

## *Cryptococcus spencermartinsiae* sp. nov., a basidiomycetous yeast isolated from glacial waters and apple fruits

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Seven strains representing a novel yeast species belonging to the genus *Cryptococcus* were isolated from different substrates from Patagonia, Argentina, and The Netherlands. Three strains were isolated from a meltwater river draining from the Frias glacier at Mount Tronador situated in Nahuel Huapi National Park (Patagonia) and four were isolated from apple surfaces in Randwijk, The Netherlands. Analysis of the D1/D2 large-subunit rRNA gene and ITS region sequences indicated that these strains represent a single species that is distinct from other species of the *Tremellales* clade. The name *Cryptococcus spencermartinsiae* sp. nov. is proposed to accommodate these strains. The type strain is CRUB 1230<sup>T</sup> (=CBS 10760<sup>T</sup> =DBVPG 8010<sup>T</sup>).

The anamorphic genus *Cryptococcus* represents a polyphyletic group of basidiomycetous yeasts, occurring in several phylogenetic lineages of the Agaricomycotina. Yeasts of this genus occur intermingled with species classified in other genera, such as *Bullera*, *Dioszegia*, *Trichosporon*, *Tsuchiyaea* and *Udeniomyces*, among the four major clades of the Tremellomycetidae (i.e. Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales) (Fell *et al.*, 2000; Scorzetti *et al.*, 2002; Okoli *et al.*, 2007).

The order Tremellales consists of seven major clusters, and presents a series of interesting taxonomic problems; e.g. many of the internal clusters do not have bootstrap support, which may reflect the heterogeneity of the order and/or the incomplete sampling of taxa (Fell *et al.*, 2000; Sampaio *et al.*, 2002). The description and characterization of new taxa seem essential to improve the knowledge of the phylogeny in the Tremellales and also for the development of a more natural classification system (Fell *et al.*, 2000; Sampaio *et al.*, 2002; Scorzetti *et al.*, 2002; Inácio *et al.*, 2005).

During two independent studies of yeasts, namely one associated with meltwaters of glacial environments in

Argentina and the second studying yeasts occurring on apples in The Netherlands, seven isolates of an unidentified yeast species were found. Analysis of sequences of D1/D2 domains of the large-subunit rRNA gene showed that the strains from Argentina and The Netherlands represented a single species that was genetically distinct from other species in the Tremellales clade (Gildemacher *et al.*, 2006; de García *et al.*, 2007). In this paper, we describe the novel species as *Cryptococcus spencermartinsiae* sp. nov., in honour of the late Portuguese yeast researcher Isabel Spencer-Martins, and in recognition of her contribution to the knowledge of yeast systematics, ecology and physiology.

The strains from Patagonia were isolated from a meltwater river originating on the Frias glacier of Mount Tronador (71° 50' W 41° 11' S) situated in Nahuel Huapi National Park, Argentina. The yeasts were isolated by filtering surface water samples as described by de García *et al.* (2007). This cold environment is characterized by a low nutrient concentration and high content of clays (Pedrozo *et al.*, 1993). Water samples were collected in February 2004. Water temperature at the moment of sampling was 5 °C. The strains from The Netherlands were isolated from the surface of Elstar apples in June 1999 in the Randwijk experimental station, Randwijk, The Netherlands (Gildemacher *et al.*, 2004, 2006). The origins of the strains studied are listed in Table 1. The yeasts were characterized

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domain of the large-subunit rRNA gene and the ITS region of strain CRUB 1230<sup>T</sup> are DQ513279 and EU249514.

**Table 1.** List of isolates of *Cryptococcus spencermartinsiae* sp. nov.

CRUB, Regional University Center of Bariloche (Centro Regional Universitario Bariloche); CBS, Centraalbureau voor Schimmelcultures.

Strain numbers	Source	Locality
CBS 10259, CBS 10261, CBS 10263, CBS 10129	Surface of apples	Randwijk, The Netherlands
CRUB 1230 <sup>T</sup> (CBS 10760 <sup>T</sup> =DBVPG 8010 <sup>T</sup> ), CRUB 1225, CRUB 1227	Meltwater river from Frias glacier	Mount Tronador, Nahuel Huapi National Park, Patagonia, Argentina

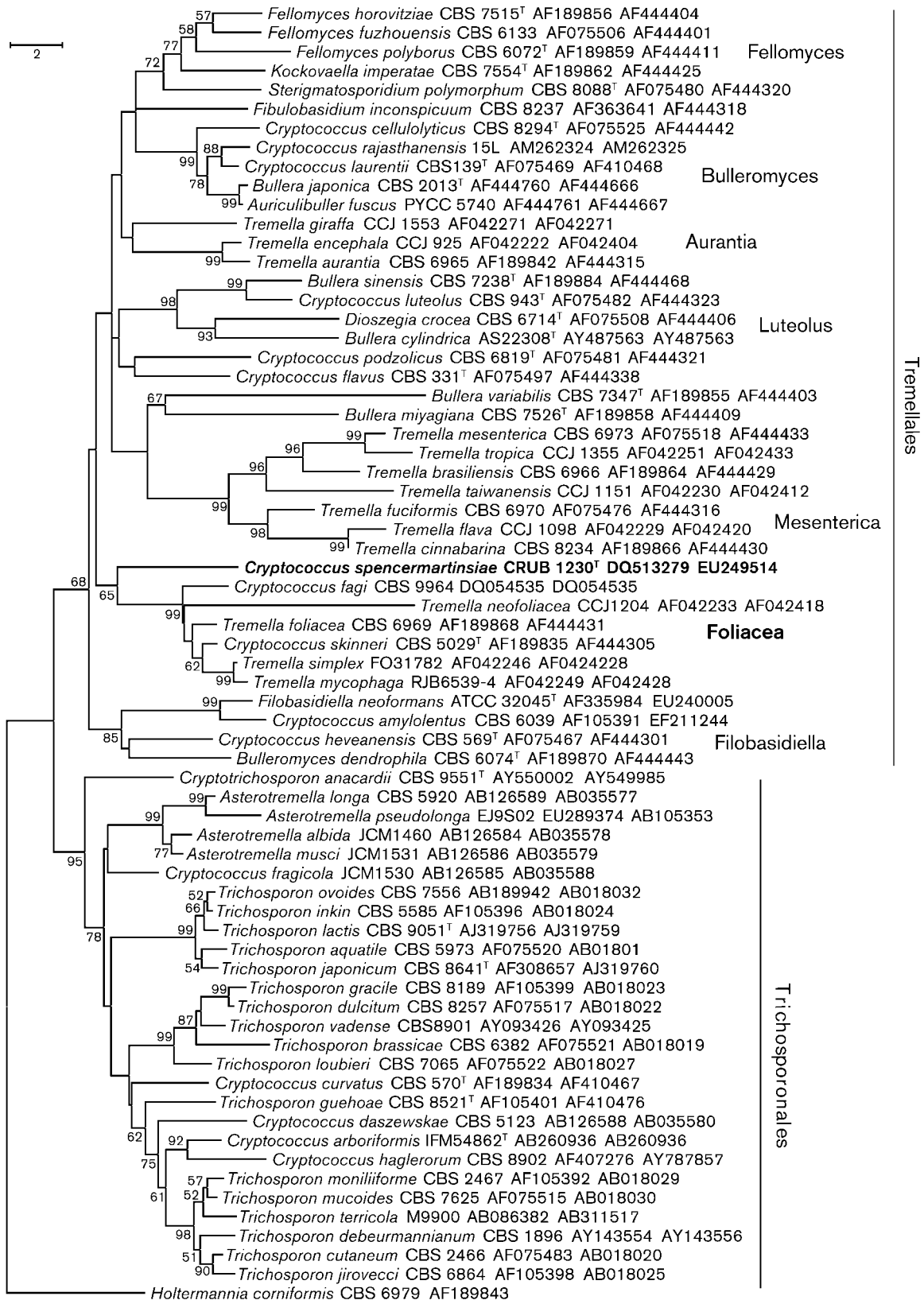
by standard methods; identification up to the genus level was performed according to morphological characteristics and physiological tests described by Yarrow (1998). Mating experiments were performed on glucose-yeast extract agar (GY, 0.2 % glucose, 0.1 % yeast extract, 2 % agar). Plates were incubated for several weeks at 20 °C and checked microscopically at regular intervals.

For PCR fingerprinting, the mini/microsatellite-primed PCR technique was applied. The protocol for DNA extraction, primers, and PCR and electrophoresis conditions were as described by Libkind *et al.* (2003). For DNA sequence analysis, DNA was amplified using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and LR6 (5'-CGCCAGTTCTGCTTACC-3'). Cycle sequencing of the 600–650 bp region at the 5' end of the 26S rRNA gene D1/D2 domains employed forward primer NL1 (5'-GCATA-TCAATAAGCGGAGGAAAAG-3') and reverse primer NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). The internal transcribed spacer (ITS) region was sequenced using the forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer ITS4 (5'-TCCTCCGCTTATGATATGC-3'). Sequencing of the D1/D2 region was performed by the Sequencing Service from STAB VIDA (Oeiras, Portugal). To estimate phylogenetic relationships on the basis of ITS and D1/D2 sequences, neighbour-joining analysis (Kimura's two-parameter model) was performed using MEGA software, version 4.0.2 (Tamura *et al.*, 2007). To test the reproducibility of the results, the Bayesian Markov chain Monte-Carlo method of phylogenetic inference was applied, as implemented in the computer program MrBayes (Ronquist & Huelsenbeck, 2003). This method allows estimation of the *a posteriori* probability that groups of taxa are monophyletic given the DNA alignment (i.e. the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). Four incrementally heated simultaneous Monte-Carlo Markov chains were run over 1 000 000 generations using the general time-reversible model (six rate classes) of DNA substitution, additionally assuming a portion of invariable sites with gamma-distributed substitution rates of the remaining sites (GTR + I + G), random starting trees, and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations resulting in an overall sampling of 10 000 trees. From those trees that were sampled after the process had reached a stationary stage (burnin=2000), a consensus tree was computed to obtain estimates for the *a posteriori* probabilities.

### Species delineation, classification and ecology

Results of the mini/microsatellite-primed PCR fingerprinting showed that all isolates shared identical DNA banding patterns and confirmed their conspecificity (data not shown). Sequence comparisons of the ITS regions and the D1/D2 domains of the large-subunit rRNA gene indicated that the strains from meltwater rivers and apple surfaces represented a novel yeast species belonging to the Foliacea cluster in the Tremellales (Fig. 1). These analyses supported the relatedness of our yeast isolates in this phylogenetic group, but did not match with any known species within the clade. Phenotypic characteristics of this yeast fit the current phenotypic circumscription of the genus *Cryptococcus* (Fell & Statzell-Tallman, 1998), since the new species can utilize D-glucuronate and *myo*-inositol, and cannot synthesize starch-like compounds. Molecular studies (D1/D2 and ITS sequence analyses) indicated that *C. spencermartinsiae* sp. nov. formed part of the Foliacea cluster (*Tremella foliacea*, *T. neofoliacea*, *T. mycophaga*, *T. simplex*, *Cryptococcus skinneri* and *C. fagi*) showing a close relationship with the genus *Tremella*, in this cluster. However, the phenotypic profile of *C. spencermartinsiae* sp. nov. differs greatly from those of the species of this cluster (data not shown). The new species described in this work was assigned to the genus *Cryptococcus* mainly because we have not found sexual reproduction (formation of mycelium or conjugation structures) among the studied strains.

Several authors have proposed to reclassify the genus *Cryptococcus* according to proper phylogenetic relationships between species of Agaricomycotina. This fact is related to the polyphyletic nature of the genus (Takashima & Nakase, 1999), the lack of support of many clusters within the Tremellales, and the unresolved issue of the relationship of many anamorphic species to teleomorphic yeasts in Tremellales (Fell *et al.*, 2000; Sampaio *et al.*, 2002; Inácio *et al.*, 2005; Okoli *et al.*, 2007; Wang *et al.*, 2007). The phylogenetic analysis of this new species indicated that it probably represents a new genus in the Tremellales related to the Foliacea cluster. However, in this case we believe that the report of a new genus should include characterization of the sexual structures. The discovery of related species would be desirable to complete the description of these new taxa. In this study only seven strains of *C. spencermartinsiae* sp. nov. were investigated, and most certainly studies with additional isolates will allow the discovery of the sexual cycle, allowing the complete characterization of this species.



**Fig. 1.** Phylogenetic placement of *Cryptococcus spencermartinsiae* sp. nov. obtained by neighbour-joining (distance K2P method) of the combined ITS and D1/D2 nucleotide datasets. Bar, substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates).

*C. spencermartinsiae* sp. nov. was isolated from a meltwater river draining from the Frias glacier at Mount Tronador, Patagonia, Argentina, and from apple surfaces in Randwijk, The Netherlands. Gildemacher *et al.* (2004) and de García *et al.* (2007) observed the capability of *C. spencermartinsiae* sp. nov. to produce different extracellular enzymes (lipase, cutinase and pectinase), which could indicate a probable association of this new species with terrestrial environments such as plants, soil or even insects. The presence of *C. spencermartinsiae* sp. nov. in a meltwater river could be the result of run-off from the surrounding terrestrial substrates; this glacial-origin river is enclosed by evergreen, rainy Valdivian forest and most of the nutrients carried out to this aquatic environment are from terrestrial sources. The primary habitat of *C. spencermartinsiae* sp. nov. could be the surface of apple fruits, since four strains were isolated from this fruit in The Netherlands. In the case of the Patagonia isolates, apple trees exist around the area where the yeasts were isolated.

*C. spencermartinsiae* sp. nov. has been isolated from two distant locations, both regions having similar temperate to cold climates, which suggests that this species could be primarily associated with the surface of fruits (apples) in temperate to cold regions. In future work we will try to establish the association between the Patagonia apple trees and *C. spencermartinsiae* sp. nov.

**Latin diagnosis of *Cryptococcus spencermartinsiae* de García, Brizzio, Boekhout, Theelen, Libkind & van Broock sp. nov.**

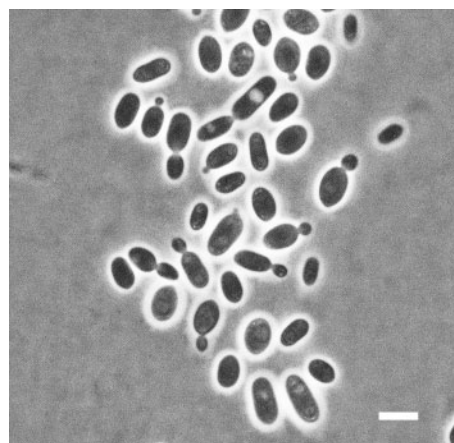
*Fungus Agaricomycotinum, Tremellomycetum, Tremellales. Cultura in agaro malti post dies 7 (18 °C) striis cremea, glabra, nitida, mucosa; margo integra. Ballistoconidia nullae. Nec pseudomycelium, nec mycelium formatur. Status sexualis non apparet. Cellulae subglobosae et ovoidae, incapsulatae, pseudomycelium et mycelium verum non-formantur. Glucosum non-fermentatur. Glucosum, sucrosum, D-galactosum, D-glucosaminum (exiguum), D-xilosum, L-arabiosum, D-arabiosum (lente), L-rhamnosum (exiguum), maltosum, trehalosum, cellobiosum, salicinum (exiguum), arbutin, melibiosum (exiguum), lactosum, raffinolum, melezitolum, ribitolum, xilitolum, D-glucitolum, D-mannitolum, galactitolum (exiguum), inositolum (exiguum), N-acetylglucosaminum (exiguum), D-glucuronatum, succinatum assimilantur. Non-assimilantur L-sorbosum, D-ribosum, sacarosum, inulinum, amyllum solubile, glycerolum, erythritolum, lactatum, glucuronatum, citratum, methanolum, ethanolum, hexadecanolum, acetoneum, ethylacetatum, 2-propanolum. Ethylaminum, lysinum, cadaverinum, glucosaminum assimilantur. Ad crescentiam vitamina externa necessaria sunt. 25 °C crescit (exiguum) neque 30 °C. In media cum 15 % NaCl, cum 1 % acido acetico aut cum 50 % glucose non-crescit. Urea finditur. Materia amyloidea iodophila formantur.*

*Typus* CRUB 1230<sup>T</sup>. *In collectione zymotica* 'Centraalbureau voor Schimmelcultures', *Trajectum ad Rhenum, sub no.* CBS 10760<sup>T</sup> *deposita est.*

**Description of *Cryptococcus spencermartinsiae* de García, Brizzio, Boekhout, Theelen, Libkind & van Broock sp. nov.**

*Cryptococcus spencermartinsiae* (spen.cer.mar.tin'si.æ N.L. gen. fem. n. *spencermartinsiae* of Spencer-Martins, referring to the late Dr Isabel Spencer-Martins, Portuguese yeast biologist, in whose honour the species is named).

Anamorphic yeast related to the subphylum Agaricomycotina, class Tremellomycetes, order Tremellales, family Tremalaceae, cluster Foliaceae. After 7 days on malt extract-yeast extract agar at 18 °C, colonies are glistening, slimy, mucoid, cream-coloured and with an entire margin. Cells are ovoidal to ellipsoidal, with a capsule, and multiply by multilateral budding. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae and true hyphae are not formed (Fig. 2). No positive mating reactions are observed. Glucose is not fermented. Glucose, sucrose, D-galactose, D-glucosamine (weak), D-ribose, D-xylose, L-arabinose, D-arabinose (slow), L-rhamnose (weak), maltose, trehalose, cellobiose, salicin (weak), arbutin, melibiose (weak), lactose, raffinose, melezitose, ribitol, xylitol, D-sorbitol, D-mannitol, galactitol (weak), *myo*-inositol (weak), *N*-acetylglucosamine (weak), D-glucuronate and succinate are assimilated. No growth occurs on L-sorbose, D-ribose, sucrose, inulin, soluble starch, glycerol, *meso*-erythritol, lactic acid, gluconate, DL-lactate, citrate, methanol, ethanol, hexadecane, acetone, ethylacetate or 2-propanol. Assimilation of nitrogen compounds: positive for lysine, D-glucosamine and cadaverine. Growth in vitamin-free medium is positive. Growth in amino-acid-free medium is positive. Growth at 25 °C is weak and at 30 °C is absent. Growth on YM agar with 10 % sodium chloride is absent. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 µg cycloheximide ml<sup>-1</sup> growth is absent. Urease activity is



**Fig. 2.** Phase-contrast micrograph of vegetative cells of *Cryptococcus spencermartinsiae* sp. nov. CRUB 1230<sup>T</sup> after 2 days on yeast extract-malt extract agar at 18 °C. Bar, 5 µm.

positive. Acid production is positive. Diazonium Blue B reaction is positive.

The type strain of *C. spencermartinsiae* sp. nov. is CRUB 1230<sup>T</sup>, recovered from the Frias meltwater river originating in the Frias glacier of Mount Tronador, Nahuel Huapi National Park, San Carlos de Bariloche, Río Negro, Argentina. The strain has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 10760<sup>T</sup> and in the Industrial Yeasts Collection of Dipartimento di Biologia Vegetale Università di Perugia, Italy, as strain DBVPG 8010<sup>T</sup>.

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