Cryptococcus agrionensis sp. nov., a basidiomycetous yeast of the acidic rock drainage ecoclade, isolated from an acidic aquatic environment of volcanic origin

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Seventy-one strains were isolated from the River Agrio–Lake Caviahue acidic aquatic environment in Argentina. Strains were isolated mainly from the most acidic section of the river (pH 1.8–2.7). According to the mini/microsatellite-primed PCR technique and physiological data, these strains are representatives of a single novel species of the genus *Cryptococcus*. Analysis of the D1/D2 region of the large-subunit rRNA gene showed that the strains belong to the order Filobasidiales of the subphylum Agaricomycotina. The novel isolates formed a phylogenetic group with *Cryptococcus ibericus*, *Cryptococcus aciditolerans* and *Cryptococcus metallitolerans*, the most closely related species. This group, which is phylogenetically related to the Gastricus clade, is considered to be an ecoclade due to its peculiar ecology and physiology. The name *Cryptococcus agrionensis* sp. nov. is proposed to accommodate these isolates, with strain CRUB 1317^T (=CBS 10799^T=JCM 15321^T) as the type strain.

The basidiomycetous yeasts of the genus *Cryptococcus* Vuillemin form a polyphyletic group comprising nearly 50 species, which are distributed in the orders Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales of the subphylum Agaricomycotina (Fell *et al.*, 2000; Bauer *et al.*, 2006; Golubev *et al.*, 2006). Species of the genus *Cryptococcus* have been isolated from a wide range of habitats, including extreme acidic aquatic environments, namely Rio Tinto (Spain), São Domingos mines (Portugal) and River Agrio–Lake Caviahue (RAC; Argentina). These environments are characterized by very low pH and high concentrations of heavy metals (López-Archilla *et al.*, 2004; Gadanho *et al.*, 2006; Russo *et al.*, 2008).

The RAC is an acidic aquatic environment created by volcanic activity (Fehn *et al.*, 2002). Its pH ranges from 1.5 at the source of the upper River Agrio (URA) to 6.4 at the lower River Agrio (LRA), 20 km downstream of the

Caviahue Lake (Russo *et al.*, 2008). In 2004, conductivity ranged from 17.86 mS cm^{−1} (URA) to 0.24 mS cm^{−1} (LRA) (Russo *et al.*, 2008). Concentrations of metals such as Fe, Mg, Mn, Al and Zn were much higher at the source of the URA than at the LRA (mg l^{−1}: 2650.0 to 6.0; 1930.0 to 10.8; 79.8 to 0.1; 921.0 to 2.2; and 7.7 to lower than measurable levels, respectively) (Gammons *et al.*, 2005).

During a study aimed at characterizing the biodiversity of the yeast community in the RAC aquatic system, 71 strains of an undescribed species of the genus *Cryptococcus* were isolated from six sampling sites along a section (approximately 30 km) of the RAC system. A detailed description of each sampling site has been presented previously by Russo *et al.* (2008). All strains showed a remarkable ability to survive in the extremely acidic waters (Russo *et al.*, 2008) suggesting that they are autochthonous to this environment. Phylogenetic analyses based on the D1/D2 region of the 26S rRNA gene showed that *Cryptococcus ibericus* was the most closely related species. Interestingly, strains isolated from the RAC were included in a clade composed of *C. ibericus*, *Cryptococcus aciditolerans* and *Cryptococcus metallitolerans*, which were isolated from acid rock drainage (ARD) of the São Domingos pyrite mine in Portugal (Gadanho & Sampaio,

Abbreviations: ARD, acid rock drainage; LRA, lower River Agrio; MSP-PCR, mini/microsatellite-primed PCR; RAC, River Agrio–Lake Caviahue; URA, upper River Agrio.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Cryptococcus agrionensis* sp. nov. CRUB 1317^T and CRUB 1364 are EU627786 and EU627788, respectively.

2009). In this report, a novel species, *Cryptococcus agrionensis* sp. nov., of the order Filobasidiales, a putative autochthonous yeast of acidic environments, is described.

Novel strains were isolated from the RAC (37° 52' S 71° 02' W) in March 2004. Yeast isolations were performed using solid MYP medium (0.7 % malt extract, 0.05 % yeast extract, 0.25 % peptone-soytone, 2.0 % agar) and acidified (containing 2.5 M H₂SO₄) solid MYP medium (Russo *et al.*, 2008). Conventional physiological and biochemical tests were performed according to Yarrow (1998). For determination of sexual compatibility, pairs of 2- to 4-day-old cultures were crossed on GSA medium (0.2 % glucose, 0.2 % peptone-soytone, 1.5 % agar), incubated at room temperature for 2 weeks and examined for production of sexual structures.

For DNA extraction, protocols described by Sampaio *et al.* (2001) and Libkind *et al.* (2003) were employed. The mini/microsatellite primed-PCR fingerprinting technique (MSP-PCR) was used according to Libkind *et al.* (2003). The minisatellite primer M13 (5'-GAGGGTGGCGTTCT-3') and the synthetic microsatellite oligonucleotide (GTG)₅ were used to obtain species-specific DNA fingerprints (Sampaio *et al.*, 2001). For sequence analysis, DNA was amplified using rRNA primers ITS5 and LR6. Cycle sequencing of the D1/D2 region at the 5' end of the 26S rRNA gene was performed according to Libkind *et al.* (2003). Sequences obtained were compared with those included in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/>). Sequence alignment was performed with MEGA 4 (Tamura *et al.*, 2007) and adjusted visually. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 4 with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 random resamplings.

C. agrionensis sp. nov. was the most abundant yeast found in the acidic waters of the RAC volcanic environment and was present at all sampling sites with pH values ranging from 1.8 to 6.7 (Russo *et al.*, 2008). However, 97 % of the isolates were obtained from the most acidic sampling sites (URA and Lake Caviahue, pH 1.8–2.7). All colonies were creamy white after the first few days of incubation at 20–22 °C in MYP agar, although after a week at 4 °C they became pale pink. Assimilation of *myo*-inositol and D-glucuronate as carbon sources, the capacity to hydrolyse urea and the inability to synthesize starch-like compounds indicated that the strains belonged to the genus *Cryptococcus* Vuillemin (Fell & Statzell-Tallman, 1998). Mating assays were always negative as reproductive structures or cell-to-cell conjugation followed by formation of mycelium were not observed, indicating that the species was anamorphic.

MSP-PCR studies of both M13 and (GTG)₅ primers suggested that all 71 strains were conspecific (Fig. 1). M13 MSP-PCR profiles were less conserved than (GTG)₅ profiles and showed some variability. Such polymorphisms

Fig. 1. MSP-PCR fingerprints of different isolates of *C. agrionensis* sp. nov. obtained with primer M13. Lane M, molecular marker.

among the novel isolates could not be related to any other ecological, physiological or molecular data. Sequence analysis of the D1/D2 region of six representative strains of *C. agrionensis* sp. nov. revealed no polymorphism (100 % similarity). Phylogenetic studies showed that *C. ibericus* SDY 22^T (=CBS 10871^T) was the most closely related strain to *C. agrionensis* sp. nov., although it is distantly related given that they differ in 35 nucleotides (Fig. 2).

Interestingly, phylogenetic analyses placed *C. agrionensis* sp. nov. in a separate clade composed of *C. ibericus* SDY 22^T, *C. aciditolerans* SDY 81^T (=CBS 10872^T) and *C. metallitolerans* SDY 190^T (=CBS 10873^T), isolated from ARD of São Domingos pyrite mine. Differences between the species isolated in São Domingos mine and *C. agrionensis* sp. nov. are shown in Table 1. Another interesting result is that *C. agrionensis* sp. nov., as observed with the three species from the São Domingos mine, proved to be highly resistant to heavy metals such as Cd²⁺, Co²⁺, Cu²⁺, Li⁺, Ni²⁺ and Zn²⁺ (unpublished data), further demonstrating the close relatedness of these species and their probable extremophile nature.

The clade composed of the three *Cryptococcus* species isolated from Portugal was designated the ARD ecoclade because of their isolation origin and distinctive ecology (Gadanhó & Sampaio, 2009). The term 'ecoclade' refers to species that are related phylogenetically and show salient physiological adaptations associated with the physico-chemical conditions present in their habitats. However, the three *Cryptococcus* species constituting the ecoclade were isolated from the same geographical region and could have been claimed to be part of a geographical clade rather than an ecoclade. The present work provides evidence that

Fig. 2. Phylogenetic placement of *C. agrionensis* sp. nov. within the genus *Cryptococcus* inferred using the neighbour-joining method (distance Kimura two-parameter method) based on an alignment of nuclear DNA sequences from the D1/D2 region of the 26S rRNA gene. *Cryptococcus arrabidenensis* was used as an outgroup. Number on the branches are bootstrap values (1000 replicates). GenBank accession numbers are given in parentheses. Branch lengths are in substitutions per site. Bar, 5 substitutions per site.

the ARD ecoclade is not restricted to the Iberian Pyrite region and demonstrates that members of this ecoclade may be found in acidic environments in general, originating both naturally and anthropically. These results further support the proposal of this phylogenetic group of yeasts as the ARD ecoclade.

Latin diagnosis of *Cryptococcus agrionensis* Russo, Libkind, Sampaio et van Broock sp. nov.

Fungus order Filobasidiales (Tremellomycetes, Agaricomycotina). In statu unicellulari cellulae ellipsoideae, 2–3 × 5–8 µm. Mycelium verum non formatur. Glucosum non fermentatur. Substrata carbonica assimilate: amyllum solubile

(exigue), arbutinum, cellobiosum, citratum, D-arabiosum, DL-lactatum, galactosum, galactitolum (exigue), D-glucuronatum, glycerolum (exigue), D-glucono-1,5-lactonum, myo-inositolum, erythritolum (lente), lactosum, L-arabiosum, L-rhamnosum, maltosum, D-mannitolum, melibiosum, mele-zitosum, raffinolum, ribitolum, D-ribosum, D-salicinum, sorbitolum, L-sorbosum, succinatum, trehalosum, D-xilosum et xilitolum (lente). Non assimilantur D-glucosaminum nec sucrosum. Substrata nitrogenica assimilate: nitritum, nitratum et cadaverinum; neque creatinum, creatininum, ethyl-aminum et L-lysinum. In media vitaminarum externarum non crescit. Non crescit 0.01 vel 0.1% cycloheximido addito. Materia amyloidea iodophila non formantur. Reactiones Diazonium caeruleum B et ureasii positivae. Crescit at 30 °C,

Table 1. Physiological and biochemical properties of *C. agrionensis* sp. nov., *C. ibericus*, *C. aciditolerans* and *C. metallitolerans*

Species: 1, *C. agrionensis* sp. nov.; 2, *C. ibericus*; 3, *C. aciditolerans*; 4, *C. metallitolerans*. Data are from this study and Gadanho & Sampaio (2009). +, Growth; –, no growth; w, weak growth.

Characteristic	1	2	3	4
Assimilation of carbon compounds:				
D-Glucosamine	–	+	+	+
L-Rhamnose	+	+	–	–
Sucrose	–	+	+	+
Melezitose	+	+	+	–
Erythritol	w	+	+	+
L-Sorbose	+	–	–	+
D-Ribose	+	–	–	+
D-Arabinose	+	–	+	–
Ribitol	+	–	+	–
Galactitol	w	–	+	–
DL-Lactate	+	–	–	–
Assimilation of nitrogen compounds:				
Potassium nitrate	+	–	–	–
Sodium nitrite	+	–	–	–
Cadaverine	+	–	–	–
Growth in/at:				
Cycloheximide (0.01 %)	–	+	+	+
35 °C	–	+	–	–
37 °C	–	+	–	–

neque 35 °C et 37 °C. *Sequentiae acidi nucleici partium* D1/D2 *submonadis majoris ribosomalis typi in collectione sequentiarum acidi nucleici* NCBI (www.ncbi.nlm.nih.gov) *numeris* EU627786 *depositae sunt*. *Positio phylogenetica* in Fig. 2 *illustratur*. *Cultura typica numero* 10799^T *in Centraalbureau voor Schimmelcultures* (CBS), *Ultrajecti, Hollandia, conservatur*.

Description of *Cryptococcus agrionensis* Russo, Libkind, Sampaio & van Broock sp. nov.

Cryptococcus agrionensis (a.gri.o.nen'sis. N.L. masc. adj. *agrionensis* of River Agrio, referring to the name of the river where most of the isolates were obtained).

Anamorphic yeast belonging to the subphylum Agaricomycotina, class Tremellomycetes, order Filobasidiales. Yeast cells after growth in MYP agar for 3 days at 20 °C are ellipsoidal (2–3 × 5–8 µm) and occur singly (Fig. 3). Budding is polar. Pure cultures are white and smooth, with entire margins. After 1 week, colonies become pale pink at 4 °C. All strains are positive for assimilation of the carbon compounds starch (weak), arbutin, cellobiose, citrate, D-arabinose, DL-lactate, galactose, galactitol (weak), D-glucuronate, glycerol (weak), D-glucono-1,5-lactone, myo-inositol, erythritol (delayed), lactose, L-arabinose, L-rhamnose, maltose, D-mannitol, melibiose, melezitose,

Fig. 3. *C. agrionensis* sp. nov. CBS 10799^T vegetative cells after 3 days in MYP agar at 20 °C. Bar, 10 µm.

raffinose, ribitol, D-ribose, D-salicin, sorbitol, L-sorbose, succinate, trehalose, D-xylose and xylitol (delayed), and the nitrogen compounds cadaverine, potassium nitrate and sodium nitrite. All strains are positive for growth at 30 °C. All strains are negative for fermentation of D-glucose, assimilation of the carbon compounds D-glucosamine and sucrose, and assimilation of the nitrogen compounds creatine, creatinine, ethylamine and L-lysine. All strains are negative for growth in vitamin-free medium, in 0.01 % and 0.1 % cycloheximide and at 35 and 37 °C, and formation of starch-like compounds. Reaction with Diazonium Blue B and urease production are positive.

The type strain is CBS 10799^T (=JCM 15321^T=CRUB 1317^T), isolated in 2004 from the RAC environment [subsurface water of the first sampling site of the upper River Agrio (pH 1.8; 37° 52' S 71° 06' W)] by G. Russo. Strain CBS 10800 (=JCM 15322=CRUB 1364), isolated in 2004 from the RAC environment [subsurface water of the second sampling site of the upper River Agrio (pH 2.2; 37° 53' S 71° 04' W)] by G. Russo, is a reference strain.

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