North American Fungi

Curvibasidium rogersii, a new yeast species in the Microbotryomycetes

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Abstract: *Curvibasidium rogersii sp. nov.* (Microbotryomycetes, Basidiomycota) is described on the basis of a strain (NRRL Y-48849) isolated from wine grapes (*Vitis vinifera*) in Washington state (USA). The taxonomic position of the strain was determined through analysis of the ITS region and the D1/D2 domains of the LSU rRNA gene. Physiological and morphological data also are presented. A basidial state was not observed.

Key words: anamorphic yeast, enology, Pacific Northwest fungi, viticulture, wine microbiology, yeast systematics, yeast taxonomy

Introduction: During a survey of yeasts occurring on wine grapes (*Vitis vinifera* L.) in central Washington state (USA), a strain was isolated that could not be assigned to any described species. Phylogenetic analysis of D1/D2 large subunit (LSU) and ITS region of nuclear ribosomal DNA (rDNA) indicated that the strain is a member of the *Curvibasidium* clade of class Microbotryomycetes (Basidiomycota: Pucciniomycotina). Herein the fungus is described as a new species of *Curvibasidium*. This paper describes morphological and physiological characteristics of this species and results of phylogenetic analyses of the D1/D2 and ITS regions.

Materials and Methods: The fungus was isolated from a sample consisting of five 'Riesling' (*Vitis vinifera* L.) grape bunches collected on September 14, 2010 from an experimental vineyard at the Irrigated Agriculture Research and Extension Center (IAREC) near Prosser, Benton County, Washington state, USA. Berries were chilled on ice until they were macerated. Macerate was streaked onto Wallerstein Labs nutrient agar (WLNA) (BD, Franklin Lakes, NJ, USA) supplemented with 100 mg/L streptomycin sulfate (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). The isolation plate was incubated at 20 – 22 °C for 5-7 days before culture purification on potato dextrose agar (PDA) (BD, Franklin Lakes, NJ, USA).

For morphological characterization, NRRL Y-48849 was grown on PDA for 7 days at 20 - 22 °C. Morphological features were characterized as described in Kurtzman et al. (2011). Photomicrographs were obtained using bright-field illumination with a Zeiss Axioskop 40 microscope and an Axiocam HRc camera using Axiovision software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

Physiology tests were performed using methods adapted from Kurtzman et al. (2011), based on the methods first outlined by Wickerham (1951).

Carbon assimilation tests were performed with 5-ml of liquid media in polypropylene 17 x 100 mm test tubes, using 36 carbon sources. Nitrogen assimilation tests of nitrate, L-lysine and cadaverine were carried out on agar cultures and in liquid media. Additional tests of 25 and 30 °C, 0.01% w/v cycloheximide and 10% w/v NaCl were performed in 5 ml of liquid media. The following strains from the USDA ARS Culture Collection (NRRL) (Peoria, IL, USA) were used to calibrate the tests (equivalent CBS strain designations are also listed): *Candida oleophila* (type strain), NRRL Y-2317^T = CBS 2219^T; *Candida oleophila*, NRRL Y-1880 = CBS 2220; *Candida railenensis*, NRRL Y-2319 = CBS 2223; *Candida saitoana* (type strain), NRRL Y-17316^T = CBS 940^T; *Metschnikowia chrysoperlae* (type strain), NRRL Y-27615^T = CBS 9803^T; *Rhodotorula nothofagi*, NRRL Y-17176^T = CBS 8166^T; and *Sporobolomyces kluyveri-nielii* (type strain), NRRL Y-17211^T = CBS 7168^T. Both the D-glucose assimilation and 25 °C growth tests were conducted without negative controls. All physiology tests were performed in triplicate, and results were confirmed at least once.

DNA was extracted using a "FastDNA® Spin Kit" (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's instructions. PCR to amplify ITS and D1-D3 LSU regions were conducted using the following primer combinations ITS1 (TCCGTAGGTGAACCTGCGG; White et al., 1990) and ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990); ITS5 (GGAAGTAAAAGTCGTAACAAGG; White et al., 1990) and ITS4; ITS5 and LR3 (CCGTGTTTCAAGACGGG; Vilgalys and Hester, 1990); and NL1 (GCATATCAATAAGCGGAGGAAAAG; Kurtzman and Robnett, 1998) and TW14 (GCTATCCTGAGGGAAACTT; Hamby et al. 1988). 25 μl reactions containing 5.0 μl of DNA template (10.0 ng/μl), 1.0 μl each of forward and reverse primers (4.0 pmol/μl), 4.0 μl of dNTP, 5.0 μl of 5x GoTaq ® Flexi Buffer (Promega, Madison, WI, USA), 1.5 μl at MgCl$_2$ (25 mmol/μl)

(Promega, Madison, WI, USA) and 0.5 µl of GoTaq ® Taq Polymerase (Promega, Madison, WI, USA) were used. PCR cycles consisted of a 4-min initial denature at 94 °C, followed by denaturing for 30-sec at 94 °C, annealing for 30 sec at 54 °C, extension for 1-min at 72 °C and a final 10 min 72 °C extension. PCR products were visualized using a 1.4% agarose gel and cleaned using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA, USA). Products were sequenced using PCR primers by ELIM Biopharm (Hayward, CA, USA). Sequencing was performed with ABI 3730 (Life Technologies, Carlsbad, CA, USA) sequencers using dye terminator methods. Chromatograms resulting from sequencing reactions were analyzed using the Chromaseq 1.0 plugin to Mesquite 2.75 (Maddison and Maddison, 2011a; Maddison and Maddison, 2011b), using the programs phred 0.071220.c (Green and Ewing, 2002) and phrap 1.090518 (Green, 2009) to call bases, assemble contigs, and assess quality. The resulting sequence was deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession JX188232.

An alignment consisting of both ITS and D1/D2 sequences from type strains of the *Curvibasidium* clade was assembled using results from Sampaio (2011a). Separate ITS and D1/D2 alignments were constructed from sequences related to NRRL Y-48849 that were identified from BLAST searches of GenBank (Altschul, 1990). The ITS sequence used for the type strain of *Leucosporidium fasciculatum,* CBS 8786[T] was obtained from the CBS database. The type strain of *Rhodotorula ingeniosa* was included in all alignments and used to root all resulting trees. Sequences were aligned using the rmcoffee mode of the program T-Coffee (Notredame et al., 2000) and edited manually. Trees were inferred from each alignment using neighbor joining (NJ) and maximum likelihood (ML) methods. MEGA 5.05 (Tamura et al., 2011) was used for NJ analysis and GARLI 2.0 (Zwickl, 2006) for ML. One thousand bootstrap replications were used for both methods. ITS and D1/D2 loci were analyzed

as separate partitions during NJ. Model choice was based on Akaike Information Criterion (AIC) (Akaike, 1974) rank using the program jModeltest (Posada, 2008; Guindon, 2003). Models are referred to using acronyms in Posada (2009). Support values are presented as percentage bootstrap scores. The scale for the ML tree is substitutions per site. Trees were visualized and rooted using the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and annotated using GIMP®.

Results: The D1/D2 sequence of NRRL Y-48849 differed by 6 bp from the type strain of the most closely-related species, *C. cygneicollum.* ITS sequences from NRRL Y-48849 and the type strain of *C. cygneicollum* differed by 8 bp. In phylogenetic analyses (Figs. 2, 3), NRRL Y-48849 is well separated from all other members of the *Curvibasidium* clade which includes several species currently classified as *Rhodotorula.* These results suggest strongly that NRRL Y-48849 represents a distinct, previously undescribed species. Physiological tests can be used to separate this species from other members of the *Curvibasidium* clade, but the differences are few and may be unreliable with inclusion of additional strains of each species. Consequently, recognition of this species should be based on diagnostic gene sequences. In view of the nucleotide divergence observed between NRRL Y-48849 and other members of the *Curvibasidium* clade, the following new species is described.

Curvibasidium rogersii Tyler B. Bourret and Dean A. Glawe *sp. nov.*
Mycobank number: MB802630

Etymology: *Curvibasidium rogersii* (ro.gers´i.i. N.L. gen. masc. sing. n. *rogersii* of Rogers, in honor of Professor J. D. Rogers for his contributions to fungal systematics).
Cultura in agaro "PDA" post dies septem ad 20-22C alba aut cremea, butyrosa, semi-nitens et

marginibus integris. Cellulae ovoideae aut cylindraceae, (2.5-)3.5-7.0 μm x 2.0-3.0(-3.5) μm, per germinationem polarem reproducentes. Hyphae et pseudohyphae non formantur. D-*glucosum,* D-*galactosum,* L-*sorbosum,* D-*xylosum,* L-*arabinosum,* D-*arabinosum (tarde), cellobiosum, salicinum, ribitolum, xylitolum,* D-*glucitolum,* D-*mannitolum, galactitolum,* D-*glucono-1,5-lactonum, 2-keto-D-gluconas (variabiliter),* D-*gluconas (variabiliter), acidum succinicum, ethanolum et acidum gallicum assimilantur.* D-*glucosaminum,* D-*ribosum,* L-*rhamnosum, sucrosum, maltosum, trehalosum, methyl α-D-glucosidum, melibiosum, lactosum, raffinosum, amylum, erythritolum, inositolum,* D-*acidum glucuronicum,* DL-*acidum lacticum, acidum citricum et acidum m-hydroxybenzoicum non assimilantur.* L-*lysinum assimilantur. Nitratum et cadaverinum non assimilantur. 25C neque 30C crescit. 0.01% cycloheximido et 10% NaCl non crescit.*

After 7 days on PDA at 20-22 °C, colonies are white or cream-colored, butyrous, moderately glossy with entire margins. Cells are ovoid to cylindrical, (2.5-)3.5-7.0 μm x 2.0-3.0(-3.5) μm; budding is polar (Fig. 1). Hyphae or pseudohyphae not produced. D-glucose, D-galactose, L-sorbose, D-xylose, L-arabinose, D-arabinose (slow), cellobiose, salicin, ribitol, xylitol, D-glucitol, D-mannitol, galactitol, D-glucono-1,5-lactone, 2-keto-D-gluconate (variable), D-gluconate (variable), succinate, ethanol and gallic acid are assimilated as carbon sources. No growth occurred with D-glucosamine, D-ribose, L-rhamnose, sucrose, maltose, trehalose, methyl α-D-glucopyranoside, melibiose, lactose, raffinose, starch, erythritol, inositol, D-glucuronate, DL-lactate, citrate, and m-hydroxybenzoate. L-lysine was assimilated as a nitrogen source. Growth was absent with nitrate or cadaverine. Growth occurred at 25 °C, but not 30 °C. No growth was present with 0.01% cycloheximide or 10% NaCl. Comparison of physiological tests of C. rogersii with those of *Rhodotorula nothofagii, Curvibasidium* species

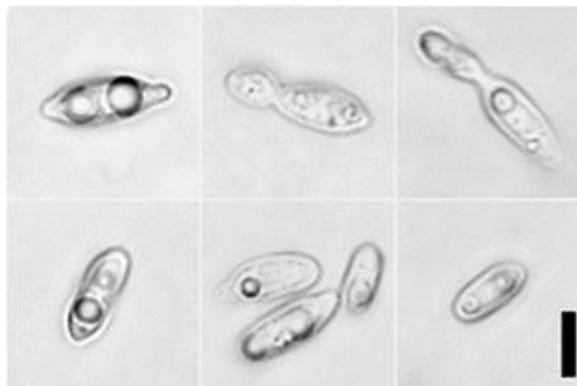

Fig. 1. Cells of *Curvibasidium rogersii* sp. nov. NRRL Y-Y-48849 after 7 days in PDA at 20-22 C. Scale bar = 5 μm.

and *Leucosporidium fasciculatum* are shown in Table 1.

Type strain: NRRL Y-Y-48849[T] (=CBS 12746[T]), isolated by T. Bourret in September 2010 from *Vitis vinifera* L. 'Riesling' at the Washington State University Irrigated Agriculture Research and Extension Center, Prosser, WA, USA. The type strain is preserved permanently in a metabolically inactive state in 10% glycerol solution at -180 °C in the ARS Culture Collection (NRRL), Peoria, Illinois, USA. Sequences of the D1/D2 and ITS rDNA loci of NRRL Y-48849 were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession JX188232.

Discussion: Sequence data supported recognition of *C. rogersii* as a distinct species and its assignment to *Curvibasidium*. The genus *Curvibasidium* was erected by Sampaio et al. (2004) to accommodate sexual states of two yeasts of the Microbotryomycetes, *C. cygneicollum* Sampaio & Golubev [anamorph: *Rhodotorula fujisanensis* (Soneda) Johnson & Phaff] and *C. pallidicorallinum* Sampaio & Golubev. Teliospores of the two species germinate to form holobasidia, a feature of yeasts otherwise unknown outside of the Agaricomycotina (Sampaio 2011). *Rhodotorula*

	C. rogersii	*C. cygneicollum*	*C. pallidicorallinum*	*R. nothofagi*	*L. fasciculatum*
D-Ribose	-	v	v	+/s	-
Trehalose	-	v	-	-	+
Raffinose	-	-	+/w	-	+
2-Keto-D-Gluconate	+/ws	-	v	-	-
D-Gluconate	+/ws	+/s	+	-	-
Citrate	-	+/w	v	+	+
Cadaverine	-	v	-	+	+
Cycloheximide 0.01%	-	v	+	+	-

Table 1. Comparison of physiological tests of *Curvibasidium rogersii* with those of *Rhodotorula nothofagii, Curvibasidium* species and *Leucosporidium fasciculatum*. +=growth; -=no growth; w=weak growth; s=slow growth. Data for species other than *C. rogersii* were obtained from Sampaio et al. (2004 and 2011) and the CBS database.

nothofagi was maintained, despite 100% D1/D2 sequence similarity with *C. pallidicorallinum* because the two species cannot be crossed and can be distinguished on the basis of ITS sequences and nuclear DNA-DNA reassociation data (Sampaio and Golubev, 2004; Sampaio, 2011b).

The D1/D2 sequence of *C. rogersii* differed by 6 bp from that of the most closely-related species, *C. cygneicollum*, while the ITS sequences differed by 8 bp. Fell et al. (2000) suggested that a 2 bp D1/D2 difference can be sufficient to distinguish species of basidiomycetous yeasts. In the *Curvibasidium* clade, *C. pallidicorallinum* and *Rhodotorula nothofagi* were regarded as distinct despite exhibiting 100% identical D1/D2 sequences to each other and a single ITS substitution (Sampaio, 2011a). The relationship between *Curvibasidium rogersii* and previously described species of *Curvibasidium* differed between phylogenetic trees. It was resolved most clearly in the combined tree which placed it in a clade with *C. cygneicollum* with strong support (Fig. 2). NJ and ML D1/D2 trees (Fig. 3) also supported separation of *C. rogersii* from other described members of the clade.

In conclusion, *C. rogersii* is a newly-recognized species and appears to be the first member of the *Curvibasidium* clade to have been found in the Pacific Northwest of the United States. Its discovery on berries from grapevine, possibly one of the most studied substrates in terms of native yeast flora (Barata et al. 2012), and in a region with a long history of mycological work by grape pathologists and others (eg., Sprague, 1953; Dugan et al., 2002), is a reminder that we still have much to learn about the diversity of yeasts on economically significant hosts. Further work is underway to assess the possible significance of this species to grape growers and enologists.
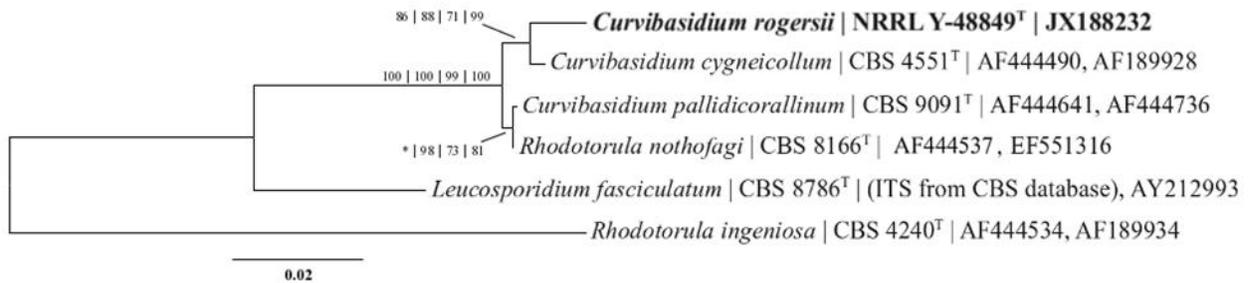
Fig. 2. Maximum likelihood ITS + D1/D2 tree of type strains in the *Curvibasidium* clade, including support values from (left and right, respectively) neighbor joining, and maximum likelihood (TVM+G, ITS; TrN+I, D1/D2). Tree is rooted with the type strain of *Rhodotorula ingeniosa*. [T] denotes type strain.
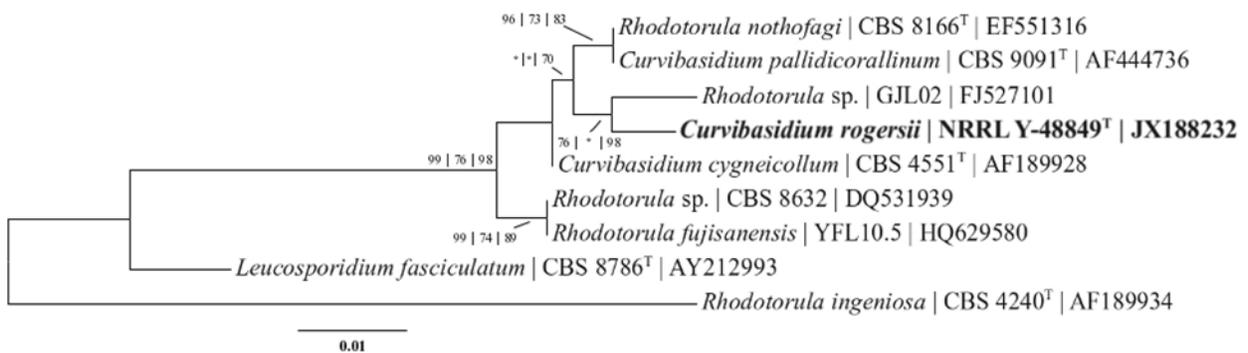
Fig. 3. Maximum likelihood D1/D2 tree of the *Curvibasidium* clade based on available GenBank data, including support values from (left and right, respectively) neighbor joining and maximum likelihood (TrN+I) trees. [T] designates type strain. Binomials for non-type strains were included with sequence data retrieved from GenBank and should be considered provisional until verified.

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