Cryptococcus terrestris sp. nov., a tremellaceous, anamorphic yeast phylogenetically related to Cryptococcus flavescens

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Cryptococcus terrestris sp. nov. (Basidiomycota, Agaricomycotina, Tremellomycetes, Tremellales) is typified by CJDX4 Y23T (=CBS 10810T =NRRL Y-48451T), isolated from forest soil in Oklahoma, USA. This species is most readily identified by the sequence of the D1/D2 domain region of the 26S rDNA and ITS (internal transcribed spacer) region. Additional strains from Oklahoma (C107DX4 Y11 =CBS 10813 =NRRL Y-48452) and Brazil (Ep11c =CBS 10812 =NRRL Y-48454; 56e =CBS 10811 =NRRL Y-48453) either had identical sequences or differed minimally. C. terrestris differs physiologically from the most closely related species, Cryptococcus flavescens, by the weak or delayed assimilation of ribose and salicin, and differs from Cryptococcus aureus by the utilization of nitrate and nitrite and growth in vitamin-free medium.

Members of the genus Cryptococcus are found in a wide variety of habitats, and are difficult to identify by traditional methods. The genus is polyphyletic and is distributed throughout the class Tremellomycetes, with representatives in all orders (Tremellales, Filobasidiales and Cystofilobasidiales) (Scorzetti et al., 2002; Hibbett et al., 2007).

Independent yeast surveys in Oklahoma (USA) and Rio Grande do Sul (Brazil) produced four isolates with very similar physiological profiles and sequences of the D1/D2 domain of the 26S rDNA and ITS (internal transcribed spacer) region. Sequences differed significantly from those of any previously described yeast species, and the isolates formed a discrete clade close to Cryptococcus flavescens. This study deals with the description of this novel species, for which the name Cryptococcus terrestris sp. nov. is proposed.

The Oklahoman strains CJDX4 Y23T and C107DX4 Y11 were isolated from a sandy loam (sand 67.5 %, silt 28.7 %, clay 3.8 %) Konawa series soil collected in the rhizosphere of a mixed hardwood (mainly Quercus spp.) forest in Payne County, Oklahoma, on 6 July 2006 (35° 59.986’ N 97° 05.089’ W) and 27 January 2007 (35° 59.949’ N 97° 05.050’ W), respectively. A subsample of the surface to 2 cm layer was incubated overnight in 50 ml M3C (Vishniac, 2002) plus an antibiotic mixture on a New Brunswick Gyrotary shaker and then sampled by spreading 0.1 ml of appropriate dilutions on M3C plus antibiotic agar. The antibiotic mixture included 160 μg penicillin G potassium salt and 100 μg streptomycin sulfate ml⁻¹ for the July sample, augmented by the addition of 32 μg gentamicin sulfate ml⁻¹ for the January sample. The Brazilian strains Ep11c and 56e were isolated during an epidemiological study of yeasts associated with a case of cryptococcal meningitis in a worker from a timber factory at Cachoeira do Sul in Rio Grande do Sul state in southern Brazil (30° 00.470’ S 52° 54.865’ W). The factory dealt mostly with wood of Pinus spp. trees, but also processed Ficus and some Eucalyptus trees. Both strains were collected within the factory area. Strain Ep11c was isolated on 26 September 2005 from dry pigeon droppings that were lying on the grass. Strain 56e was isolated from mixed sawdust on 12 December 2005. Appropriate dilutions of the samples were plated on YEPD agar (glucose 2 %, peptone 1 %, yeast extract 0.5 %, agar
2 %) with 34 μg chloramphenicol ml⁻¹ and incubated at 30 °C for 10 days.

Morphological and physiological characterization was performed according to standard methods described by Yarrow (1998) and Barnett et al. (2000) and according to Vishniac (1985), with similar results regardless of the methodology used. NaCl tolerance was additionally assessed spectrophotometrically following growth in (shaken) glucose (0.5 % w/v), peptone (0.5 %), yeast extract (0.3 %) liquid medium with various concentrations of added NaCl (0–16 %) for up to 100 h at room temperature (about 22–23 °C).

Total DNA was extracted and processed according to Ramos et al. (2001). The D1/D2 domain of the 26S rDNA was sequenced using primers NL1 (5' - GCATATCAAT - AAGCGGAGGAAAAG) and NL4 (5' - GGTCCGTGGTTTCAAGACGG), while the ITS region was sequenced using primers ITS1 (5' - TCCGTAGTTAAGCTCGGG) and ITS4 (5' - TCTTCCGCTTATTGATATGC) according to Kurtzman & Robnett (1998) and Scorzetti et al. (2002). Sequences were obtained with an Amersham MegaBACE 1000 automated sequencer using standard protocols at the facilities of the Brazilian Genome Network at Center of Biotechnology (Chbiot-UFRGS-RS). 26S rDNA D1/D2 domain sequencing of strains CJDX4 Y23³ and C107DX4 Y11 was performed by Laragen Inc. (Los Angeles, CA, USA). Alignments were made with the STADEN package (Staden et al., 2000). Phylogenetic analysis was performed with MEGA 4 (Tamura et al., 2007). Phylogenetic trees were constructed by using the neighbouring-joining method and bootstrapping was done based on 1000 random samplings.

MS-PCR fingerprinting followed the protocols described in Sampaio et al. (2001), using the primer (GTG)₅. Gel electrophoresis images were acquired with the GelDoc XR System software (Bio-Rad).

Phylogenetic analysis based on the 26S rDNA D1/D2 domain and ITS sequences showed that strains CJDX4 Y23³, C107DX4 Y11, Ep11c and 56e clustered in a group most closely related to C. flavescens and Cryptococcus aures, anamorphic yeasts within the Tremellales (Figs 1 and 2). Trees constructed using maximum parsimony shared a similar topology to those using neighbour joining (data not shown). Bootstrap values supporting the C. terrestis clade were high in both the D1/D2 (96 %) and ITS (95 %) trees. Thus, C. terrestis is phylogenetically distinct. C. terrestis is related more distantly to Auriculibuller fuscus, Bullera japonica and Bullera pseudoalba and still more distantly to Cryptococcus laurentii and Cryptococcus rajasthanensis. The isolates are therefore assigned to the genus Cryptococcus, as they fail to produce the ballistospores characteristic of Bullera and Auriculibuller, and to the order Tremellales. This generic assignment is somewhat unfortunate, as the genus Cryptococcus is presently polyphyletic (Fell & Statzell-Tallman, 1998; Fell et al., 2000; Sugita et al., 2000).

The sequences of the 26S rDNA D1/D2 region of strains CJDX4 Y23³, C107DX4 Y11, Ep11c and 56e have at least 8 substitutions compared with the type strain of C. flavescens and 10 substitutions compared with the type strain of C. aures, while the ITS region has 11 and at least 22 substitutions compared with the same strains (the other strains that appear in the same cluster as C. terrestis are discussed below). The most genetically divergent strain within these four isolates is 56e, with 3 substitutions from CJDX4 Y23³ in the D1/D2 domain and 1 substitution in the ITS region. The definition of species on the basis of nucleotide differences in the D1/D2 and ITS regions is not straightforward (Fell et al., 2000; Scorzetti et al., 2002). Anamorphic species are generally defined with reference to the identical or closely similar sequences that typically characterize sexually reproducing species. Rare exceptions to such similarity occur among both basidiomycetous and ascomycetous teleomorphic species; Sporidiobolus salmonicolor mating strains can have 7 substitutions in the D1/D2 and 26 in the ITS region (Scorzetti et al., 2002), and two of some 37 strains of Clavispora lusitaniae differed by 32 substitutions in the D2 domain (Lachance et al., 2003). The sequence differences that characterize the C. terrestis cluster are sufficient by the usual criteria for establishing an anamorphic species. However, the present species delineation does not depend upon nucleotide differences alone. The C. terrestis cluster is also defined by physiological and biochemical tests that differentiate this species from the most phylogenetically similar species, C. flavescens and C. aures (Table 1). The four strains were identical in response to the conventional items of physiological profiling, another reason for including strain 56e in C. terrestis.

The D1/D2 and ITS trees (Figs 1 and 2) also make it clear that many strains identified as C. laurentii occur in the C. flavescens and C. terrestis clades, and are not conspecific with the type strain of C. laurentii, being presumably misidentified. An example is [C. laurentii] CBS 8645, recently reclassified by Takashima et al. (2003) as C. flavescens. The same happens with some strains identified as C. flavescens that appear in the C. terrestis clade. Since most of these strains, including those identified as Cryptococcus sp. in the C. terrestis clade, have only one sequence (D1/D2 or ITS) available in GenBank, and no other information is available, this investigation did not attempt to revise their taxonomy. Cryptococcus sp. CBS 8372, which has 6 substitutions in the D1/D2 region and 7 substitutions in the ITS region compared with the type strain of C. terrestis, is differently and somewhat ambiguously positioned in the two trees. It was therefore not included in C. terrestis. The novel species C. terrestis is described on the basis of the four isolates that consistently grouped together in both the D1/D2 and ITS trees: CJDX4 Y23³, C107DX4 Y11, Ep11c and 56e.

The occurrence of C. terrestis in two such widely separated locations (Oklahoma and southern Brazil) may be explained by a fortuitous dispersion or by a genuinely broad range of distribution. While aerial transport can take yeasts, like other microbes, over long distances, the
occurrence of more than one strain in each location suggests that this species is indigenous to both. The fact that other strains possibly belonging to or phylogenetically very close to \( C. \) terrestris (see Fig. 1) have been found in other places around the world also suggests a broad range of occurrence. Oklahoma and southern Brazil differ considerably in mean annual temperature and rainfall, two environmental factors that account for nearly half of the geographical distribution of soil yeasts (see Vishniac, 2006). The Oklahoma site has a mean annual temperature of 14.55 °C and mean annual rainfall of 93.23 cm; Cachoeira do Sul is characterized by 19 °C mean annual temperature and 200 cm mean annual rainfall. Furthermore, the two sets of \( C. \) terrestris strains are also not genetically identical. MS-PCR fingerprinting with primer GTG5 produced geographically distinct patterns (Fig. 3). Fingerprinting with this primer usually gives species-specific patterns, but there are other examples of pattern differentiation among geographically separated strains belonging to the same species (Caruso et al., 2002; Libkind et al., 2007). In spite of the different fingerprinting profiles, the identical phenotypic characteristics and the D1/D2 and ITS sequences indicate that the Oklahoman and Brazilian isolates belong to the same species.
Description of *Cryptococcus terrestris* sp. nov.

*Cryptococcus terrestris* (ter.res'tris. L. adj. *terrestris* associated with soil, the substrate from which the type strain was isolated).

After 3 days of growth in liquid YEPD medium (glucose 2 %, peptone 1 %, yeast extract 0.5 %) at 25 °C, cells are globose to ovoid and occur singly or in pairs (3–7 μm) (Fig. 4). Sexual reproduction has not been observed; asexual budding is monopolar. Sediment is formed. On YM agar after 3 days at 25°C, colonies are smooth, mucous to butyrous, glistening and cream-coloured with an entire margin. After 15 days in slide cultures on cornmeal agar, pseudomycelium or mycelium is not formed. Fermentation ability is negative. L-Arabinose, D-glucose, l-arabinose, arbutin (weak), cellobiose, citrate (weak), erythritol (delayed or weak), ethanol, galactitol, galactonate, D-galactose, D-galacturonate, D-glucitol, D-glucuronate, 2- and 5-keto- D-glucuronate, 2- and 5-ketogluconate, D-glucose, D-glucosamine, N-acetyl D-glucosamine, protocatechuic acid, quinic acid, L-sorbose, vanillic acid and xylan were assimilated. Gallic acid, gentisic acid, D-glucosamine, N-acetyl D-glucosamine, protocatechuic acid, quinic acid, L-sorbose, vanillic acid and xylan were assimilated.

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**Fig. 2.** Phylogenetic tree showing placement of *C. terrestris* sp. nov. among related basidiomycetous yeasts, derived from neighbour-joining analysis of the ITS region. Bootstrap analysis was done based on 1000 random samplings. *B. pseudoalba* CBS 7227T, *Cryptococcus cellulolyticus* CBS 8294T, *C. laurifolius* CBS 139T, *C. rajasthanensis* 15LT and *C. anemochoreius* CBS 10258T were designated the outgroup in this analysis. Sequences from uncultured fungi were not used in the analysis. Type strains are highlighted in bold. Bar, 0.01 substitutions per nucleotide position.
are not assimilated. Cadaverine, ethylamine, L-lysine, sodium nitrite and D-tryptophan are utilized. Creatine, creatinine, D-glucosamine and potassium nitrate are not utilized. Vitamins are not required for growth. No growth in the presence of 0.01 or 0.1% cycloheximide. No growth in the presence of 10 or 16% NaCl. No growth occurs on 50% glucose/yeast extract broth. Starch-like substances are produced. Diazonium blue B reaction is positive. Urease activity is positive. Growth at 30°C, but not at 37°C.

The type strain, CJDX4 Y23T (=CBS 10810T =NRRL Y-48451T), was isolated from a soil sample in Oklahoma, USA. Strains C107DX4 Y11 (=CBS 10813 =NRRL Y-48452), also isolated in Oklahoma, and Ep11c (=CBS 10812 =NRRL Y-48454) and 56e (=CBS 10811 =NRRL Y-48453), isolated in southern Brazil, also belong to the species.

**Latin diagnosis of Cryptococcus terrestris sp. nov.**

nitritum, et d-tryptophanum assimilantur, sed non cretini-
num, creatinium, d-glucosaminum, kalli nitratum. Vitamina 
exerna ad crescendentiam necessaria non sunt. In medio cum 
50 % glucos non crescit. In medio cum 10 % et 16 % NaCl 
non crescit. Materia amyloidea fornatur. Reactio Diazonii 
coerulei B positivum. Ureum finditur. Crescit in 30 °C, non 
crescit in 37 °C. Typus CJDX4 Y23T (=CBS 10810T 
=NRRL Y-48451T), isolatus ex terra, in Oklahoma, 
preservatus in Centraalbureau voor Schimmelcultures, 
Utrecht, The Netherlands et in ARS Culture Collection.

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