

Saitoella coloradoensis sp. nov., a new species of the Ascomycota, subphylum Taphrinomycotina

Cletus P. Kurtzman · Christie J. Robnett

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Abstract *Saitoella coloradoensis* sp. nov. (NRRL YB-2330, CBS 12360, type strain, MycoBank accession number 563858) is described. This new member of the phylum Ascomycota, subphylum Taphrinomycotina was isolated from insect frass occurring in an Engelmann spruce (*Picea engelmannii*) that was growing in Colorado, USA. Multigene sequence analysis showed that *S. coloradoensis* is distinct from *Saitoella complicata*, the only other known species of *Saitoella*. The two species may be separated phenotypically from growth reactions on D-xylose, ribitol and methyl- α -D-glucoside. Asexual reproduction is by budding and both species produce thick-walled, spherical cells that appear morphologically similar to the ascogenous cells formed in plant host tissue by species of *Protomyces* and some species of *Taphrina*. The thick-walled cells did not form ascospores but did produce buds when placed on fresh growth media.

Keywords New yeast · *Saitoella* · Taphrinomycotina

Introduction

The monotypic yeast genus *Saitoella* was described by Goto et al. (1987) with the type species *S. complicata* based on two strains that had been isolated from soil collected in Laya, Bhutan. The strains were initially classified from phenotype as *Rhodotorula glutinis* (Goto and Sugiyama 1970). Later, it was discovered that the isolates gave a negative diazonium blue B color reaction, typical of ascomycetous yeasts (Sugiyama et al. 1985), and that the cell wall ultrastructure was characteristic of the Ascomycota (Goto et al. 1987). Because *S. complicata* synthesizes carotenoid pigments, forms coenzyme Q_{10} and lacks xylose in cellular hydrolysates, it resembles the plant pathogenic yeast-like genera *Protomyces* and *Taphrina* (Sugiyama et al. 1985; Sugiyama and Hamamoto 2011).

Phylogenetic analysis of genes for nuclear small subunit rRNA (Nishida and Sugiyama 1993), domains D1 and D2 of large subunit rRNA (Kurtzman and Robnett 1998) and multigene sequence analysis (Sugiyama et al. 2006) placed *Saitoella*, *Protomyces* and *Taphrina* in the same clade, which also includes *Schizosaccharomyces*, *Pneumocystis* and *Neolecta*. Sugiyama et al. (2006) noted from multigene sequence analysis that the position of *Saitoella* in the subphylum Taphrinomycotina is not strongly resolved, but the genus may be most closely related to *Protomyces* and *Taphrina*. Recently, the new genus *Archaeorhizomyces* was described for strains isolated from coniferous

C. P. Kurtzman (✉) · C. J. Robnett
Bacterial Foodborne Pathogens and Mycology Research
Unit, National Center for Agricultural Utilization
Research, Agricultural Research Service, U.S.
Department of Agriculture, 1815 North University Street,
Peoria, IL 61604, USA
e-mail: cletus.kurtzman@ars.usda.gov

ectomycorrhizal root tips, and multigene sequence analysis placed this new taxon in the Taphrinomycotina (Rosling et al. 2011). The preceding studies and that of James et al. (2006) resolved the phylum Ascomycota into three major clades or subphyla. The subphylum Taphrinomycotina encompasses the above noted seven genera and is basal to the subphyla Saccharomycotina and Pezizomycotina, which are sister taxa. Budding yeasts such as *Saccharomyces* are placed in the Saccharomycotina, whereas “filamentous” species, such as *Aspergillus* and *Neurospora* are members of the Pezizomycotina (Hibbett et al. 2007).

The present widespread practice of identifying yeasts newly isolated from nature by DNA sequence analysis has resulted in the discovery of many new species, but additional strains of *Saitoella* have not been isolated and the genus is still known only from the original two cultures obtained from Himalayan soil (Goto and Sugiyama 1970) that formed the basis for description of *S. complicata* (Goto et al. 1987). During characterization of unidentified yeasts maintained in the ARS Culture Collection, a new species of *Saitoella* was recognized from D1/D2 LSU rRNA gene sequence analysis. The genetic separation between this species and *S. complicata* was supported by additional gene sequences. This new species, which was isolated from insect frass in an Engelmann spruce (*Picea engelmannii*) growing in Colorado, USA, is described here.

Materials and methods

The proposed new species of *Saitoella*, NRRL YB-2330, was isolated by L. J. Wickerham from insect frass collected in 1950 by staff of the U.S. Forest Service from an Engelmann spruce (*Picea engelmannii*) growing in the White River National Forest, Meeker, CO, USA. The composition of the culture media used and procedures for conducting growth and sugar fermentation tests were by standard methods (Kurtzman et al. 2011). Growth tests were conducted in liquid media and incubated at 25°C for four weeks. Cultures of NRRL YB-2330 and *S. complicata* NRRL Y-17804 were tested for ascospore germination on YM, 5% malt extract, RG and water agar media, as well as on water agar containing 1.5 cm² sections of leaves from mulberry (*Morus rubra*) and lily-of-the-valley (*Convallaria majalis*) and branch tips with needles from a

Colorado blue spruce (*Picea pungens*). Freshly collected leaf sections and spruce bud tips were placed in tubes of water agar and autoclaved for 20 min at 121°C. The agar surface was inoculated with cells near the protruding leaf sections.

Methods for DNA isolation and sequencing of genes for domains 1 and 2 (D1/D2) of large subunit (LSU) rRNA, mitochondrial small subunit (Mt SSU) rRNA, translation elongation factor (EF-1 α) and the internal transcribed spacers 1 and 2 (ITS 1 and 2) were reported earlier (Kurtzman and Robnett 1998, 2003). Briefly, following DNA isolation, each of the regions to be sequenced was amplified by PCR and the resulting amplicons were sequenced using the ABI BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI 3730 automated DNA sequencer according to the manufacturer's instructions. For phylogenetic analysis, sequences were visually aligned. Phylogenetic relatedness among species was determined from the maximum parsimony and neighbor-joining programs of PAUP*4.063a (Swofford 1998). Bootstrap support for the phylogenetic trees was determined from 1,000 replicates. The species compared and the GenBank accession numbers for the genes sequenced are given in Table 1.

Results and discussion

NRRL YB-2330 was initially recognized as a member of the genus *Saitoella* from its D1/D2 LSU rRNA gene sequence. The strain differed from NRRL Y-17804, type strain of *S. complicata*, by four nucleotide differences in D1/D2 (Table 2). To further determine extent of divergence between the two strains, sequences for ITS, EF-1 α and Mt SSU were compared. Surprisingly, the two strains differed by only two ITS substitutions, but differences in EF-1 α and Mt SSU were substantially greater (Table 2). Yeast species often differ from one another by 1% or greater substitutions in D1/D2 and ITS, but some closely related species may be unresolved by D1/D2 and/or ITS. For example, *Meyerozyma guilliermondii* and *Candida carpophila*, which differ by a single D1/D2 nucleotide, show 55% nuclear DNA relatedness as measured by reassociation (Vaughan-Martini et al. 2005), and this latter assessment of genome similarity suggests that the two taxa represent separate species. In contrast, *Trichosporon montevidense* and *T. domesticum* are unresolved by ITS, but are easily separated from

Table 1 Species of *Saitoella* and reference taxa compared

| Species | Strain no. | GenBank accession no. ^a | | | |
|----------------------------------|---------------------------|------------------------------------|----------|---------------|----------|
| | | D1/D2 | ITS | EF-1 α | Mt SSU |
| <i>Saitoella complicata</i> | NRRL Y-17804 CBS 7301 | JN161156 | JN161162 | JN161159 | JN161164 |
| <i>Saitoella coloradoensis</i> | NRRL YB-2330 CBS 12360 | JN161157 | JN161161 | JN161160 | JN161163 |
| <i>Botryozyma nematodophila</i> | NRRL Y-17705 CBS 7426 | DQ442709 | – | JN161158 | – |
| <i>Protomyces inouyei</i> | IAM 14512 | AY548294 | – | DQ497611 | – |
| <i>Taphrina wiesneri</i> | IFO 7776 | AY548292 | – | DQ479936 | – |
| <i>Schizosaccharomyces pombe</i> | NRRL Y-12796 CBS 356 | DQ442711 | – | EU014774 | – |

CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IAM Institute of Applied Microbiology, University of Tokyo, Japan; IFO Institute for Fermentation, Osaka, Japan; NRRL ARS Culture Collection, Peoria, IL, USA

^a Sequences for *P. inouyei* and *T. wiesneri* are those reported by James et al. (2006)

their D1/D2 sequences, from which Scorzetti et al. (2002) interpreted the presence of two species. Multi-strain comparisons by Fonseca and Rodrigues (2011) for *Taphrina*, which is more closely related to *Saitoella* than species in the preceding examples, have suggested that strains of a *Taphrina* species may show as many as two nucleotide substitutions in D1/D2 and ITS sequences. Thus, separation of *S. complicata* and the proposed new species of *Saitoella* is uncertain from substitutions in D1/D2 and ITS, but analysis of additional gene sequences supports the presence of two species among the *Saitoella* strains examined. The extent of substitutions between the two taxa for EF-1 α and Mt SSU is typical of differences seen for closely related species in the genera *Saccharomyces* (Kurtzman and Robnett 2003) and *Komagataella* (Kurtzman 2009).

In view of the separation of NRRL YB-2330 from *S. complicata* by multigene sequence analysis (Fig. 1; Table 2), and the absence of a demonstrable ascospore state, as described below, the following new species is proposed for the anamorphic genus *Saitoella*.

Latin diagnosis of *Saitoella coloradoensis*
Kurtzman et Robnett, sp. nov.

In agaro YM post dies 3 ad 25°C, cultura est butyrosa et glabra; post dies 10, cultura est butyrosa et salmonea. Cellulae sunt ellipsoideae vel elongatae, 3–4.5 \times 5–8.5 μ m, singulae et binae. Chlamydo sporae

formantur globosae, 6–12 μ m. In agaro morphologico post dies 7 ad 25°C, nec pseudohyphae nec hyphae formantur. Asci non formantur. Glucosum non fermentatur. Assimilantur glucosum, inulinum, sucrosus, raffinosis, trehalosus, maltosus, melezitosis, amyllum solubile, cellobiosus, salicinum, D-xylosus, D-arabiosus (infirmus), ethanolum, glycerolum, D-glucitolium (infirmus), acidum succinicum (infirmus) et potassii nitras. Non assimilantur melibiosus, galactosus, lactosus, methyl- α -D-glucosidus, L-sorbosus, L-rhamnosus, L-arabiosus, D-ribosus, methanolum, erythritolum, ribitolium, galactitolium, D-mannitolium,

Table 2 Extent of nucleotide differences between *S. complicata* and *S. coloradoensis* for gene sequences of D1/D2 LSU rRNA, ITS, EF-1 α and Mt SSU rRNA

| Species pair/ NRRL no. | D1/D2 (575) ^a | ITS (579) ^a | EF-1 α (906) ^a | Mt SSU (574) ^a |
|------------------------------------|-----------------------------|---------------------------|-------------------------------------|------------------------------|
| <i>S. complicata</i> Y-17804 | | | | |
| <i>S. coloradoensis</i> YB-2330 | 4s | 2s | 14s | 56 (24s, 32i) ^b |

^a The number of nucleotides per aligned sequence is given in parentheses following the gene designation. ITS includes ITS1-5.8S-ITS2. Differences between the strains compared include substitutions (s) and indels (i)

^b Based on the sequence alignment used in this study, there are 56 differences between the Mt SSU rRNA gene sequences for the two species. These include 24 substitutions and 32 indels of which three represent single nucleotides, six represent two groups of three and one represents 23 contiguous nucleotides

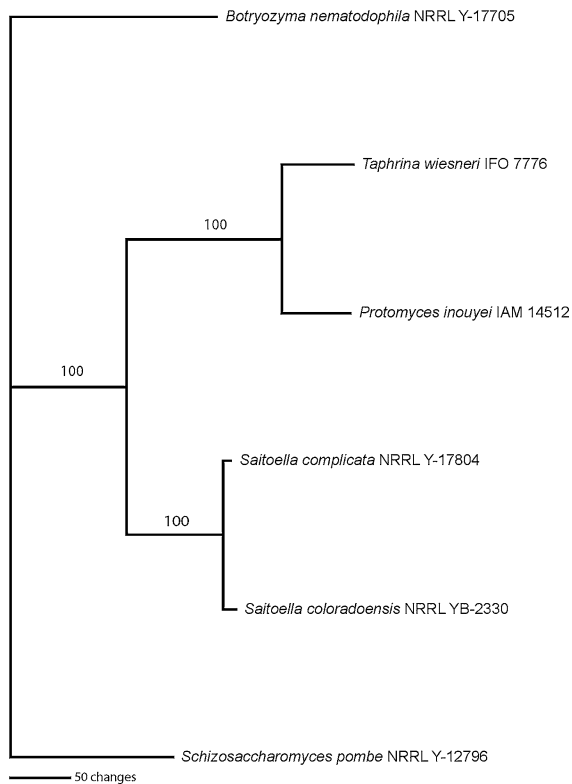


Fig. 1 Phylogenetic relatedness of *Saitoella complicata* and *S. coloradoensis* and their placement near representative species of the neighboring genera *Protomyces* and *Taphrina* as represented by the single most parsimonious tree determined from maximum parsimony analysis of concatenated gene sequences from D1/D2 LSU rRNA and EF-1 α . The analysis included 1,533 characters of which 266 were parsimony informative. Consistency index = 0.879, rescaled consistency index = 0.612, homoplasy index = 0.121. Bootstrap values are from 1,000 replicates. *Schizosaccharomyces pombe* (Taphrinomycotina) was the designated outgroup, but an identical tree resulted when *Botryozyma nematodophila* (Saccharomycotina) was selected as the outgroup species. Neighbor-joining analysis with the Kimura 2-parameter correction gave an identical tree. See text for discussion of phylogenetic placement of *Saitoella* within the Taphinomycotina

myo-inositolum, DL-acidum lacticum, acidum citricum, D-gluconatum, D-glucosaminum, N-acetyl-D-glucosaminum, hexadecanum, 2-keto-D-gluconatum, 5-keto-D-gluconatum, et saccharatum. Amylum non formatur. Vitamina externa ad crescentiam non necessaria. Gelatinum non liquescit; esters non fiunt; pellicula non fiunt. Crescit in medio 10% sodii chloridium/5% glucosum. Augmentum in temperatura 37°C non fiunt. Species nova a speciebus aliis sequentibus nucleotiditis nucleus submonas largus D1/D2 rRNA gene, ITS rDNA, mitochondrial submonas parvus rRNA gene et

factore elongationis-1 α gene distinguenda. Typus: NRRL YB-2330 (CBS 12360) designat stirpem typicam. Isolata a dejectus coleopterorum in *Picea engelmannii*, Meeker, Colorado, USA. Depositata in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

Description of *Saitoella coloradoensis* Kurtzman & Robnett, sp. nov.

Growth on YM agar

After 3 days at 17°C, cells divide by budding, which is polar or nearly polar (Fig. 2). Cells are ellipsoid to elongate, 3–4.5 \times 5–8.5 μ m, and occur singly or as budded pairs. Colony growth is white, butyrous and glistening. After 7–10 days, the growth becomes light pinkish–red, which then darkens to a salmon color. Thick-walled cells, which we have termed chlamydospores, are 6–12 μ m in diameter and form within 3–7 days (Fig. 2).

Cells of *S. complicata* are similar to *S. coloradoensis* in size, 3–4 \times 6–7 μ m, and morphology (Fig. 2). Colony growth is also similar, but cultures of *S. complicata* tend to form the reddish color a few days earlier. Thick-walled chlamydospores, 7–15 μ m in diameter, are also formed by *S. complicata* within 3–7 days (Fig. 2).

Dalmat plate culture on morphology agar

After 7 days at 17°C, growth under the coverglass showed neither hyphae nor pseudohyphae.

Tests for ascospore formation

Saitoella coloradoensis and *S. complicata* were examined for production of ascospores. Cultures of NRRL YB-2330 and NRRL Y-17804 were incubated at 5°, 17° and 25°C on YM, 5% malt extract, RG and water agar for 7 months with initial examination twice weekly for 2 weeks followed by biweekly examination for the remainder of the incubation time. Ascospore formation was not detected in either species. The two taxa were also mixed and incubated at 5°, 17° and 25°C on YM, 5% malt extract, RG and water agar media for 2 months but there was no evidence of conjugation or ascospore formation.

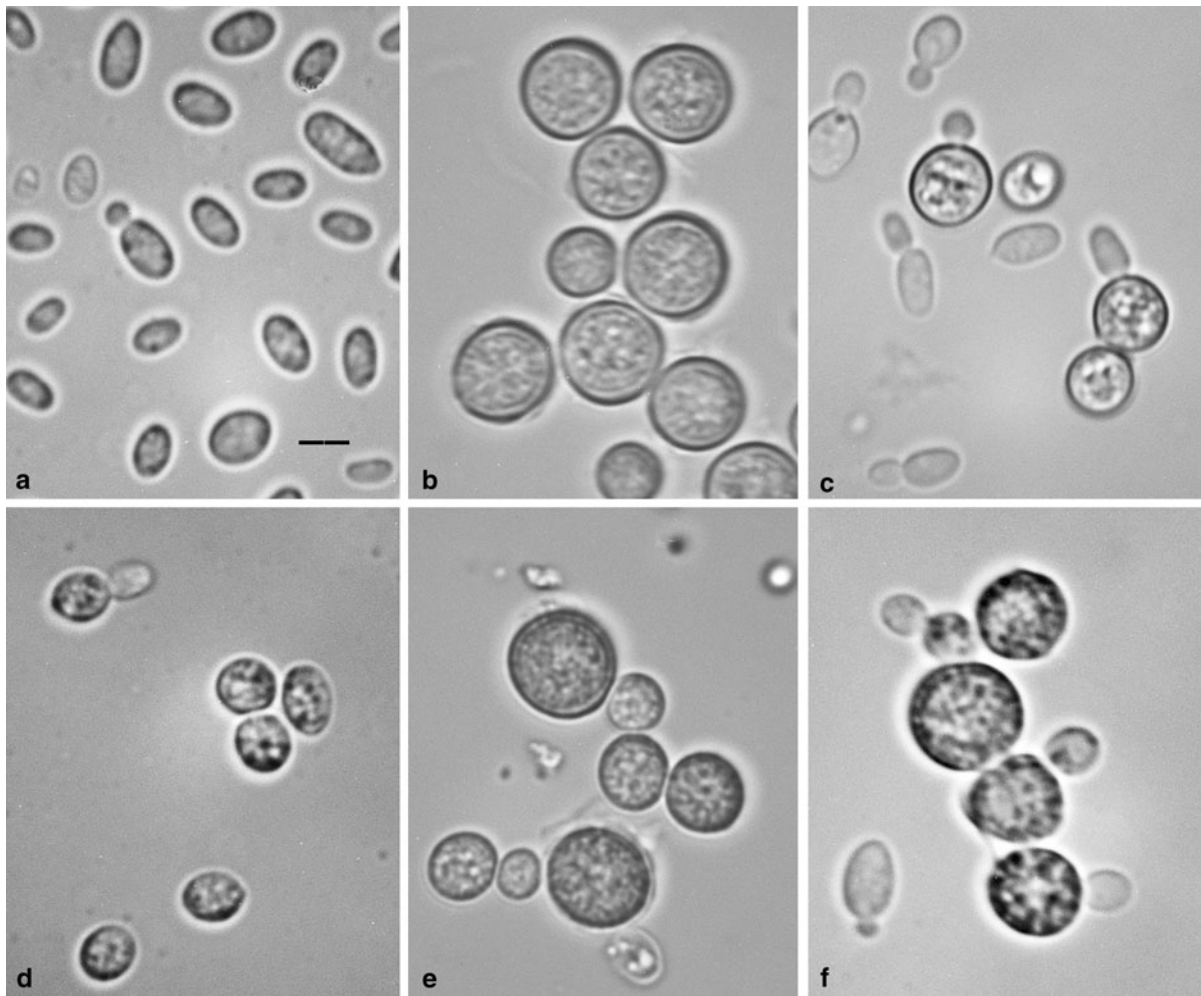


Fig. 2 Cell morphology of *Saitoella coloradoensis* and *S. complicata*. *S. coloradoensis* NRRL YB-2330. **a** Budding cells, YM agar, 3 days, 17°C. **b** Chlamydo-spores, 5% malt extract agar, 5 weeks, 17°C. **c** Budding chlamydo-spores, 1 day, YM agar, 17°C, after transfer of chlamydo-spores from 5% malt extract

agar, 14 weeks, 17°C. Buds from chlamydo-spores have given rise to additional budding cells. *S. complicata* NRRL Y-17804. **d** Budding cells. **e** Chlamydo-spores. **f** Budding chlamydo-spores. Conditions for **d**, **e**, **f**, are the same as for **a**, **b**, **c**, respectively. Bar = 5 µm for all *photographs*

Formation of large, thick-walled chlamydo-spores, which are filled with small lipid droplets, began after 3–10 days incubation at 5°, 17°, 25°, 28° and 32°C (Fig. 2). Chlamydo-spores were formed by both species, but the description of *S. complicata* did not report their presence (Goto et al. 1987). Chlamydo-spores formed most abundantly on 5% malt extract agar followed by YM and RG agars, respectively. Chlamydo-spores that formed at 17°C appeared unchanged after several months of further incubation, but chlamydo-spores formed at temperatures of 25°C or greater showed loss of internal structure after a few weeks followed by breakage of the cell wall. The chlamydo-spores that

formed on 5% malt extract agar at 17°C were transferred after 1 month to YM, 5% malt extract and RG agar media and incubated at 5°, 17° and 25°C for 3 months. Ascospores did not form under these conditions. Usually, 12–24 h after transfer of chlamydo-spores to fresh media, 10–20% of the chlamydo-spores formed ellipsoid to elongate buds that continued to bud producing typical vigorously growing yeast cultures (Fig. 2). Often, the very small oil droplets in the chlamydo-spores coalesced into larger droplets, but pressure applied to the coverglass on the microscope slide under observation caused the chlamydo-spores to rupture and fragment the sometimes cell-like larger

lipid droplets. In an additional attempt to stimulate ascospore formation, chlamydospores from 2 month growth on 5% malt extract agar, 17°C, were inoculated onto water agar slants containing sections of autoclaved leaves from mulberry (dicot) and lily-of-the-valley (monocot) and the branch tips of Colorado blue spruce (monocot) because autoclaved plant material such as sections of carrots, cucumbers and leaves stimulate ascospore formation in some yeasts (Kurtzman et al. 2011). These preparations were incubated at 5°, 17° and 25°C for 4 months, but the only change was occasional bud formation by the chlamydospores. However, for species of *Taphrina* and *Protomyces*, a specific living host plant is required for ascospore formation (Fonseca and Rodrigues 2011; Kramer 1973), and the same may be true for *Saitoella*, if it is a plant pathogen.

Fermentation and growth reactions

The growth reactions for *S. coloradoensis* and *S. complicata* are given in Table 3. Neither species fermented glucose, galactose, maltose, sucrose, lactose or raffinose. Assimilation reactions for the two species differ somewhat allowing their separation from reactions on methyl- α -D-glucoside, D-xylose, D-arabinose, ribitol, D-glucitol and 10% NaCl/5% glucose. Results for *S. complicata* are similar to those reported by Sugiyama and Hamamoto (2011), except that in our tests, growth was absent with mannitol and D-arabinose, but present with soluble starch.

Type

NRRL YB-2330, the type strain, is preserved as a lyophilized preparation in the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and as CBS 12630, with the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The MycoBank accession number for this strain is 563858. The strain had been isolated by L. J. Wickerham from insect frass in an Engelmann spruce growing in Colorado, USA, and remained unidentified until the present study.

Etymology

The species name *coloradoensis* was selected to denote the origin of the type strain from the U.S. State of Colorado.

Table 3 Comparative physiological characteristics of *Saitoella complicata* and *S. coloradoensis*

| Physiological test ^a | <i>S. complicata</i> NRRL Y-17804 | <i>S. coloradoensis</i> NRRL YB-2330 |
|---------------------------------|--------------------------------------|---|
| Fermentation: absent | | |
| <i>Growth</i> | | |
| Glucose | + | + |
| Inulin | + | + |
| Sucrose | + | + |
| Raffinose | + | + |
| Melibiose | – | – |
| Galactose | – | – |
| Lactose | – | – |
| Trehalose | + | + |
| Maltose | + | + |
| Melezitose | + | + |
| Methyl- α -D-glucoside | + | – |
| Soluble starch | + | + |
| Cellobiose | + | + |
| Salicin | + | + |
| L-Sorbose | – | – |
| L-Rhamnose | – | – |
| D-Xylose | – | + |
| L-Arabinose | – | – |
| D-Arabinose | – | w |
| D-Ribose | – | – |
| Methanol | – | – |
| Ethanol | + | + |
| Glycerol | + | + |
| Erythritol | – | – |
| Ribitol | + | – |
| Galactitol | – | – |
| D-Mannitol | – | – |
| D-Glucitol | – | w |
| <i>myo</i> -Inositol | – | – |
| DL-Lactate | – | – |
| Succinate | w | w |
| Citrate | – | – |
| D-Gluconate | – | – |
| D-Glucosamine | – | – |
| <i>N</i> -Acetyl-D-glucosamine | – | – |
| Hexadecane | – | – |
| Nitrate | + | + |
| Vitamin-free | + | + |
| 2-Keto-D-gluconate | – | – |
| 5-Keto-D-gluconate | – | – |
| Saccharate | – | – |

Table 3 continued

| Physiological test ^a | <i>S. complicata</i> NRRL Y-17804 | <i>S. coloradoensis</i> NRRL YB-2330 |
|---------------------------------|--------------------------------------|---|
| 10% NaCl/5% glucose | – | + |
| Starch formation | – | – |
| Gelatin liquefaction | – | – |
| Growth at 32°C | + | + |
| Growth at 37°C | – | – |

^a – negative, + positive, w weak

The genus *Saitoella* was first shown from single gene phylogenetic analyses to be a member of the Taphrinomycotina (Nishida and Sugiyama 1993; Kurtzman and Robnett 1998; Kurtzman and Sugiyama 2001), and this placement was supported by multigene sequence analysis (Sugiyama et al. 2006). These analyses suggest, as indicated in Fig. 1, that *Saitoella* appears closely related to *Protomyces* and *Taphrina*. The latter two genera are plant pathogens that are only known to form an ascospore state in the tissue of their hosts, but they produce a budding asexual state that is easily grown on agar media and the colonies become pigmented pinkish or red as also seen for *Saitoella* (Goto and Sugiyama 1970; Goto et al. 1987). For *Taphrina*, dikaryotic subcuticular hyphae diploidize and asci are directly produced on the hyphae by some species, but for other species asci arise from thick-walled ascogenous cells (Mix 1949; Kramer 1973; Fonseca and Rodrigues 2011). Species of *Protomyces* form chlamydospore-like ascogenous cells in host tissue that germinate to form multispored asci (Reddy and Kramer 1975). Pavgi and Mukhopadhyay (1969) reported formation of chlamydospores on agar media by *Protomyces macrosporus* but ascospore formation did not occur. Whether the chlamydospores formed by *Saitoella* are environmentally resistant asexual cells or analogous to the morphologically similar ascogenous cells typically produced by *Protomyces* and some species of *Taphrina* in host plant tissue is unknown, but their formation raises the possibility that *Saitoella* may be a plant pathogen. Many of the described species of *Protomyces* are known only from herbarium specimens and their identity is uncertain, which raises the possibility that some of these taxa may be misidentified species of *Saitoella*. However, testing this hypothesis will require sequence analysis of all described *Protomyces* species as well as sequence

analysis of the poorly characterized plant pathogenic genera *Burenia*, *Protomyces*, *Taphridium* and *Volkartia*, which are not known from culture, but are morphologically similar to *Protomyces* (Reddy and Kramer 1975; Kurtzman 2011). Of still further interest concerning the possible habitat of *Saitoella* is the study of Allison et al. (2010). In this work, DNA was cloned from soil sampled in an Alaskan (USA) boreal forest. Of the 433 fungal sequences obtained, three (GU212336, GQ892426, GQ892425) were identical to the D1/D2 LSU rRNA gene sequence of *S. complicata*.

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