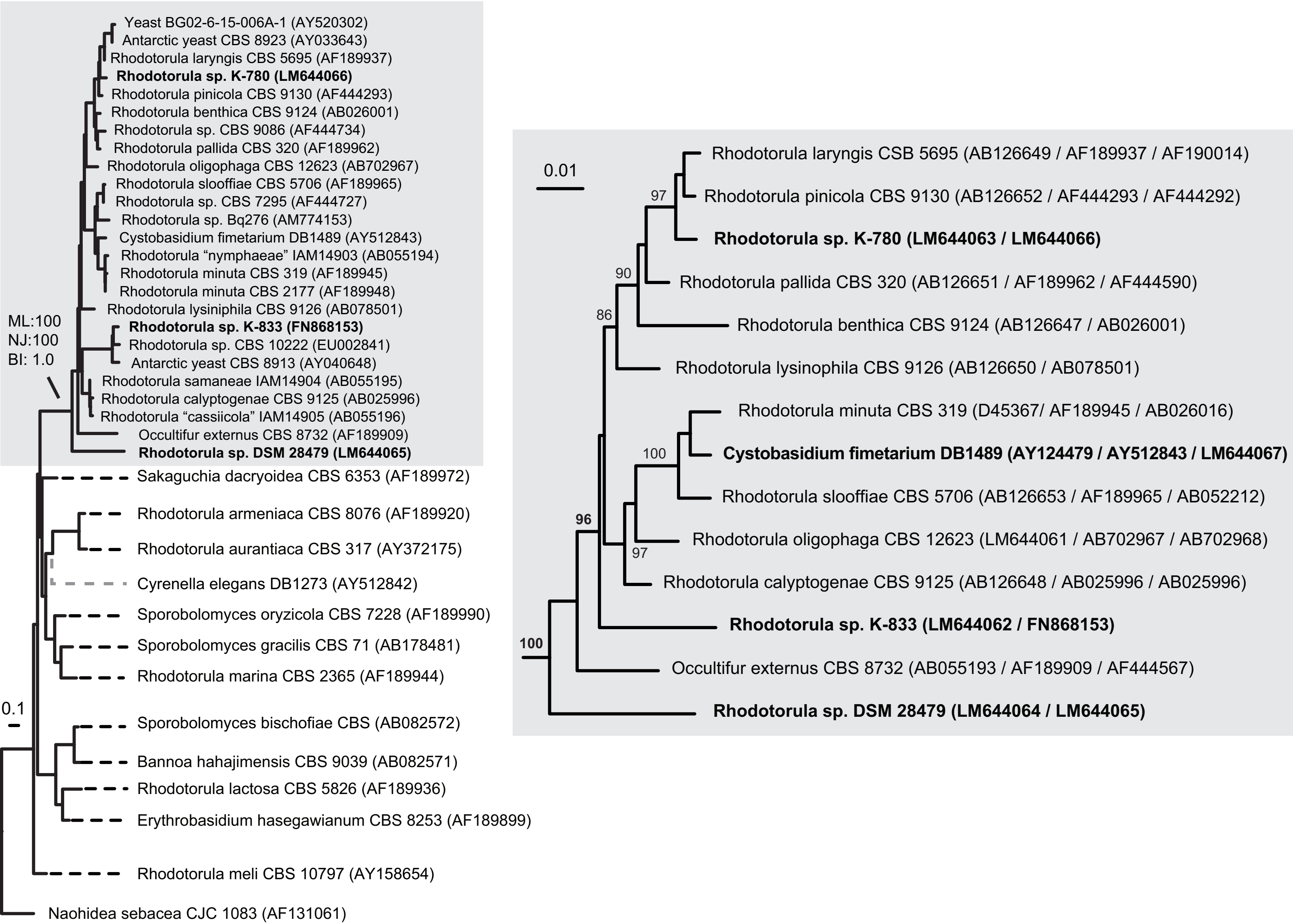
Yeast BG02-6-15-006A-1 (AY520302)
Antarctic yeast CBS 8923 (AY033643)
Rhodotorula laryngis CBS 5695 (AF189937)
Rhodotorula sp. K-780 (LM644066)
Rhodotorula pinicola CBS 9130 (AF444293)
Rhodotorula benthica CBS 9124 (AB026001)
Rhodotorula sp. CBS 9086 (AF444734)
Rhodotorula pallida CBS 320 (AF189962)
Rhodotorula oligophaga CBS 12623 (AB702967)
Rhodotorula slooffiae CBS 5706 (AF189965)
Rhodotorula sp. CBS 7295 (AF444727)
Rhodotorula sp. Bq276 (AM774153)
Cystobasidium fimetarium DB1489 (AY512843)
Rhodotorula “nymphaeae” IAM14903 (AB055194)
Rhodotorula minuta CBS 319 (AF189945)
Rhodotorula minuta CBS 2177 (AF189948)
Rhodotorula lysiniphila CBS 9126 (AB078501)
ML:100
NJ:100
BI: 1.0
Rhodotorula sp. K-833 (FN868153)
Rhodotorula sp. CBS 10222 (EU002841)
Antarctic yeast CBS 8913 (AY040648)
Rhodotorula samaneae IAM14904 (AB055195)
Rhodotorula calyptogenae CBS 9125 (AB025996)
Rhodotorula “cassiicola” IAM14905 (AB055196)
Occultifur externus CBS 8732 (AF189909)
Rhodotorula sp. DSM 28479 (LM644065)
Sakaguchia dacryoidea CBS 6353 (AF189972)
Rhodotorula armeniaca CBS 8076 (AF189920)
Rhodotorula aurantiaca CBS 317 (AY372175)
Cyrenella elegans DB1273 (AY512842)
Sporobolomyces oryzicola CBS 7228 (AF189990)
Sporobolomyces gracilis CBS 71 (AB178481)
Rhodotorula marina CBS 2365 (AF189944)
0.1
Sporobolomyces bischofiae CBS (AB082572)
Bannoa hahajimensis CBS 9039 (AB082571)
Rhodotorula lactosa CBS 5826 (AF189936)
Erythrobasidium hasegawianum CBS 8253 (AF189899)
Rhodotorula meli CBS 10797 (AY158654)
Naohidea sebacea CJC 1083 (AF131061)
0.01
Rhodotorula laryngis CSB 5695 (AB126649 / AF189937 / AF190014)
97
Rhodotorula pinicola CBS 9130 (AB126652 / AF444293 / AF444292)
Rhodotorula sp. K-780 (LM644063 / LM644066)
90
Rhodotorula pallida CBS 320 (AB126651 / AF189962 / AF444590)
86
Rhodotorula benthica CBS 9124 (AB126647 / AB026001)
Rhodotorula lysinophila CBS 9126 (AB126650 / AB078501)
Rhodotorula minuta CBS 319 (D45367/ AF189945 / AB026016)
100
Cystobasidium fimetarium DB1489 (AY124479 / AY512843 / LM644067)
Rhodotorula slooffiae CBS 5706 (AB126653 / AF189965 / AB052212)
96
Rhodotorula oligophaga CBS 12623 (LM644061 / AB702967 / AB702968)
97
Rhodotorula calyptogenae CBS 9125 (AB126648 / AB025996 / AB025996)
Rhodotorula sp. K-833 (LM644062 / FN868153)
100
Occultifur externus CBS 8732 (AB055193 / AF189909 / AF444567)
Rhodotorula sp. DSM 28479 (LM644064 / LM644065)

Figure 2

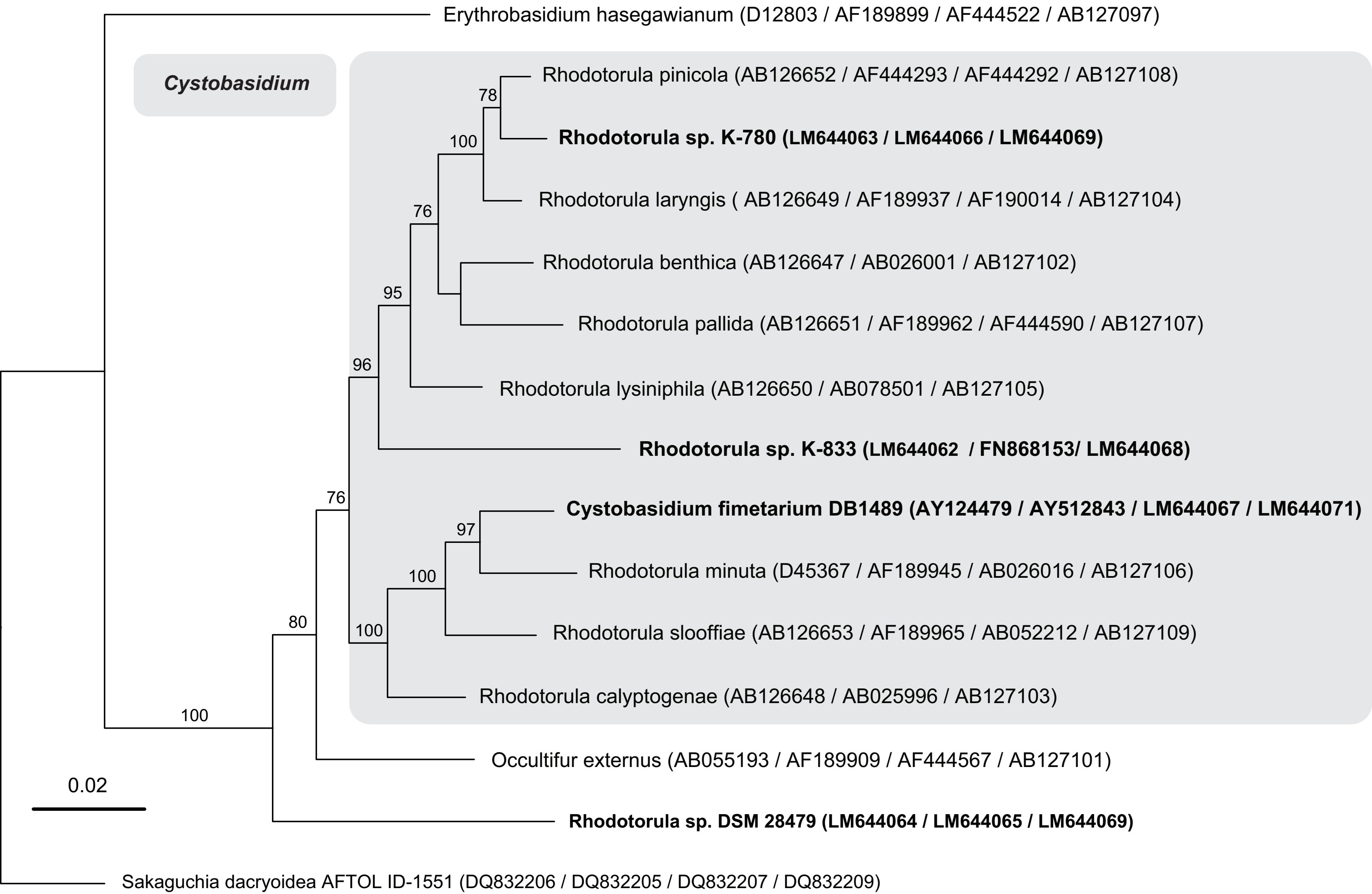
Erythrobasidium hasegawianum (D12803 / AF189899 / AF444522 / AB127097)
Cystobasidium
Rhodotorula pinicola (AB126652 / AF444293 / AF444292 / AB127108)
78
Rhodotorula sp. K-780 (LM644063 / LM644066 / LM644069)
100
Rhodotorula laryngis (AB126649 / AF189937 / AF190014 / AB127104)
76
Rhodotorula benthica (AB126647 / AB026001 / AB127102)
95
Rhodotorula pallida (AB126651 / AF189962 / AF444590 / AB127107)
96
Rhodotorula lysiniphila (AB126650 / AB078501 / AB127105)
Rhodotorula sp. K-833 (LM644062 / FN868153/ LM644068)
76
Cystobasidium fimetarium DB1489 (AY124479 / AY512843 / LM644067 / LM644071)
97
Rhodotorula minuta (D45367 / AF189945 / AB026016 / AB127106)
100
80
Rhodotorula slooffiae (AB126653 / AF189965 / AB052212 / AB127109)
100
Rhodotorula calyptogenae (AB126648 / AB025996 / AB127103)
100
Occultifur externus (AB055193 / AF189909 / AF444567 / AB127101)
0.02
Rhodotorula sp. DSM 28479 (LM644064 / LM644065 / LM644069)
Sakaguchia dacryoidea AFTOL ID-1551 (DQ832206 / DQ832205 / DQ832207 / DQ832209)

Figure 3

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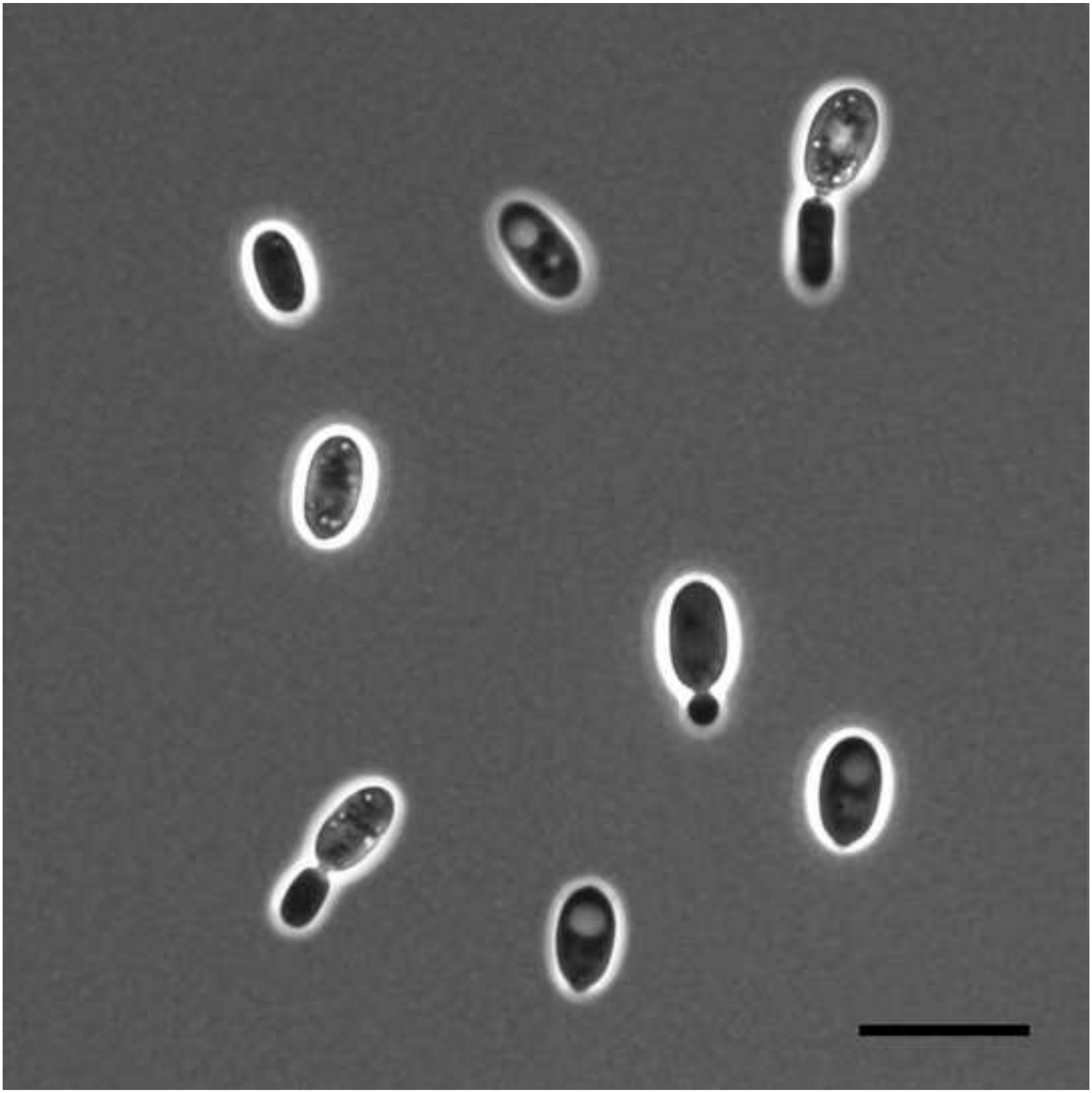

Figure 4

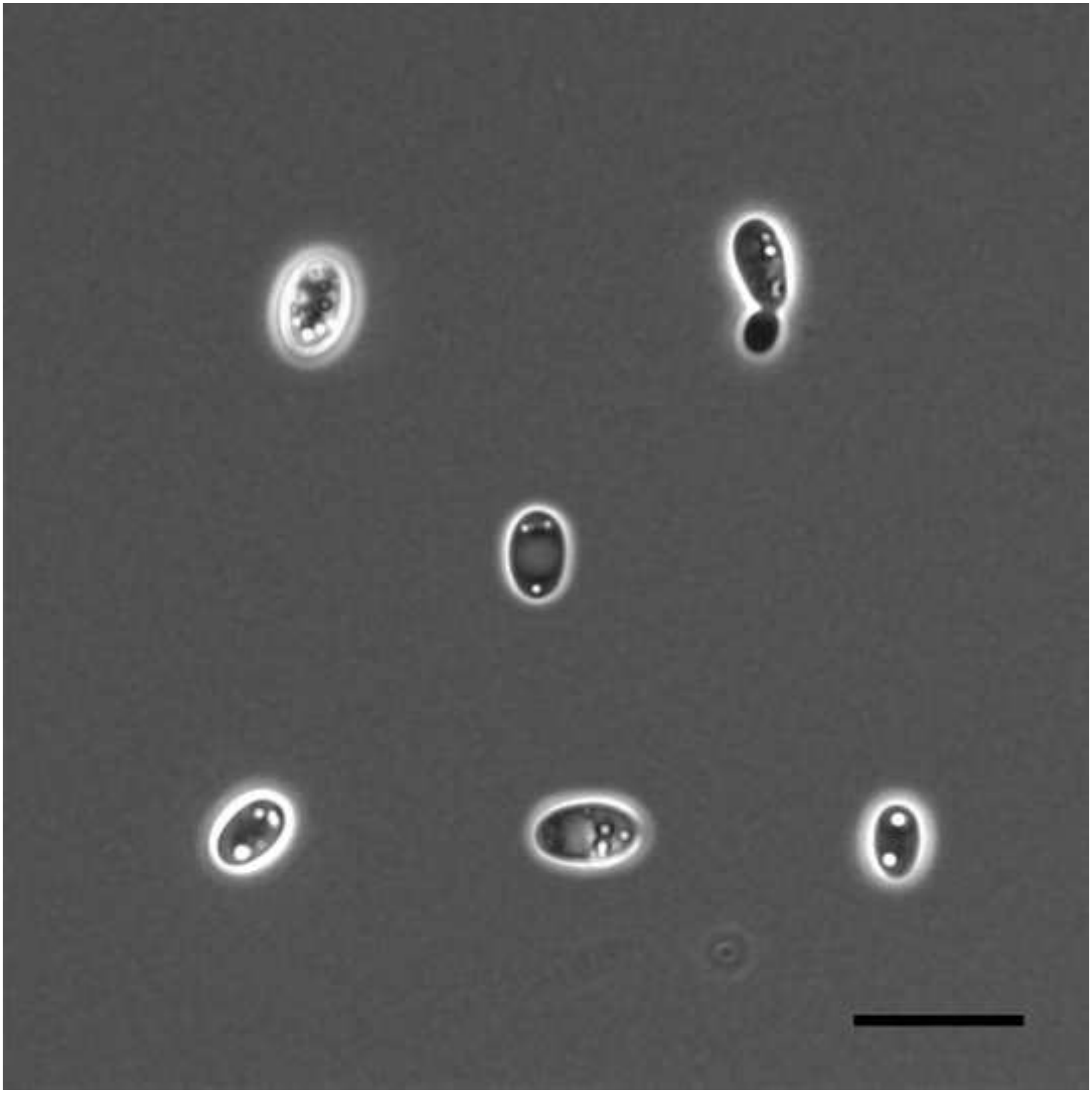

Table 1 – Primers used in this study to amplify and sequence fragments of the gene encoding translation elongation factor 1 alpha (*TEF-1*).

Primer name	Primer sequence	Reference
CBEF-a08r	TCGYTCYATCTTCTCKAGGAGRGT	this study
CBEF-04r	GTGACRACCATACCRGCCTTGATG	this study
CBEF03f	CATCCGGHTTCATCAARAAGGTCGG	this study
EF-df	AAGGAYGGNCARACYCGNGARCAYGC	Rehner & Buckley (2005)
EF-gr	GCAATGTGGGCRGTRTGRCARTC	Rehner & Buckley (2005)
983F	GCYCCYGGHCAYCGTGAYTTYAT	Rehner & Buckley (2005)
1953R	CCRGCRACRGTRTGTCTCAT	Rehner & Buckley (2005)
TEF-1154F	CGAGGCTGGTATCTCCAAGGATGG	this study
TEF-1150F	AGTTCGAGGCTGGTATCTCCAAGG	this study

Table 2 – Key and Some Discrimination Growth Tests of the Species Belonging to the Order Cystobasidiales (according Kurtzman et. al., 2011; Satoh et al., 2013; this study).

	Nitrate	*myo*-Inositol	D -Glucoronate	Sucrose	Maltose	Lactose	Cellobiose	Er ythritol	L-Sorbose	Raffinose	Melezitose	D -Arabinose	Glucosamine	Salicin	Galic acid	Veratric acid	Growth at 37^{o} C
Cystobasidium benthicum	-	+	+	+	+	+	+	+	+	+	+	+	-	-	?	?	+
C. calyptogenae	-	+	+	-	+	+	+	+	-	+	+	+	-	+	?	?	+
C. fimetarium	-	-	+	V	-	+	+	-	-	-	-	+	-	+	+	+	-
C. laryngis	-	-	+	+	-	-	+	-	V	-	+	+	-	+	-	-	-
C. lysinophilum	-	-	+	+	+	+	+	+	-	+	+	+	-	-	?	?	-
C. minuta	-	-	+	+	-	+	+	-	+	-	+	+	-	+	+	+	-
C. oligophagum	-	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	+
C. pallidum	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
C. pinicola	-	+	+	-	+	+	+	-	-	+	+	+	-	+	?	?	-
C. psychaquaticum sp. nov.	-	-	+	+	-	-	V	-	V	-	+	V	-	+	-	+	-
C. ritchieii sp. nov.	-	-	+	+	-	-	+	-	+	-	+	+	+	+	+	+	-
C. slooffiae	-	-	+	+	-	+	+	-	+	-	+	+	-	V	?	?	-
Occultifur externus	-	-	+	+	+	+	+	-	+	-	+	+	-	+	-	+	-