Rhodotorula subericola sp. nov., an anamorphic basidiomycetous yeast species isolated from bark of *Quercus suber* (cork oak)

C. Belloch,[1] M. Villa-Carvajal,[2] M. L. Álvarez-Rodríguez[3] and J. J. R. Coque[3]

Correspondence
C. Belloch
belloch@iata.csic.es

[1]Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, 46100 Burjassot, Valencia, Spain

[2]Centro Tecnológico AINIA, Parque Tecnológico de Valencia, 46980 Paterna, Valencia, Spain

[3]Instituto de Biotecnología de León (INBIOTEC), Parque Científico de León, 24006 León, Spain

Two yeasts strains, Y-31^T and Y-20B, pertaining to a previously unknown yeast species were isolated from bark of cork oak in Spain. Physiological characterization revealed a pattern of assimilation of carbon and nitrogen compounds compatible with members of the genus *Rhodotorula*. From sequence analysis of the D1/D2 region of the 26S rRNA gene, *Rhodotorula cycloclastica* and *Rhodotorula philyla* were related to the unknown species. Phylogenetic reconstruction based on the D1/D2 region of the 26S rRNA gene showed that the novel species clustered in a branch together with *R. cycloclastica*. The name *Rhodotorula subericola* sp. nov. is proposed, with isolate Y-31^T (=CECT 11976^T=CBS 10442^T) the type strain of this novel taxon in the *Microbotryum* lineage, subclass Microbotryomycetidae, class Urediniomycetes of basidiomycetous yeasts.

During a survey of microbial biota in the manufacturing process of cork stoppers in Spain, several bacteria, yeasts and filamentous fungi were isolated and identified (Álvarez-Rodríguez *et al.*, 2002, 2003; Villa-Carvajal *et al.*, 2004). Physiological characterization and RFLPs of the ITS–5.8S rDNA region indicated that yeast isolates Y-31^T and Y-20B represented unique yeasts. Analysis of the 26S rRNA D1/D2 domain sequences of strains Y-31^T and Y-20B showed them to be identical and that they could not be ascribed to a recognized species. BLAST analysis yielded the highest similarity value, 95 %, with three strains of *Rhodotorula* sp. CBS 8445^T, CBS 8446^T, CBS 8447 and CBS 8448^T, and the species *Rhodotorula philyla*, therefore Villa-Carvajal *et al.* (2004) concluded that isolates Y-31^T and Y-20B represented a novel species in the genus *Rhodotorula* although formal description was not given. Scorzetti *et al.* (2002) placed these *Rhodotorula* species in a tight cluster in the *Microbotryum* lineage and, recently, Thanh *et al.* (2004) described *Rhodotorula cycloclastica*, *Rhodotorula retinophila* and *Rhodotorula terpenoidalis* to accommodate CBS 8447 and CBS 8448^T, CBS 8446^T and CBS 8445^T, respectively.

In this study, phenotypic characterization of strains Y-31^T and Y-20B and phylogenetic analysis based on the sequences of the D1/D2 region of the 26S rRNA gene and ITS–5.8S rDNA region were performed. The name *Rhodotorula subericola* sp. nov. is proposed to accommodate these two strains.

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA and ITS–5.8S rRNA gene sequences of strain CECT 11976^T are AY296052 and DQ870625, respectively.

Collection site and isolation of the strains

Cork was sampled in the province of Badajoz (Extremadura, Spain). Badajoz is in the west of Spain and occupies a surface area of 1500 km^2. The weather is continental with influence from the Atlantic Ocean, characterized by mild winters with minimum mean temperatures of 3.2 °C and maximum mean temperatures of 13.9 °C. Summers are warm with minimum mean temperatures of 17.0 °C and maximum mean temperatures of 34.3 °C. Badajoz has a rainy season from October to April with precipitation between 50 and 80 mm, while the rest of the year it is below 30 mm. Cork is produced by *Quercus suber* L. (Fam. *Cupuliferae*) and consists of the exterior layers of the bark beneath the epidermis, which develop extraordinarily in these species, becoming thick and of the peculiar spongy consistency which characterizes cork. Cork is extracted every 11 years from the trees. Then, cork planks are stored in the fields for some time, even years, before they are used to make cork stoppers.

Yeast strains Y-31^T and Y-20B were isolated in Spain from bark of cork oak at the initial stages of the manufacturing process of cork stoppers (Álvarez-Rodríguez *et al.*, 2003).

Phenotypic characterization of yeast isolates

Morphology of the yeasts was studied using a light microscope (Nikon Eclipse E800). Physiological and morphological characterization of the strains was performed according to standard methods (Yarrow, 1998).

Sequencing and phylogenetic analysis

Yeast cells picked from 48-h-old colonies were directly used in PCR reactions. The D1 and D2 domains of the 26S rRNA gene were amplified using the external primers NL-1 and NL-4 (O'Donnell, 1993), and the internal transcribed spacers ITS and the 5.8S ribosomal gene were amplified using the external primers ITS-1 and ITS-4 (White *et al.*, 1990). PCR reactions were performed in a PROGENE thermocycler (Techne) as follows: a first denaturation step at 95 °C for 5 min, followed by 40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 30 s, with a final extension of 10 min at 72 °C. The PCR products were cleaned with the Perfectprep Gel Cleanup (Eppendorf) and then directly sequenced using the BigDye terminator version 3.1 cycle sequencing kit (Applied Biosystems), following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer, model 310. Primers NL-1 and NL-4 were used in the sequencing reactions to read both DNA strands of D1 and D2 domains of the 26S rRNA gene, and primers ITS-1 and ITS-4 were used in the sequencing reactions to read both DNA strands of the ITS1–5.8S–ITS2 rDNA region.

Sequences of the D1/D2 region of the 26S rRNA gene were edited and assembled using MEGA version 3.1 software (Kumar *et al.*, 2004), and then subjected to a GenBank BLASTN search to retrieve sequences of closely related taxa.

The sequences of the D1/D2 region of the 26S rRNA gene were included in a multiple alignment generated using MEGA version 3.1. The Kimura two-parameter model was used for distance correction, and the neighbour-joining method (Saitou & Nei, 1987) was used for phylogenetic inference. Support for tree branches was evaluated by bootstrap analysis from 1000 heuristic searches (MEGA version 3.1). Sequence AF189901 representing the D1/D2 ribosomal region of *Kondoa aeria* CBS 8352^T was used as the tree outgroup.

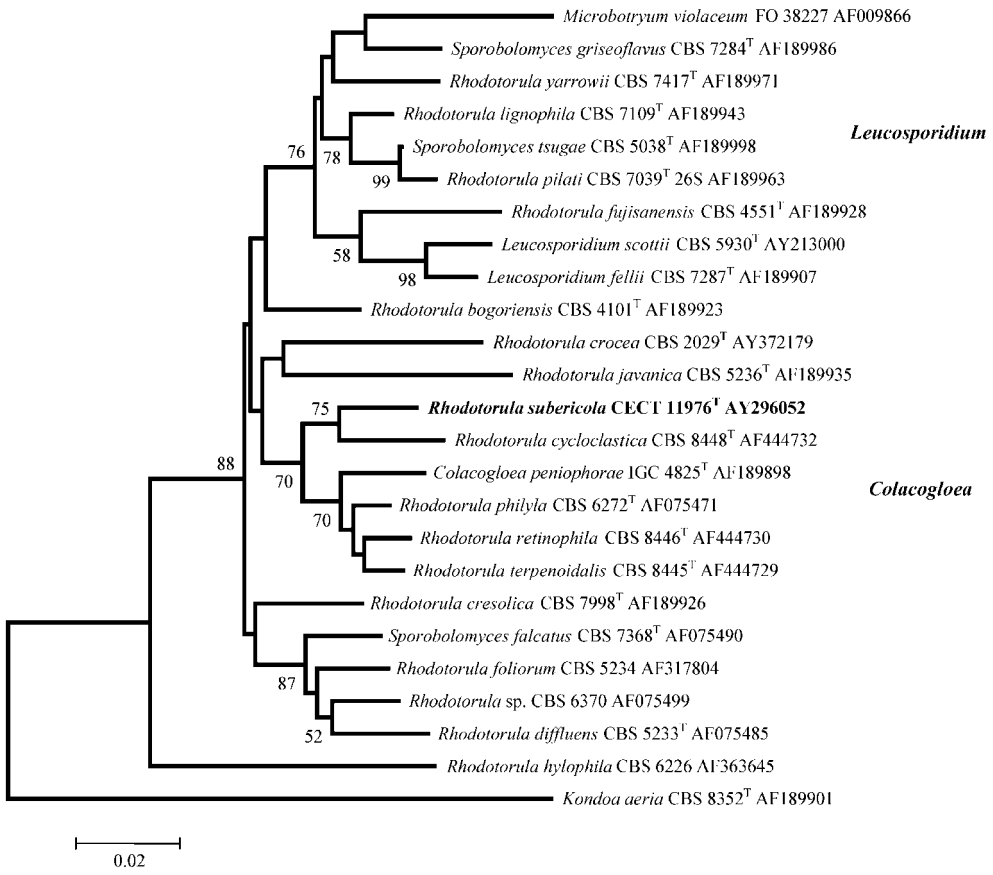

Fig. 1. Phylogenetic tree reconstructed using the neighbour-joining method, depicting relationships between species in the *Microbotryum* lineage. The tree was constructed based on nucleotide divergences in the D1/D2 region of the 26S rRNA gene using the program MEGA 3.1. Bootstrap values for frequencies less than 50 % are not given. GenBank accession numbers of sequences are given after the species names. Outgroup: *Kondoa aeria*.

Phylogenetic position of Y-31^T and Y-20B and phenotypic comparison with closely related species

Sequence variability within Y-31^T and Y-20B was absent in the D1/D2 region of the 26S rRNA gene and the ITS–5.8S rDNA region.

Phylogenetic analysis based on the D1/D2 region sequences indicated that the closest relative (75 % bootstrap) to *R. subericola* was *R. cycloclastica* (Fig. 1). Alignment of the D1/D2 region sequences of the novel isolates and related taxa revealed 25 bp substitutions in 644 positions (96 % sequence identity) between the novel isolates and *R. cycloclastica*. These species cluster in a group (70 % bootstrap) with *R. retinophila*, *R. terpenoidalis*, *Colacogloea peniophorae* and *R. philyla* in the *Microbotryum* lineage. The *Microbotryum* lineage pertains to the subclass Microbotryomycetidae in the class Urediniomycetes of basidiomycetous yeasts (Scorzetti *et al.*, 2002). The class Urediniomycetes is divided into four lineages: *Microbotryum*, *Sporidiobolus*, *Erythrobasidium* and *Agaricostilbum*. Scorzetti *et al.* (2002) divided the *Microbotryum* lineage into two weak clades, namely *Colacogloea* and *Leucosporidium*. Phylogenetic reconstructions including most of the members in the *Microbotryum* lineage (Alvaro Fonseca, 2004) produced a tree similar to the one reconstructed by Scorzetti *et al.* (2002). In the present study, the *Microbotryum* lineage appears to be divided into three clusters, *Leucosporidium* (76 % bootstrap), *Colacogloea* (70 % bootstrap) and a third cluster (87 % bootstrap) composed of the species *Sporobolomyces falcatus*, *Rhodotorula foliorum*, *Rhodotorula* sp. CBS 6370 and *Rhodotorula diffluens*. The lineage *Microbotryum* includes, in addition to members of the genera *Rhodotorula*, species from the genera *Sporobolomyces*, *Leucosporidium* and *Colacogloea*. Other members of the polyphyletic anamorphic genus *Rhodotorula* can also be found in the lineages *Erythrobasidium* and *Sporidiobolus* Scorzetti *et al.* (2002).

Physiological differences between the members of the cluster where Y-31^T and Y-20B were included ranged from four to eight properties (Table 1). Y-31^T and Y-20B were the only members of the group able to use galactose as sole carbon source and nitrate as sole nitrogen source. Y-31^T and Y-20B were investigated for basidiospore formation on cornmeal agar, 5 % malt extract agar and sucrose-yeast extract agar, but after 6 weeks of growth and observation no basidiospores were found. A loopful of cells of each isolate was mixed on a plate of cornmeal agar to obtain a dikaryotic hyphal phase, but after several days of observation no hyphal growth was detected, therefore we concluded that mating did not occur between the two isolates.

Table 1. Physiological properties differentiating *R. subericola* sp. nov. from phylogenetically related species

1, *R. subericola* sp. nov.; 2, *R. cycloclastica*; 3, *R. retinophila*; 4, *R. terpenoidalis*; 5, *R. philyla*. +, Positive; −, negative; V, variable; S, slow; W, weak. Physiological properties common to all species were assimilation of D-glucose, trehalose, succinate, citrate, glucitol, mannitol, ribitol, ethanol and urease, and all grew on vitamin-free medium. All species were negative for assimilation of sorbose, cellobiose, lactose, melibiose, raffinose, L-arabinose, D-ribose, D-glucosamine, inulin, soluble starch, L-rhamnose, glycerol, erythritol, galactitol, inositol, methanol and salicin. No species produced deeply pigmented colonies (orange, pink or red), produced starch-like compounds or grew on NaCl plus 5 % glucose.

Characteristic	1	2*	3*	4*	5†
Assimilation of:					
D-Galactose	+	−	−	−	−
Maltose	−	−	−	+	−
Sucrose	+	−	−	+	−
Melezitose	−	−	−	+	−
D-Arabinose	S	−	+	+	−
D-Xylose	+	−	+	+	V
DL-Lactate	−	−	W	W	−
D-Gluconate	+	−	+	+	+
Methyl α-D-glucoside	−	−	−	+	−
D-Glucuronate	+	−	+	−	+
Nitrate	+	−	−	−	−
Growth at 37 °C	−	−	+	W	−

*Data from Thanh *et al.* (2004).
†Data from Fell & Statzell-Tallman (1998).

Latin diagnosis of *Rhodotorula subericola* Belloch, Villa-Carvajal, Álvarez-Rodríguez *et* Coque sp. nov.

In medio liquido dextrosum et peptonum et extractum levidinis continenti post 3 *dies ad* 25 °C *cellulae sunt singulae aut binae, ovoidae aut cylindricae*, 1.5–3 × 3.5–7 μm. *Post unum mensem sedimentum adest. In agaro malti cultura aquosa mucosescens vel glutinosescens, partim cremea, impellucida, glabra, nitida. Pseudomycelium non formantur. Fermentatio*

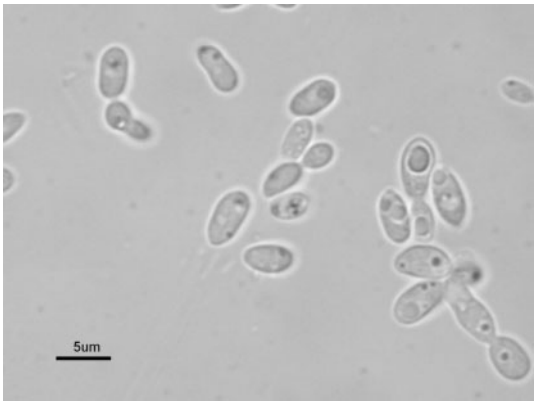

Fig. 2. Vegetative cells of Y-31^T (=CECT 11976^T) grown on malt extract for 3 days at 25 °C. The image was taken with a digital Nikon DXM1200F camera under light field mode. Bar, 5 μm.

nulla. D-*Glucosum*, *sucrosum*, α-*trehalosum*, D-*glucitolum*, D-*mannitolum*, *ribitolum*, *ethanolum*, D-*gluconatum*, D-*glucosaminum*, *N*-*acetyl*-D-*glucosaminum*, D-*glucuronatum*, *citratum*, *succinatum*, *xylitolum assimilantur*, *at non* D-*galactosum*, L-*sorbosum*, *cellobiosum*, *lactosum*, *maltosum*, *melibiosum*, *melezitosum*, *raffinosum*, *inulinum*, *amylum solubile*, D-*arabinosum*, L-*arabinosum*, D-*ribosum*, D-*xylosum*, L-*rhamnosum*, *glycerolum*, *erythritolum*, L-*arabinitolum*, *galactitolum*, *meso*-*inositolum*, *methanolum*, DL-*lactatum*, *methyl* α-D-*glucosidum*, *salicinum*, *hexadecanum nec saccharatum. Glucosminum*, L-*lysinum*, *natrium nitricum et natrium nitrosum assimilantur neque ethylaminum et imidazolum. Sine vitaminis externis supplementis crescens. Maxima temperatura crescentiae*: 30 °C. *Gelatinum non liquescit et arbutinum non finditur. Acidum non formantur. Non crescit in* 50 % *glucoso. Materia amyloidea non formantur. Ureum hydrolysatur.*

Typus: Y-31^T (=CECT 11976^T=CBS 10442^T), *ex suber isolata. Holotypus lyophilus conservatur in collectione culturarum* Colección Española de Cultivos Tipo, Hispania.

Description of *Rhodotorula subericola* Belloch, Villa-Carvajal, Álvarez-Rodríguez & Coque sp. nov.

Rhodotorula subericola [su.be.ri′co.la. L. n. *suber* -*eris* the cork-oak, cork-tree, cork; L. suff. -*cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *subericola* inhabitant of cork tree, cork].

In GPY medium after 3 days at 25 °C, cells are ovoid to cylindrical (1.5–3 × 3.5–7 μm) and occur singly or in parent–bud pairs (Fig. 2). After 1 month at 25 °C, sediment is present. Streak culture on malt agar is viscous to mucoid, partly hyaline, partly creamish-opaque, smooth and glistening. No true hyphae or pseudohyphae develop in Dalmau plate cultures. Fermentation is negative. It assimilates D-glucose, sucrose, α-trehalose, D-arabinose, D-xylose, D-glucitol, D-mannitol, ribitol, ethanol, citrate (weak), succinate, D-gluconate, D-glucosamine, *N*-acetyl-D-glucosamine, D-glucuronate and xylitol. Does not assimilate D-galactose, L-sorbose, cellobiose, lactose, maltose, melibiose, melezitose, raffinose, inulin, soluble starch, L-arabinose, D-ribose, L-rhamnose, glycerol, erythritol, L-arabinitol, galactitol, inositol, methanol, DL-lactate, methyl α-D-glucoside, salicin, hexadecane or saccharate. Assimilation of nitrogen compounds: positive for nitrate, nitrite, L-lysine and glucosamine, negative for ethylamine and imidazole. Growth in vitamin-free medium is positive. Growth at 25 and 30 °C is positive. Growth at 37 °C is negative. Gelatin liquefaction and arbutin hydrolysis are negative. Acid formation on chalk agar is negative. Growth on 50 % glucose/yeast extract is negative. No starch-like substance is produced. Urease activity is positive.

The type strain, Y-31^T (=CECT 11976^T=CBS 10442^T), was isolated from a cork sample in Spain.

References

Álvarez-Rodríguez, M. L., Lopez-Ocaña, L., Lopez-Coronado, J. M., Rodriguez, E., Martinez, M. J., Larriba, G. & Coque, J.-J. R. (2002). Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by O methylation of 2,4,6-trichlorophenol. *Appl Environ Microbiol* **68**, 5860–5869.

Álvarez-Rodríguez, M. L., Belloch, C., Villa, M., Uruburu, F., Larriba, G. & Coque, J.-J. R. (2003). Degradation of vanillic acid and production of guaiacol by microorganisms isolated from cork samples. *FEMS Microbiol Lett* **220**, 49–55.

Alvaro Fonseca, J. I. (2004). Reinstatement of *Rhodotorula colostri* (Castelli) Lodder and *Rhodotorula crocea* Shifrine & Phaff, former synonyms of *Rhodotorula aurantiaca* (Saito) Lodder. *FEMS Yeast Res* **4**, 557–561.

Fell, J. W. & Statzell-Tallman, A. (1998). *Rhodotorula* F.C. Harrison. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 800–827. Edited by C. Kurtzman & J. W. Fell. Amsterdam: Elsevier Science Publishers.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Bioinformatics* **5**, 150–163.

O'Donnell, K. (1993). *Fusarium* and its near relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*, pp. 225–233. Edited by D. R. Reynolds & J. W. Taylor. Wallingford, UK: CAB International.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Scorzetti, G., Fell, J. W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res* **2**, 495–517.

Thanh, V. N., Smit, M. S., Moleleki, N. & Fell, J. W. (2004). *Rhodotorula cycloclastica* sp. nov., *Rhodotorula retinophila* sp. nov., and *Rhodotorula terpenoidalis* sp. nov., three limonene-utilizing yeasts isolated from soil. *FEMS Yeast Res* **4**, 857–863.

Villa-Carvajal, M., Coque, J.-J. R., Alvarez-Rodriguez, M. L., Uruburu, F. & Belloch, C. (2004). Polyphasic identification of yeasts isolated from bark of cork oak during the manufacturing process of cork stoppers. *FEMS Yeast Res* **4**, 745–750.

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. Kurtzman & J. W. Fell. Amsterdam: Elsevier Science Publishers.