Cryptococcus ibericus sp. nov., *Cryptococcus aciditolerans* sp. nov. and *Cryptococcus metallitolerans* sp. nov., a new ecoclade of anamorphic basidiomycetous yeast species from an extreme environment associated with acid rock drainage in São Domingos pyrite mine, Portugal

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In this report, we describe three novel asexual basidiomycetous yeast species, *Cryptococcus aciditolerans* sp. nov. (type strain CBS 10872^T = SDY 081^T), *Cryptococcus ibericus* sp. nov. (type strain CBS 10871^T = SDY 022^T) and *Cryptococcus metallitolerans* sp. nov. (type strain CBS 10873^T = SDY 190^T), which were isolated from acid rock drainage collected at the São Domingos mine in southern Portugal. Phylogenetic analysis of molecular sequence data indicated that the novel species belong to the order Filobasidiales of the class Tremellomycetes and form a well-separated clade, next to *Cryptococcus gastricus* and *Cryptococcus gilvescens*. Since the novel species also share a peculiar ecology, being able to thrive under extreme environmental conditions characterized by very low pH and high concentrations of heavy metals, we designate this combination of phylogenetic and ecological characteristics as an ecoclade.

Microbial oxidation of iron pyrite was first demonstrated by Bryner *et al.* (1954). Subsequently, several authors studied this transformation and it was found that, besides the bacteria of the *Ferrobacillus*–*Thiobacillus* group, the microbial colonizers of acid mine water included yeasts, flagellates and amoebae (Ehrlich, 1963). Among the yeasts, those that formed pink colonies (probably *Rhodotorula*) and arthroconidia (*Trichosporon*) were the most prominent in a copper mine in the USA (Ehrlich, 1963). Beck (1960), who studied iron-oxidizing bacteria isolated from acidic leach water, pioneered the microbial investigation of acid mine waters and revealed a unique community of micro-organisms, supported by a peculiar ecology, which is based on chemosynthetic autotrophy.

More recently, López-Archilla *et al.* (2001) rediscovered the distinctive ecology of this type of environment in investigations carried out in Rio Tinto, on the Spanish side of the Iberian pyrite belt (IPB). This research

highlighted the extremely low pH (mean 2.2) and the high concentrations of heavy metals (2.3 g Fe l⁻¹; 0.22 g Zn l⁻¹, 0.11 g Cu l⁻¹) (López-Archilla *et al.*, 2001) and an unsuspected microeukaryotic diversity that surpassed that of the prokaryotes (Amaral Zettler *et al.*, 2002). These studies challenged earlier ideas about the phylogenetic range of organisms able to live in extreme environments and prompted us to analyse with more detail the yeast community associated with acid rock drainage (ARD) in IPB ecosystems. Therefore, a comparative study was conducted in Rio Tinto and in São Domingos, on the Portuguese side of the IPB (Gadanho *et al.*, 2006). Interestingly, the yeast communities of the two localities showed a remarkable resemblance, and eight of the 13 most abundant species (species with more than five isolates) found at São Domingos were also detected at Rio Tinto. In total, 27 yeast species were detected, 48% of which were judged to represent undescribed taxa.

Another interesting type of extreme environment is formed by acidic volcanic waters that share with acid mine waters a similar physico-chemical composition, although their genesis and dynamics are not linked to human activities. The yeast community of a volcanic acidic river, Rio Agrio,

Abbreviations: ARD, acid rock drainage; IPB, Iberian pyrite belt.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 regions of the 26S rRNA genes of strains SDY 81^T, SDY 190^T and SDY 22^T are respectively AY731789–AY731791.

and the recipient lake Caviahue (RAC) in Patagonia, Argentina, was recently studied and found to be similar to the IPB yeast communities (Russo *et al.*, 2008). Five species, two of them undescribed, were common to IPB and RAC environments. Taken together, these studies support the claim that a distinct assemblage of yeasts is autochthonous to such environments.

In a continuation of our characterization of the ARD yeast community, we focus here on a group of yeasts previously isolated in São Domingos (Gadanho *et al.*, 2006) that belong to the order Filobasidiales of the class Tremellomycetes (Basidiomycota). In this report, we describe three novel species, *Cryptococcus ibericus* sp. nov. (previously designated *Cryptococcus* sp. 1), *Cryptococcus aciditolerans* sp. nov. (*Cryptococcus* sp. 2) and *Cryptococcus metallitolerans* sp. nov. (*Cryptococcus* sp. 3), that are phylogenetically close to *Cryptococcus gastricus* and *Cryptococcus gilvescens*.

Yeast isolation, preliminary characterization and sequence analysis

All strains of the three novel species were isolated in 2002 during a study of the yeast communities associated with ARD in the abandoned São Domingos pyrite mine (Gadanho *et al.*, 2006). For yeast isolation, 500 ml water was filtered through 0.45 µm pore-size sterile nitrocellulose filters (for details see Gadanho *et al.*, 2006).

For morphological characterization, cultures were grown on MYP agar (1^l: 7.0 g malt extract, 0.5 g yeast extract, 2.5 g peptone-soytone, 15.0 g agar) at room temperature (20–23 °C) and studied with phase-contrast optics using an Olympus BX50 microscope. Physiological and biochemical characterization was performed according to the techniques described by Yarrow (1998). Additional assimilation tests with aldaric acids and aromatic compounds were performed as described by Fonseca (1992) and Sampaio (1999), respectively.

Sequences of the D1/D2 region of the large-subunit rRNA gene obtained previously (Gadanho *et al.*, 2006) were aligned with reference sequences from the Filobasidiales retrieved from the GenBank database. Alignments were made using CLUSTAL_X 1.83 (Thompson *et al.*, 1997) and corrected visually. Phylogenetic trees were computed with the PAUP software (Swofford, 2001), using the neighbour-joining method (Saitou & Nei, 1987). Distances between sequences were calculated using Kimura's two-parameter model (Kimura, 1980) and bootstrap analysis (Felsenstein, 1985) was based on 1000 random resamplings.

Phylogenetic placement and ecological significance

As shown in Fig. 1, the three novel species described in this study are phylogenetically related and form a well-supported clade. Interspecific nucleotide sequence variability of the D1/D2 region was observed for the novel

species. Four nucleotide substitutions were observed between *C. aciditolerans* sp. nov. CBS 10872^T and *C. metallitolerans* sp. nov. CBS 10873^T and 22–24 substitutions were detected between these strains and *C. ibericus* sp. nov. CBS 10871^T. In the ITS regions, 6 differences were recorded between *C. aciditolerans* sp. nov. CBS 10872^T and *C. metallitolerans* sp. nov. CBS 10873^T, while these two strains and *C. ibericus* sp. nov. CBS 10871^T showed a number of mismatches and gaps too large to obtain a reliable alignment. As shown in Fig. 1, the closest relatives of the novel species are *C. gilvescens* and *C. gastricus*.

Multiple isolates were obtained for the three species (19 isolates for *C. aciditolerans* sp. nov. and 21 isolates each for *C. ibericus* sp. nov. and *C. metallitolerans* sp. nov.). Collections were done in different periods of the year and at different sampling sites in São Domingos which were previously designated Open Pit, Achada do Gamo and Pond A (Gadanho *et al.*, 2006). Considering the prevailing physico-chemical parameters of these sites, they were recorded as sites of low, intermediate and high environmental stress (see Fig. 5 of Gadanho *et al.*, 2006). Table 1 depicts the occurrence of the three novel species in the various sampling sites during the survey. *C. aciditolerans* strains were found in Achada do Gamo, that had a pH of 1.8 and Fe and Cu concentrations greater than 18 and 1.5 g l⁻¹, respectively, and was the most extreme acidic environment surveyed. This species was considered one of the three most tolerant yeast species found in São Domingos (Gadanho *et al.*, 2006). *C. aciditolerans* and *C. ibericus* strains were collected in different seasons of the year and were also detected in Rio Tinto (Gadanho *et al.*, 2006). *C. metallitolerans* strains were obtained only in the autumn sampling in the Open Pit and in Pond A. Altogether, our results suggest that these yeasts are autochthonous to ARD environments, since they have not been found in other environments and were repeatedly isolated from ARD waters. Ongoing experiments focusing on adaptations to extreme acidic environments have revealed polymetallic resistance profiles for the three species described in this report. The novel species were found to be resistant to increased concentrations of iron, arsenite, arsenate, cadmium, copper and zinc and, in contrast to control yeasts, were able to grow in sterilized ARD water supplemented with carbon and nitrogen sources (data not shown).

The closest relatives of the novel species, *C. gilvescens* and *C. gastricus*, have been isolated from substrates unrelated to ARD environments (soil, rumen and the stomach of a tuberculosis patient). More than 50 nucleotide substitutions were detected between the three novel species and *C. gilvescens* and *C. gastricus*. Therefore, we use the designation 'ecoclade' to refer to this peculiar combination of separate phylogenetic placement and distinct ecology. Examples of other species forming ecoclares related with extreme environments are the psychrophiles *Rhodotorula glacialis* and *Rhodotorula psychrophila* recently described by Margesin *et al.* (2007).

Fig. 1. Phylogenetic tree of strains of *Cryptococcus aciditolerans* sp. nov., *Cryptococcus metallitolerans* sp. nov. and *Cryptococcus ibericus* sp. nov. and related taxa of the Filobasidiales. Neighbour-joining analysis of an alignment of the D1/D2 region of the 26S rRNA gene. The topology was rooted with the sequences of *Trichosporon cutaneum* CBS 2466^T, *T. jirovecii* CBS 6864^T and *T. moniliiforme* CBS 2467^T. Numbers on branches are bootstrap percentages from 1000 replicates. GenBank accession numbers are indicated. *F.*, *Filobasidium*; *T.*, *Trichosporon*; *B.*, *Bullera*. Bar, 0.01 nucleotide substitutions per site.

Table 1. Occurrence of strains of the novel species in three sampling sites

The sampling sites can be classified as high (Achada do Gamo), intermediate (Pond A) or low (Open Pit) environmental stress based on measurements of pH and heavy metals. ND, Not determined.

Sampling site	<i>C. ibericus</i>	<i>C. metallitolerans</i>	<i>C. aciditolerans</i>
Spring			
Open Pit	—	—	—
Pond A	+	—	—
Achada do Gamo	—	—	—
Summer			
Open Pit	+	—	—
Pond A	+	—	+
Achada do Gamo	ND	ND	ND
Autumn			
Open Pit	—	+	+
Pond A	+	+	—
Achada do Gamo	—	—	+

Phenotypic properties

The members of the novel species form cream-coloured colonies when incubated at room temperature. However, *C. aciditolerans* sp. nov. and *C. metallitolerans* sp. nov. show faint pink pigmentation of the colonies after 1 week at 4 °C.

Mating experiments involving various isolates of each of the three novel species were performed on cornmeal agar at 18 °C and were always negative. The phenotypic characteristics that differentiate these novel species are shown in Table 2. For *C. aciditolerans*, the relevant phenotypic properties are the assimilation of D-arabinose and galactitol. For *C. metallitolerans*, the assimilation of L-sorbose, D-ribose and D-gluconate is distinctive and, for *C. ibericus*, salient tests are the assimilation of L-rhamnose, methyl α-D-glucoside and the ability to grow at 35 °C. None of these species was able to assimilate nitrate or nitrite as sole nitrogen source. This phenotypic characteristic is rare in the Filobasidiales and, up to now, has been found only in *C. gilvescens*, *C. gastricus* and *Cryptococcus magnus*. Therefore, except for *C. magnus*, which is included in the floriforme clade (Scorzetti *et al.*, 2002), all nitrate-negative species are allocated to the gastricus clade and in the ARD ecoclade. Phenotypic differentiation between all the

Table 2. Phenotypic characteristics that differentiate nitrate-/nitrite-negative species of the Filobasidiales

Species: 1, *C. aciditolerans* sp. nov.; 2, *C. metallitolerans* sp. nov.; 3, *C. ibericus* sp. nov.; 4, *C. gilvescens*; 5, *C. gastricus*; 6, *C. magnus*. Data for reference species were obtained from the Centraalbureau voor Schimmelcultures. +, Positive; v, variable between strains; –, negative.

Characteristic	1	2	3	4	5	6
Carbon assimilation						
L-Sorbose	–	+	–	–	–	+
D-Ribose	–	+	–	–	–	–
D-Arabinose	+	–	–	–	–	+
L-Rhamnose	–	–	+	v	+	–
Methyl α -D-glucoside	–	–	+	–	+	+
Melezitose	+	–	+	+	+	+
Ribitol	+	–	–	–	–	–
Xylitol	+	+	–	–	+	+
Galactitol	+	–	–	–	–	–
D-Gluconate	–	+	–	+	+	+
Citric acid	+	–	+	–	+	–
Growth at 35 and 37 °C	–	–	+	–	–	–

nitrate-/nitrite-negative species of the Filobasidiales can be accomplished as shown in Table 2. These characteristics, together with their inability to assimilate nitrate or nitrite, enable the differentiation of the novel *Cryptococcus* species from the remaining yeasts of the Filobasidiales. Furthermore, except for *Filobasidium uniguttulatum*, the members of the Filobasidiales are able to utilize several aromatic compounds (Sampaio, 2004). None of the novel species was able to grow with any of the aromatic compounds tested as sole carbon sources.

Latin diagnosis of *Cryptococcus ibericus* Gadanho et Sampaio sp. nov.

In agar MYP post 5 dies ad 22 °C cellulae ovals (2.5–4.0 × 3.0–5.0 µm), singulae, aut binae. Cellulae polariter gemmantes, binae cohaerentes. Cultura crenea, viscosa, margine integra, nec pseudohyphae nec hyphae formantur. Teleomorphe ignota. Characteres biochemici physiologici in tabula 2 describuntur. Typus: SDY 022^T (=CBS 10871^T).

Description of *Cryptococcus ibericus* Gadanho et Sampaio sp. nov.

Cryptococcus ibericus (i.be'ri.cus. L. masc. adj. *ibericus* Iberian, relating to the geographical site of isolation of the first strains, the Iberian Peninsula).

After 5 days of growth on MYP agar at 22 °C, cells are ovoid (2.5–4.0 × 3.0–5.0 µm) and occur singly or in pairs. Budding is polar. Colonies are creamy on MYP agar, butyrous and with entire margins. No pseudomycelium or true hyphae are formed. The following carbon compounds

are assimilated: D-glucose, D-galactose, D-glucosamine, D-xylose, L-arabinose, L-rhamnose, sucrose, maltose, trehalose, methyl α -D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, erythritol, D-glucitol, D-mannitol, *myo*-inositol, glucono- δ -lactone, D-glucuronate, succinic acid and citric acid. No growth occurs on L-sorbose, D-ribose, D-arabinose, inulin, soluble starch, glycerol, ribitol, galactitol, D-gluconate, DL-lactate, ethanol or methanol. Assimilation of the nitrogen compounds nitrate, nitrite, ethylamine, creatine, creatinine and cadaverine is negative. D-Glucosamine is assimilated. Growth occurs in the presence of 100 mg cycloheximide l⁻¹ but not 1000 mg l⁻¹. Urea hydrolysis is positive. Diazonium blue B reaction is positive. No starch-like substances are produced. Growth occurs at 35 and 37 °C but not at 40 °C.

The type strain, SDY 022^T (=CBS 10871^T), was isolated from ARD collected in Pond A, Moitinha, São Domingos mine, Portugal, in April 2002. Additional strains were obtained from the same site in different seasons of the year and also from the Open Pit (September and December 2002). Strains belonging to this species were also obtained from the Rio Tinto, Spain.

Latin diagnosis of *Cryptococcus aciditolerans* Gadanho et Sampaio sp. nov.

In agar MYP post 5 dies ad 22 °C cellulae ovals (3.5–5.5 × 4.0–6.5 µm), singulae, aut binae. Cellulae polariter gemmantes, binae cohaerentes. Cultura crenea, viscosa, margine integra, nec pseudohyphae nec hyphae formantur; post 10 dies 4 °C subrosea. Teleomorphe ignota. Characteres biochemici physiologici in tabula 2 describuntur. Typus: SDY 081^T (=CBS 10872^T).

Description of *Cryptococcus aciditolerans* Gadanho et Sampaio sp. nov.

Cryptococcus aciditolerans (a.ci.di.tol'er.ans. N.L. neut. n. *acidum* acid; L. pres. part. *tolerans* tolerating; N.L. part. adj. *aciditolerans* acid-tolerating).

After 5 days of growth on MYP agar at 22 °C, cells are ovoid to ellipsoid (3.5–5.5 × 4.0–6.5 µm) and occur singly or in pairs. Budding is polar. Colonies are creamy on MYP agar, butyrous and with entire margins. Old cultures incubated at 4 °C become slightly pink. No pseudomycelium or true hyphae are formed. The following carbon compounds are assimilated: D-glucose, D-galactose, D-glucosamine, D-xylose, L-arabinose, D-arabinose, sucrose, maltose, trehalose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, erythritol, ribitol, xylitol, D-glucitol, D-mannitol, *myo*-inositol, galactitol, glucono- δ -lactone, D-glucuronate, succinic acid and citric acid. No growth occurs on L-sorbose, D-ribose, L-rhamnose, methyl α -D-glucoside, inulin, soluble starch, glycerol, D-gluconate, DL-lactate, ethanol or methanol. Assimilation of the nitrogen compounds nitrate, nitrite, ethylamine, creatine, creatinine and cadaverine is negative. D-Glucosamine is assimilated.

Growth occurs in the presence of 100 mg cycloheximide l^{-1} but not 1000 mg l^{-1} . Urea hydrolysis is positive. Diazonium blue B reaction is positive. No starch-like substances are produced. Growth occurs at 30 °C, but not at 35 °C.

The type strain, SDY 081^T (=CBS 10872^T), was isolated from ARD collected in Pond A, Moitinha, São Domingos mine, Portugal (September 2002). Additional strains were obtained from the same site in different seasons of the year and also from the Open Pit and at Achada do Gamo (December 2002).

Latin diagnosis of *Cryptococcus metallitolerans* Gadanho et Sampaio sp. nov.

In agar MYP post 5 dies ad 22 °C cellulae ovaes (3.5–5.5 × 4.0–6.5 µm), singulae, aut binae. Cellulae polariter gemmantes, binae cohaerentes. Cultura crenea, viscosa, margine integra, nec pseudohyphae nec hyphae formantur; post 10 dies 4 °C subrosea. Teleomorpha ignota. Characteres biochemici physiologici in tabula 2 describuntur. Typus: SDY 190^T (=CBS 10873^T).

Description of *Cryptococcus metallitolerans* Gadanho et Sampaio sp. nov.

Cryptococcus metallitolerans (me.ta.li.to'ler.ans. L. n. *metallum* metal; L. pres. part. *tolerans* tolerating; N.L. part. adj. *metallitolerans* metal-tolerating).

After 5 days of growth on MYP agar at 22 °C, cells are ovoid to ellipsoid (3.5–5.5 × 4.0–6.5 µm) and occur singly or in pairs. Budding is polar. Colonies are creamy on MYP agar, butyrous and with entire margins. Old cultures incubated at 4 °C become slightly pink. No pseudomycelium or true hyphae are formed. The following carbon compounds are assimilated: D-glucose, D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, sucrose, maltose, trehalose, cellobiose, salicin, melibiose, lactose, raffinose, erythritol, xylitol, D-glucitol, D-mannitol, myo-inositol, glucono-δ-lactone, D-gluconate, D-glucuronate and succinic acid. No growth occurs on D-arabinose, L-rhamnose, methyl α-D-glucoside, melezitose, inulin, soluble starch, ribitol, glycerol, galactitol, DL-lactate, citric acid, ethanol or methanol. Assimilation of the nitrogen compounds nitrate, nitrite, ethylamine, creatine, creatinine and cadaverine is negative. D-Glucosamine is assimilated. Growth occurs in the presence of 100 mg cycloheximide l^{-1} but not 1000 mg l^{-1} . Urea hydrolysis is positive. Diazonium blue B reaction is positive. No starch-like substances are produced. Growth occurs at 30 °C, but not at 35 °C.

The type strain, SDY 190^T (=CBS 10873^T), was isolated from ARD collected at Achada do Gamo, São Domingos mine, Portugal (December 2002). Additional strains were obtained from the Open Pit (December 2002).

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