System. Appl. Microbiol. 22, 97–105 (1999)

http://www.urbanfischer.de/journals/sam

SYSTEMATIC AND APPLIED MICROBIOLOGY

Polyphasic Taxonomy of a Novel Yeast Isolated from Antarctic Environment; Description of *Cryptococcus victoriae* sp. nov.

Mª Jesús Montes[1], Carmela Belloch[2], Monica Galiana[2], Mª Dolores Garcia[2], Carmen Andrés[1], Santiago Ferrer[1], Jose Mª Torres-Rodriguez[3], and Jesús Guinea[1]

[1]Unidad de Microbiología, Facultad de Farmacia, Universidad de Barcelona, España
[2]Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, España
[3]Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Espanya

Received December 4, 1998

Summary

In 1992 some samples of mosses, lichens and soils were collected from Botany Bay, Southern Victoria Land (77° 01′ S 162° 32′ E) and, as a result of a routine screening programme some yeasts were isolated. One of them, designated as strain G5, showed marked differences when compared to other antarctic yeasts. According to morphological and physiological characteristics, we were able to identify the strain G5 as a yeast belonging to the genus *Cryptococcus*. Some characteristics of this genus are the growth response to myo-inositol, celobiose, raffinose and D-glucuronate, no-fermentation, the absence of mycelium and pseudomycelium, asexual reproduction, Diazolium blue B test (DBB) and urea hydrolisis positive and the growth without vitamines. This strain (G5) formed cream colonies of slimy appearance with cells of 3×2 µm in size, that grew between 4 °C and 20 °C. The G+C content of strain G5 was 50.3 mol%. The molecular characterization by whole-cell proteins and RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (5.8S-ITS region), revealed that this strain was different from other antarctic species of this genus. The phylogenetic tree deduced from the 5.8S rRNA gene sequence showed the strain G5 as a member of the genus *Cryptococcus*, clearly separated from other basidiomycetous yeasts. On the basis of the physiological, genotypical and phylogenetical data, the new isolate G5 was described as *Cryptococcus victoriae*, sp. nov., with the type strain G5 (= CECT 11114).

Key words: Antarctic yeast – *Cryptococcus victoriae* – G+C – whole-cell protein electrophoresis, – 5.8S-ITS RFLP – 5.8S rRNA gene sequence – phylogeny

Introduction

The genus *Cryptococcus* is a heterogeneous group of non-fermentative, encapsulated yeast species, which includes strains isolated from diverse substrates such as food, air, water, soil, insects, and several human pathogens (Kreger-Van Rij, 1984). Most do not produce a sexual state and are identified only by their asexual reproductive state (anamorph). Due to the lack of a known sexual state, most of the species belonging to the genus *Cryptococcus* are defined by a combination of morphological and physiological characteristics, which include the absence of pseudomycelia and red pigmented colonies, lack of fermentation, production of amylose and ultrastructure of the septal pore (Kreger-Van Rij, 1984; Barnett et al., 1990).

Attending mainly to the features above, the G+C content and DNA/DNA reassociation some new species of *Cryptococcus* isolated from antarctic environments, have been described in recent years (Baharaeen and Vishniac, 1982; Vishniac and Baharaeen, 1982; Vishniac and Hempfling, 1979a and b; Vishniac and Kurtzman, 1992).

Morphological and cultural characteristics, as well as biochemical properties are the principal criteria used in yeast taxonomy for the classification of yeast species, and among them, the electrophoretic whole-cell protein patterns have revealed as a convenient and valuable tool for the establishment of intra- and intergeneric relationships (Vancanneyt et al., 1991 and 1994).

Recent studies have demonstrated that taxonomy of yeasts based exclusively on anamorphic features is artificial, may lead to the misclassification of species and does not reflect true phylogenetic relationships (Kurtzman, 1993 and 1994; Vilgalys and Hester, 1990; White et al., 1990). The most significant contribution to the re-

0723-2020/99/22/01-097 $ 12.00/0

construction of the basidiomycetous yeast evolutionary tree has come from ribosomal DNA sequencing (FELL and KURTZMAN, 1990; GUEHO et al., 1989; KWONG-CHUNG and CHANG, 1994; MITCHELL et al., 1992; SUH and SUGIYAMA, 1993; VAN DER PEER et al., 1992; WILMOTTE et al., 1993). The internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene are known for their value as evolutionary markers, since they contain both low and high conserved regions and exhibit far greater interspecific differences than 18S and 25S rRNA genes (BELLOCH et al., 1998; GARDES and BRUNS, 1993; GUILLAMON et al., 1998; JAMES et al., 1996; LEE and TAYLOR, 1992; MITCHELL et al., 1992; MOLINA et al., 1992; NGUYEN et al., 1997; ODA et al., 1997; REDECKER et al., 1997; VALENTE et al., 1996; WINGFIELD et al., 1996).

In this comparative study we used phenotypic, genotypic and phylogenetic data. The whole-cell protein electrophoresis and the restriction analysis of the 5.8S rRNA gene and ITS1 and ITS2 spacers (5.8S-ITS region), were used to determine the similarity between related species of the genus *Cryptococcus*, and the comparison of 5.8S rRNA gene sequences to infer a phylogenetic tree reflecting their relationships with other basidiomycetous yeast. Our results show that all antarctic species described by VISHNIAC et al., (BAHARAEEN and VISHNIAC, 1982; VISHNIAC and BAHARAEEN, 1982; VISHNIAC and HEMPFLING, 1979 a and b; VISHNIAC and KURTZMAN, 1992), represent a very close group of species. However, the strain G5 (= CETC 11114), appears in a separate branch, constituting a different species, which we propose as the new species: *Cryptococcus victoriae*.

Materials and Methods

Yeast strains: Table 1 shows the 15 type strains representing the *Cryptococcus* species isolated from antarctic soil, obtained from the Spanish Type Culture Collection (CECT, University of Valencia, Spain).

The isolate G5 was recovered from a sample of mosses, lichens and soil collected at Botany Bay, Cape Geology, Granite Harbour, Southern Victoria Land, Antarctica (77° 01′ S, 162° 32′ E) (PAYNE et al., 1975), and was isolated on dextrose-agar plates supplemented with 30 µg/ml of tetracycline and 20 µg/ml of dieldrin (acaricide) aerobically incubated at 4, 14, 22 and 30 °C for three weeks.

Morphology: Cell size and morphology were determined by scanning electron microscopy (Hitachi S 3200), from a culture on Sabouraud-Dextrose broth at 15 °C. Negative staining was used in order to demonstrate capsule presence or absence; the cells stained with nigrosine were viewed using a phase contrast microscope.

Physiological tests: The physiological characterization of the strain G5 was carried out according to the methods currently used in yeast taxonomy (KREGER-VAN RIJ, 1984; BARNETT et al., 1990), complemented with some other tests, as growth on sunflower seeds at 4 °C. The yeast identification computer program by VELAZQUEZ et al. (1993) was also used to perform the identification of the strain G5.

Serotyping: Agglutination reactions with specific antisera anti-A, B, C and D of *Cryptococcus neoformans* (Chipto check System, Iatron, Japan) were also carried out.

SDS-Page of whole-cell proteins: Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed by using the method of KERSTERS and DE LEY (1980), later modified by KERSTERS (1985). Whole-cell protein samples were prepared from five days old cultures at 15 °C, according to the method of POT and KERSTERS (1991).

DNA Composition: The base DNA composition was determined according to MARMUR and DOTY (1962) and MANDEL and MARMUR (1968).

DNA preparation and amplification: Total genomic DNA was obtained according to the method described by QUEROL et al. (1992) with slight modifications, and diluted to 50–100 ng/µl. The Internal Transcribed Spacer regions (ITS 1 and ITS 2) and 5.8S rRNA gene were amplified in an Eppendorf 3130 Thermal Cycler, by using the primer pairs ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCT-

Table 1. *Cryptococcus* yeast strains utilised in the present study.

Species	Strain designation	Isolation source
C. albidosimilis	CECT 11117 = NRRL Y-17463	soil, Antarctica
C. antarcticus	CECT 11118 = NRRL Y-17461	soil, Antarctica
C. asgardensis	CECT 11088 = NRRL Y-17202	soil, Antarctica
C. baldrensis	CECT 11087 = NRRL Y-17203	soil, Antarctica
C. consortionis	CECT 11142 = NRRL Y-17204	soil, Antarctica
C. friedmanii	CECT 11161 = CBS 7160	soil, Antarctica
C. hempflinglii	CECT 11089 = NRRL Y-17207	soil, Antarctica
C. lupi	CECT 11083 = NRRL Y-17187	soil, Antarctica
C. socialis	CECT 11160 = CBS 7158	soil, Antarctica
C. tyrolensis	CECT 11090 = NRRL Y-17188	soil, Antarctica
C. vishniacii var. *asocialis*	CECT 11985 = NRRL Y-17190	soil, Antarctica
C. vishniacii var. *vladimirii*	CECT 11086 = NRRL Y-17209	soil, Antarctica
C. vishniacii var. *wolfii*	CECT 11084 = NRRL Y-17210	soil, Antarctica
C. vishniacii var. *vishniacii*	CECT 11082 = NRRL Y-17208	soil, Antarctica
C. wrightensis	CECT 11091 = NRRL Y-17189	soil, Antarctica
Cryptococcus sp.	strain G5 = CECT 11114	soil, Antarctica

CBSCBS – Centraalbureau voor Schimmelcultures, Delft, The Netherlands; CECT – Colección Española de Culticos Tipo, Spanish Type Culture Collection, University of Valencia, Valencia, Spain; NRRL – Northern Regional Research Laboratory, US Department of Agriculture, Peoria, Illinois, USA.

Table 2. Biochemical and physiological characteristics of G5 and reference strains of the *Cryptococcus* genus. *C. asgardensis* (C1), *C. baldrensis* (C2), *C. hempflingii* (C3), *C. lupi* (C4), *C. tyrolensis* (C5), *C. wrightensis* (C6), *C. vishniacii* var. *vishniacii* (C7), *C. vishniacii* var. *vladimirii* (C8), *C. vishniacii* var. *asocialis* (C9), *C. vishniacii* var. *wolfii* (C10), *C. albidosimilis* (C11), *C. antarcticus* (C12), *C. consortionis* (C13), *C. friedmanii* (C14), *C. socialis* (C15).

Character	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	G5
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+
D-Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
L-Sorbose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+
L-Arabinose	+	+	–	d	+	–	d	+	–	d	+	–	d	+	+	+
D-Arabinose	–	–	–	–	–	–	d	d	–	–	–	–	–	–	–	+
L-Rhamnose	d	–	–	–	–	–	–	d	d	d	+	+	–	–	–	+
Sucrose	+	+	+	+	+	+	+	+	+	d	+	+	d	v	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Trehalose	d	d	d	d	d	d	d	d	d	d	d	+	d	+	+	+
Cellobiose	–	+	–	+	–	+	–	+	–	–	+	+	–	+	+	+
Arbutin	d	+	+	d	+	+	d	+	d	d	+	+	d	+	+	+
Melibiose	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	+
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Raffinose	+	+	d	+	d	+	+	+	+	–	–	+	d	–	+	+
Melezitose	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	+
Inulin	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	+
Starch	+	+	d	+	+	+	+	+	+	+	+	+	–	+	+	+
Glycerol	d	d	d	d	–	d	d	d	d	–	+	+	–	–	–	+
Erythritol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Ribitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Xylitol	–	–	–	–	–	–	–	–	–	–	+	d	–	–	–	+
D-Glucitol	–	–	–	–	–	–	–	–	–	–	+	d	–	–	–	+
D-Mannitol	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+
Galactitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
myo-Inositol	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+
2-Keto-D-Gluconate	+	+	+	+	+	+	+	+	+	+	+	+	d	+	d	+
5-Keto-D-Gluconate													?	–	?	+
D-Gluconate	–	–	–	–	–	–	–	–	–	–	+	+	–	d	–	+
D-Glucuronate	+	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+
DL-Lactato	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Succinate	–	–	–	–	–	–	–	–	–	d	+	+	–	–	d	+
Citrate	–	–	–	–	–	–	–	–	–	–	+	–	–	d	–	+
Methanol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ethanol	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–
Propane 1,2 diol	–	–	–	–	–	–	–	–	–	–	?	?	–	?	–	–
Butane 2,3 diol	–	–	–	–	–	–	–	–	–	–	?	?	–	?	–	–
Nitrate	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+
Nitrite	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Ethylamina	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
L-Lysine	–	–	–	–	–	d	–	–	–	d	+	d	–	–	–	+
Cadaverine	–	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–
Creathinie	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+
Creatine	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+
Imidazole	–	–	–	–	–	–	–	–	–	–	?	?	–	–	–	–
Growth w/o vitamine	+	+	+	v	+	v	v	?	?	?	–	+	d	d	d	+
Growth at 25 °C	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
Growth at 30 °C	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
0,01% Cycloheximide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0,1% Cycloheximide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1% Acetic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
50% Glucose	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Starch Formation	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–
Urea Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+
DBB reaction	++	+	–	d	–	+	+	+	+	+	+	+	–	+	–	+

+ positive; – negative; d – weak response; ? no data available; v – variable according to BARNETT et al. (1990).

Table 3. 5.8S rRNA gene sequences obtained from the EMBL library.

Species	EMBL accession number
Filobasidium floriforme	M94515
Filobasidium uniguttulatus	M94520
Filobasidium capsuligenum	M94513
Cystofilobasidium capitatum	M94512
Cystofilobasidium bisporidii	M94511
Filobasidiella depauperata	M94514
Filobasidiella neoformans	L14067
Cryptococcus neoformans	L14068

TATTGATATGC 3') described by WHITE et al. (1990). PCR reactions were as follows: initial denaturation at 94 °C for 5 minutes, 35 cycles of 94 °C 40 seconds, 55 °C 40 seconds and 72 °C 30 seconds, with a final 10 minutes step at 72 °C.

Restriction analysis of PCR products: The PCR products were digested, without further purification, with 7 restriction endonucleases (Boehringer-Mannheim): the four-cutter enzymes *Alu*I, *Cfo*I, *Hae*III, *Taq*I and *Tru*9I, the 5pb recognising enzyme *Hin*fI, and *Eco*RI that has a 6 pb recognition site. The restriction fragments were separated by electrophoresis on 3% agarose gels with TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0). After electrophoresis, gels were stained with ethidium bromide (0.5 mg/ml), and the DNA bands visualised under UV light and compared against a 100 bp ladder DNA standard (Gibco-BRL).

Restriction site map was obtained by means of double digests, and lately verified by comparison with the ITS-5.8S region sequences of the species *C. antarcticus*, *C. friedmanii*, *C. vishniacii* and *C. victoriae* determined in this study.

DNA cleaning and direct sequencing: DNA amplification products were purified using the GeneClean Purification Kit (Bio101) and directly sequenced using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) in an Applied Biosystems model 373A Automatic DNA Sequencer. The primers ITS1 and ITS4 together with two additional primers, ITS2 (5' GCTGCGTTCTTCATCGATGC 3') and ITS3 (5' GCATCGATGAACGCAGC 3') were used to obtain the sequence of both DNA strands.

Phylogenetic tree reconstruction using the 5.8S rRNA gene sequence: The 5.8S rRNA gene sequences of species representing the genera *Cystofilobasidium*, *Filobasidium* and *Filobasidiella* were obtained from the EMBL data library and their accession numbers are shown in Table 3. The 5.8S rRNA gene sequences of the strains used for the phylogenetic analysis were aligned automatically using the multiple-sequence alignment program CLUSTAL W (THOMPSON et al., 1994), and were subsequently manually corrected. The genetic distances were calculated using the Kimura two-parameter model of the program MEGA v. 1.0 (KUMAR et al., 1993). An unrooted phylogenetic tree was constructed by using the Neighbour-Joining method (SAITOU and NEI, 1987) contained in MEGA v. 1.0 program. The confidence values of branches were determined by performing a bootstrap analysis in which 1000 replicates were used.

Results

Morphology

The strain G5 (= CECT 11114) is a yeast which gives rise to slimy colonies, whose cellular size ranges between 3 µm in length and 2 µm in diameter (Figure 1). Cells are encapsulated (Figure 2). Sexual reproduction has not been observed. Neither mycelium nor pseudomycelium is formed.

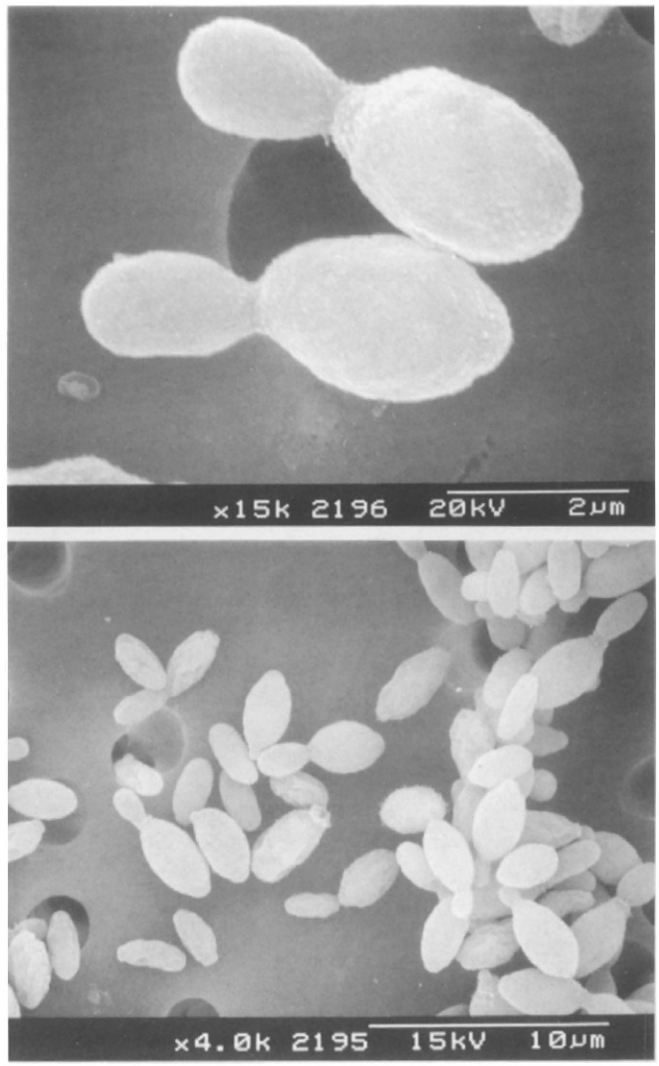

Fig. 1. Morphology of strain G5 cells as determined by scanning electron microscopy.

Phenotypic characterization

Table 2 shows the phenotypic characteristics of strain G5 and of the 15 type strains representative of the *Cryptococcus* antarctic species. Most strains assimilate D-xylose, maltose, arbutin, melezitose, starch and 2-keto-gluconate, though sometimes weakly. Growth response to myo-inositol, cellobiose, and raffinose were variable. All the strains assimilate D-glucuronate as a sole carbon souce, indicating that our results are in agreement with the suggestion of GOLUBEV et al. (1974) to use this test as diagnostic character for *Cryptococcus* species.

Only *C. albidosimilis* grew at 30 °C although growth at 25 °C was detected in *C. antarcticus* also. All the strains are inhibited by cycloheximide and acetic acid. Growth on

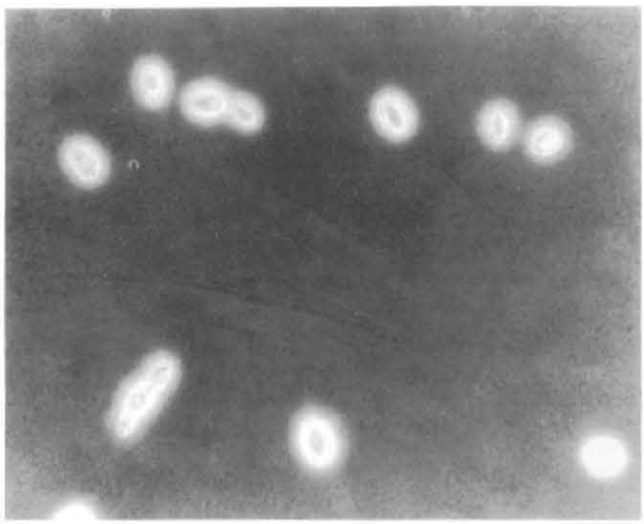

Fig. 2. Presence of capsule in strain G5, cells stained with nirgrosine and viewed using a phase contrast microscope. (Magnification ×200).

50% glucose was detected in *C. friedmanii* only. The hydrolysis of urea and the DBB test are positive for the majority of the strains. The comparison of the physiological traits indicates that strain G5 is similar to *C. albidosimilis* and *C. antarcticus*. Finally, production of melanic pigment has not been observed on sunflower seeds at 4 °C.

Based on the results above, as well as absence of fermentation and sexual reproduction, indicated that the isolate G5 is a member of the genus *Cryptococcus*.

Serology

All the results of the tests with specific antisera anti-A, B, C and D of *Cryptococcus neoformans* are negative.

SDS-Page of whole-cell proteins

The total protein profile (Figure 3) indicates the singularity of the isolated strain G5, confirming the differences between the species *C. albidosimilis* and *C. antarcticus*, which share more physiological characteristics with G5.

DNA base composition

The G+C content determined in this study, 50.3 mol%, is in the range of the other antarctic *Cryptococcus* species.

DNA amplification and restriction analysis

The PCR products from all strains were 620 bp long, except for *C. friedmanii*, 650 bp and strain G5, 525 bp. After PCR amplification and without further purification, the amplified fragments were digested with the following restriction endonucleases: *Alu*I, *Cfo*I, *Eco*RI, *Hae*III, *Hin*fI, *Taq*I and *Tru*9I. The map of the amplified region of the rDNA (Figure 4) shows the location of oligonucleotide primers and the cleavage sites of the restriction endonucleases. The position of the different restriction sites was common to the majority of the species, with the exception of the species *C. antarcticus* and *C. friedmanii*,

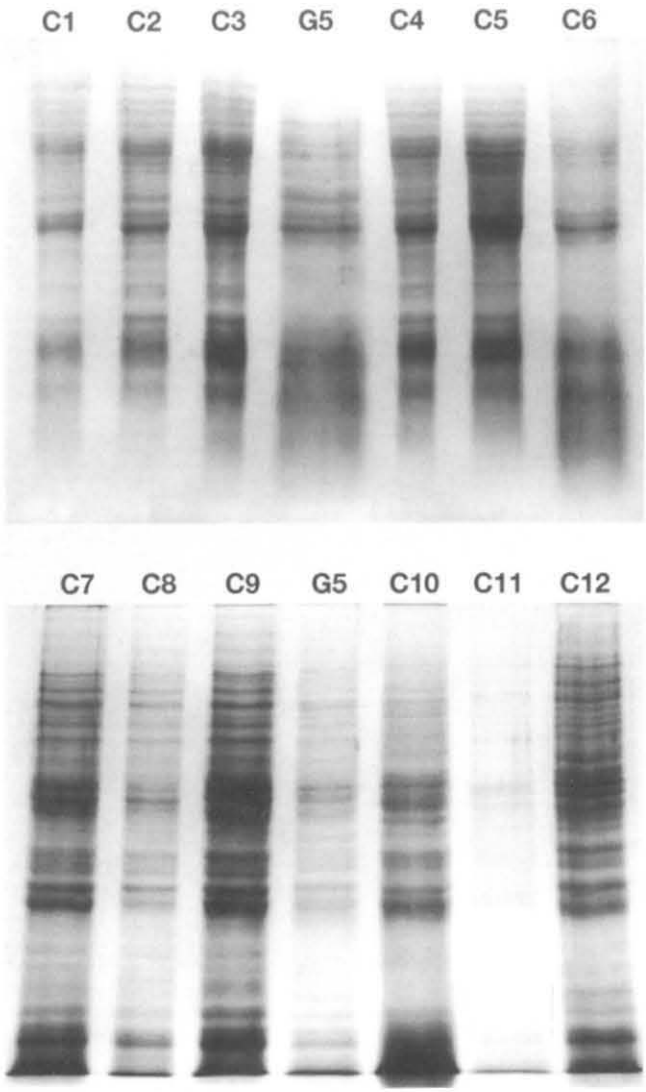

Fig. 3. Profiles of protein after PAGE of: *C. lupi* (C1), *C. hempflingii* (C2), *C. vishniacii* var. *vishniacii* (C3), G5, *C. tyrolensis* (C4), *C. baldrensis* (C5), *C. wrightensis* (C6), *C. socialis* (C7), *C. asgardensis* (C8), *C. friedmanii* (C9), G5, *C. antarticus* (C10), *C. albidosimilis* (C11), *C. consortionis* (C12).

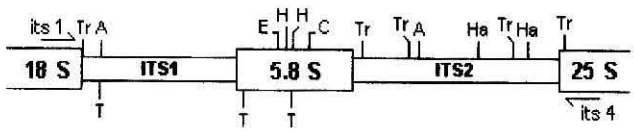

Fig. 4. Restriction map of the 5.8S rRNA gene and ITS regions from the 16 species of the genus *Cryptococcus*. Abbreviations for the restriction enzymes are A: *Alu*I; C: *Cfo*I; E: *Eco*RV; H: *Hin*fI; Ha: *Hae*III; T: *Taq*I; Tr: *Tru*9I.

that lacked the *Tru*9I site immediately after the 5.8S rDNA gene, and the presence of an additional *Tru*9I site located in the ITS1 region of *C. friedmanii*. The strain G5 (= CECT 11114), presented a very distinct restriction pattern, lacking most of the restriction sites located along the internal transcribed spacers ITS1 and ITS2.

Sequence analysis

The differences deduced by RFLP analysis of the PCR products from the species *C. antarcticus*, *C. friedmanii*, *C. vishniacii* and strain G5, were confirmed by comparison of the ITS1 and ITS2 regions and 5.8S rRNA gene sequences. The aligned sequences form these species are presented in Figure 5. Our results show that comparison of the ITS1 regions produces similarity values ranging from 83% to 96% between the most similar species *C. antarticus*, *C. vishniacii* and *C. friedmanii*. However, lower similarity values were calculated when comparing strain G5 (= CECT 11114) with the other species (61% to 62%). The analysis of ITS2 sequences revealed higher similarity values, ranging from 96% to 98% between the most similar species, while comparison of these species with strain G5 (= CECT 11114) produces values of 73% (higher than for ITS1), in spite of the 60 bp deletion in strain G5 (= CECT 11114).

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C. antarcticus   AAGGATCATT AATGATTG-- TATGCCTGTC GAGCTTGCTC ACAGGATTTT ACTCATATCC
C. friedmanii    .......... .....A.ACA G......... .......... ..G..C...C TA.......
C. vishniacii    .......... .......... A.C.T..... .......... .....C..A. ---.......
C. victoriae     .......... ...------.. AG....CTCT ..TGCAAA.. .TT..G..AG -------...T

C. antarcticus   ATAACACCTG TGCACTTGTC GG-ATGGCTT AGTGAAGACC G--CAAGGTT GGAAC---TA
C. friedmanii    .......... .........T ......TTC. ..A..C.TAG .GTT..ACC. .A.GTCAA..
C. vishniacii    .......... .......... ..G....... .......... .......... ...T......
C. victoriae     GC----T..C .T.G.AA.AA .A.GG.TT.C CA.AC.C... ...-TGAAC. .TGG....--

C. antarcticus   TCCATCTACT T-TACATAAC AATCCTGTAA CAAATGTAGT CTTATTATAA CATAATAAAA
C. friedmanii    GT....C... .AC..TA... ...AA..... .......... .......... ..A.......
C. vishniacii    ...G..... .......... .......... .......... .......... ..........
C. victoriae     .T.GG.C.TC A.---CA... TG.TA-.... TG......A. A.C..A.C.- -.A..C....

C. antarcticus   CTTTCAACAA CGGATCTCTT GGCTCTCGCA TCGATGAAGA ACGCAGCGAA ATGCGATAAG
C. friedmanii    ...C..... .......... .......... .......... .......... ..........
C. vishniacii    ...C..... .......... .......... .......... .......... ..........
C. victoriae     ....T..... .......... .......... .......... .......... ..........

C. antarcticus   TAATGTGAAT TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACCTT GCGCTCC-TT
C. friedmanii    .......... .......... .......... .......... .......... ..........
C. vishniacii    .......... .......... .......... .......... .......... .......C..
C. victoriae     .......... .......... .......... .......... .......... ....-..T..

C. antarcticus   GGTATTCCGA GGAGCATGCC TGTTTGAGTG TCATGAAAAC CCTCAACCTT AGATTGGTTA
C. friedmanii    .......... .......... .......... .......... .......... ..........
C. vishniacii    .......... .......... .......... .......... .......... ..........
C. victoriae     .......... A.G....... .......... .........-- ........CC .CT.G....T

C. antarcticus   TTTGACCTTT CTTTGGCTTG GATTTGGACG TTTGCCGATG CTAAGTCGGC TCGTCTTAAA
C. friedmanii    .......... .......... .......... .......... TC........ ..........
C. vishniacii    AC--....C. .......... .......... .......... A......... ..........
C. victoriae     ..----GCC. GAGC..TGGT .TA....GT. ..GC.T--.. ....A--... ...C......

C. antarcticus   AGTAATAGCT GGATCTGTCT CGCGACATGG TTTGACTTGG CGTAATAAGT ATTTCGCTAA
C. friedmanii    .......... .......... .......... .......... .......... ..........
C. vishniacii    .......... .......... .......... .......... .......... ..........
C. victoriae     GAC.TA...- ------AC.. T.-..TG.AA .AC.TT.CAT .C.TC.GG.. GGC.-.A...

C. antarcticus   GGACATCTTC GGATGGCCGC GTTGCAGGAC TAAAGACCGC TTTCTAATCC ATTGATCTTC
C. friedmanii    .......... .........CT .......... .......... .......... ..........
C. vishniacii    .......... .......... .......... .......... .......... ..........
C. victoriae     ---------- ---------- ---------- ---------- ---------- ------.CC.

C. antarcticus   GGATTAATAT TCTTGACATC TGGCCTCAAA TCAGGTAGGA CTACCCGCTG AACTTAA
C. friedmanii    .......... ..A.A..... .......... .......... .......... ..-....
C. vishniacii    .........C .......... .......... .......... .....-.... .......
C. victoriae     ----AC.... ..A...--.. .......... .........G .......... .......
```

Fig. 5. Alignment of the 5.8S gene and ITS regions of *C. antarcticus*, *C. friedmanii*, *C. vishniacii* and *C. victoriae*. The 5.8S rRNA gene is in bold characters.

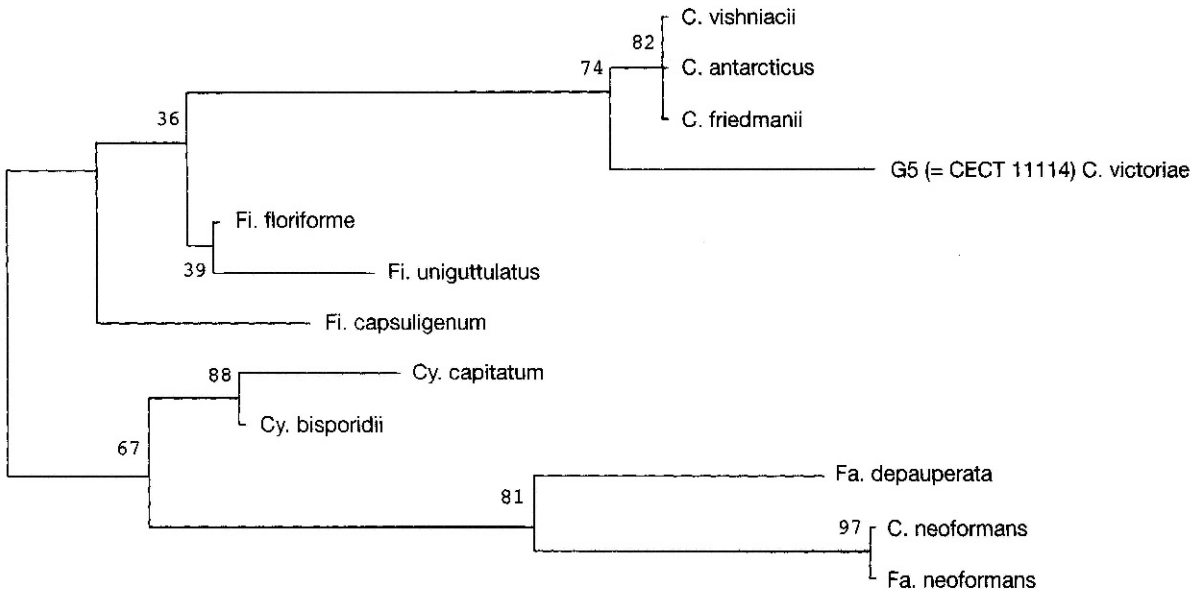

Fig. 6. Neighbour-Joining tree based on nucleotide divergences, estimated according to Kimura two-parameter model, from the 5.8S rRNA gene sequences. The numbers on the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1000 runs.

Phylogenetic analysis

Because of the significant differences when comparing strain G5 (= CECT 11114) ITS regions with those from the other antarctis strains, we have elaborated a phylogenetic tree by using only the 5.8S gene sequences obtained in this study, together with the sequences from other basidiomycetous yeast from the Genbank (Table 3). All sequences were aligned and subsequently used to infer a phylogenetic tree. Figure 6 shows the Neighbour-Joining (NJ) tree derived from the 5.8S rRNA gene sequences, where the numbers on the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1000 runs. The four represented genera are grouped independently in different branches of the phylogenetic tree. The antarctic *Cryptococcus* species, alongside the strain G5, are included in a clearly separated and significative group (74%), that appears closer to *Filobasidium* than to *Cystofilobasidium* and *Filobasidiella*, even though the latter includes two strains pertaining to the *C. neoformans* species.

Discussion

Various taxonomic parameters of *Cryptococcus* species were studied and a huge amount of data was collected. The total number of taxa, phenotypic characteristics, G+C content, and protein profile, place strain G5 (= CECT 11114) as a yeast integrated in the genus *Cryptococcus*.

Considering our results from RFLP analysis of the amplified rDNA region, there are not great differences between *C. vishniacii* and the species of the so-called *C. vishniacii* complex (VISHNIAC and BAHARAEEN, 1982), including the *C. vishniacii* varieties and the species *C. asgardensis, C. baldrensis, C. hempflingii, C. tyrolensis* and *C. wrightensis*. These conclusions are in agreement with the phylogenetic tree, derived from rRNA sequences of the large subunit, for basidiomycetous yeasts (KURTZMAN, 1994) where all the species described by VISHNIAC et al. (1982) are grouped together.

Moreover, the ITS region sequences revealed higher variability than we deduced by restriction analysis, for this reason the sequencing of the ITS regions of the species involved in the *C. vishniacii* complex would be a valuable information for the clarification of this closely related group.

However, the primary conclusion from this work, is the description of a new *Cryptococcus* species: *C. victoriae* (type strain G5 = CECT 11114), that being isolated from the same environment than the other antarctic species of the genus (BAHARAEEN and VISHNIAC, 1982; VISHNIAC and BAHARAEEN, 1982; VISHNIAC and HEMPFLING, 1979; VISHNIAC and KURTZMAN, 1992), displays low similarity levels with them when we compare their 5.8S rRNA gene and ITS sequences. In the phylogenetic tree deduced by the 5.8S rRNA gene sequences, the new described species *C. victoriae* also seems to be different from any other species pertaining to the basidiomycetous genus closely related to *Cryptococcus*, such as *Filobasidium, Filobasidiella* and *Cystofilobasidium*.

Latin description of *Cryptococcus victoriae* sp. nov.
(Victoria-victoriae pertaining to Victoria land)

Cryptococcus victoriae efformat colonias, agaro colore cremeo. Micat et de mucosae natura est; in medis liquidis minus crescit. Cellulae eius morphologiam ellipsoidalem habent, ca. 3×2 µm. Nec pseudomycelium nec mycelium formatur, calore ex 4 °C usque ad 20 °C. Nulla fermentatio datur. Assimilitat: L-arabinosam, D-arabinosam, arbutinam, acidum citricum, fructosam, creatinam, creatininam, eritritolem, D-galactitolem, D-glucitolem, D-galactosam, glucosam, glicerolum, glucuronicum, gluconata, inulinam, inositolum, lactosam, melibiosam, melicitosam, D-mannitolum, threalosum, L-ramnosum, D-ribitolum, succinatum, D-xylitolum.

Cum nitrato crescit, etiam crescit sine vitaminis, urea finditur et diazolium blue respondet, G+C, ca. 50.3 mol% glucosae nunquam crescit, nec in 0.01%–0.1% cicloheximidae.

Eius molecularis distinctio per archetypum totarum proteinarum, per determinationem RPLP de RNAr 5.8S et descriptarum sequentiarum internarum ribosomarum (5.8S-ITS region) manifeste ostendit singularitatem huius stirpis coram omnibus speciebus huis generis.

Standard description of *Cryptococcus victoriae* sp. nov.

This species is typified by G5 (= CECT 11.114) which was isolated by screening programme on samples of mosses, lichens and soils from Botany Bay (Southern Victoria Land, Antarctica). It develops cream and slimy colonies in Sabouraud agar, with cells of 3×2 µm. Cells are encapsulated (Figure 2). Pseudomycelia and mycelia are not formed. Sexual reproduction is unknown.

Growth occurs from 4 °C to 20 °C; optimal growth is at 15 °C. Fermentation does not occur. Compounds assimilated: D-Glucose, D-Galactose, D-Xilose, L-Arabinose, D-Arabinose, L-Rhamanose, Sucrose, Maltose, Trehalose, Cellobiose, Arbutin, Melibiose, Lactose, Raffinose, Melezitose, Inulin, Starch, Glycerol, Erythritol, Xylitol, D-Glucitol, D-Manitol, Galactitol, myo-Inositol, 2-Keto-D-Gluconate, 5-Keto-D-Gluconate, D-Gluconate, Succinate, Citrate, Nitrate, L-Lysine, Creathinine and Creatine.

Growth is inhibited by 0.01% of Cicloheximide. Diazolium blue B test, urea hidrolysis and growth without vitamines are positive.

G+C content of nuclear DNA is 50.3 mol%.

The phylogenetic tree deduced from the 5.8S rRNA gene sequence showed *Cryptococcus victoriae* as a new species of the genus *Cryptococcus*.

Acknowledgements

We are grateful to Dra. J. CASTELLVÍ and Dr. B. SCHROETER for the antarctic samples suministred. We thank also Dr. E. BARRIO for his useful help on phylogenetic analysis. This research was financed by grant ANT 98-1701-E from CICYT project and by 1997 SGR 00403 project from II Pla de Recerca de Catalunya.

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Corresponding author:
Dr. JESÚS GUINEA, Unidad de Microbiología. Facultad de Farmacia. C/ Juan XXIII s/n 08028 Barcelona, España.