Cryptococcus rajasthanensis sp. nov., an anamorphic yeast species related to *Cryptococcus laurentii*, isolated from Rajasthan, India

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Two novel anamorphic yeast strains (S-15L^T and 3-C1) were isolated from the inflorescences of plants collected in two different towns in Rajasthan State, India. Sequencing of the D1/D2 domains of the large-subunit (LSU) rDNA and the internal transcribed spacer (ITS) regions suggested they are strains of the same species. Phenotypic characteristics such as the absence of fermentation, the absence of sexual structures and ballistoconidia, the assimilation of *myo*-inositol and D-glucuronate, and positive Diazonium blue B and urease reactions indicated that these strains belong to the genus *Cryptococcus*. The novel strains differed from *Cryptococcus laurentii* in six physiological tests and differed from other related species in more than six tests. A phylogenetic analysis of the sequences of the D1/D2 domains of the LSU rDNA and the ITS regions placed these strains in the *Bulleromyces* clade within the order Tremellales, with *C. laurentii* as their closest described relative. The novel strains showed 1.6 and 7.5 % divergence in the D1/D2 domain of the LSU rDNA and ITS regions, respectively, with respect to *C. laurentii*. The divergence from other species was more than 3 % for the D1/D2 domain and more than 9 % for the ITS region. On the basis of the phenotypic and molecular data, strains S-15L^T and 3-C1 represent a novel species within the genus *Cryptococcus*, for which the name *Cryptococcus rajasthanensis* sp. nov. is proposed. The type strain is S-15L^T (=MTCC 7075^T = CBS 10406^T).

The basidiomycetous yeast genus *Cryptococcus* Vuillemin is characterized by the following features: a lack of sexual structures and ballistoconidia, the utilization of D-glucuronate, the absence of fermentation, the presence of xylose in cell hydrolysates and positive reactions in the diazonium blue B and urease tests (Fell & Statzell-Tallman, 1998). The genus is known to be polyphyletic, containing members of the orders Tremellales, Filobasidiales, Trichosporonales and Cystofilobasidiales (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). The taxonomic heterogeneity of two so-called 'ubiquitous' *Cryptococcus* species, *Cryptococcus albidus* and *Cryptococcus laurentii*, was reported in some earlier studies (Vancanneyt *et al.*, 1994; Takashima & Nakase, 1999; Fonseca *et al.*, 2000; Sugita *et al.*, 2000). Sequence analysis of the D1/D2 domain of the large-subunit (LSU) rDNA and the internal

transcribed spacer (ITS) region has resulted in the description of several novel *Cryptococcus* species that were previously thought to be members of either *C. albidus* or *C. laurentii* on the basis of the results of phenotypic analyses (Takashima *et al.*, 2003; Middelhoven, 2005; Golubev *et al.*, 2006).

Members of the genus *Cryptococcus* are heterogeneous in terms of their nutritional abilities and have been isolated from diverse habitats. However, there are only few reports of *Cryptococcus* species having been isolated from flowers. *Cryptococcus gattii*, an emerging pathogen of humans, was isolated from different parts of *Eucalyptus* trees, including the flowers (Chakrabarti *et al.*, 1997; Ellis & Pfeiffer, 1990). *Cryptococcus* species were found to be the predominant yeasts (23 out of 69) in flowers in Germany (Herzberg *et al.*, 2002); most of them were related to *Cryptococcus victoriae*, followed by *Cryptococcus diffluens* and *C. albidus*. Only one strain (CBS 9007) was shown to be related to *C. laurentii*. Hong *et al.* (2002) isolated four strains related to *C. victoriae* and one related to *Cryptococcus luteolus* from flowers in Korea.

During a survey of yeasts from flowers from the Ganganagar and Rawatbhata regions of Rajasthan State in India, more

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DBJ accession numbers for the sequences of the D1/D2 domain of the LSU rDNA and the ITS region of strain S-15L^T are AM262324 and AM262325, respectively, and that for the combined D1/D2 domain and ITS region sequence of strain 3-C1 is AM262981.

A neighbour-joining phylogenetic tree for strains S-15L^T and 3-C1 and related species based on LSU rDNA sequences is available as a supplementary figure in IJSEM Online.

than 70 yeast strains belonging to various genera were isolated. Conventional methods and sequencing of the D1/D2 domain of the LSU rDNA suggested that five isolates were related to members of the genus *Cryptococcus*. Two isolates (S-15L^T and 3-C1) were related to *C. laurentii*, two isolates (S-1C and S-1D) were related to *C. victoriae* and one strain (2-B2) was identified as *C. diffluens*. Strain S-15L^T was isolated from an inflorescence of *Digera* sp., and strain 3-C1 was isolated from a different locality (about 1000 km away from the first location) from an inflorescence of *Andrographis echinoides* (see Table 1). On the basis of conventional morphological and physiological tests, these strains were shown to belong to the genus *Cryptococcus*. No hyphae or pseudohyphae were observed on cornmeal agar even after 30 days. No sexual structures or ballistoconidia were observed on cornmeal agar, 2 % malt agar, 5 % malt agar or potato dextrose agar (PDA) after 1 month. Molecular characterization of these yeast strains through sequencing of the D1/D2 domains of LSU rDNAs and the ITS regions suggested that S-15L^T and 3-C1 are strains of the same species and confirmed their close relationship with *C. laurentii*. It has been suggested that basidiomycetous yeast strains that differ by more than 1 % sequence divergence in the D1/D2 domain and the ITS region can be considered to be strains of different species (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). Strains S-15L^T and 3-C1 differ from *C. laurentii* by 1.6 % sequence divergence in the D1/D2 domain and 7.5 % variation in the ITS region, and also showed six differences in physiological tests. All of these data support the assignment of these strains to a novel species of the genus *Cryptococcus*.

Strains S-15L^T and 3-C1 were isolated from 1 g samples of inflorescences homogenized in 9.0 ml sterile water; serial dilutions were made and spread on yeast extract/peptone/dextrose (YEPD) plates, supplemented with chloramphenicol (50 mg l⁻¹) and streptomycin (30 mg l⁻¹) to suppress bacterial growth. The strains were isolated from YEPD plates at 25 °C after 4 days incubation, purified, stored in 10 % glycerol at -70 °C and in liquid nitrogen for long-term maintenance. The methods used to determine the morphological, physiological and biochemical properties were performed as described by Yarrow (1998). The type strain of *C. laurentii*, MTCC 2898^T (=CBS 139^T), was used for comparison of the phenotypic characteristics. Assays for observation of sexual structures were performed on

cornmeal agar, 2 % malt agar, 5 % malt agar and PDA, with plates being incubated at 20 and 25 °C for 1 month in pure and mixed cultures.

Isolation of genomic DNA and PCR amplification of ITS regions and D1/D2 domains of LSU rDNAs were done as described previously (Prasad *et al.*, 2005). The amplified fragments were purified by using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Direct sequencing of gel-purified PCR products was performed with the ABI BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). Both strands of the PCR product were sequenced. Sequencing reactions were purified by using ethanol and 125 mM EDTA precipitation. Processing of the samples for loading onto an ABI 3130 xl Genetic Analyzer (Applied Biosystems) was performed according to the instructions of the manufacturer. A sequence-similarity search was done using GenBank BLASTN (Altschul *et al.*, 1997). Sequences of closely related taxa were retrieved and aligned using the CLUSTAL X program (Thompson *et al.*, 1997). For the neighbour-joining analysis (Saitou & Nei, 1987), distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Strains S-15L^T and 3-C1 differ from *C. laurentii* in six physiological tests. The novel strains assimilate nitrite, citrate (weakly), erythritol (weakly) and sorbose (weakly), produce starch-like compounds (weakly) and cannot grow at 35 °C, whereas *C. laurentii* assimilates only erythritol (not nitrite, citrate or sorbose), shows pronounced production of starch-like compounds, and shows growth at 35 °C. The novel strains showed more than six phenotypic differences with respect to other related species (Table 2). Strains S-15L^T and 3-C1 have identical sequences in D1/D2 domain and differ by one base substitution in the ITS region, suggesting that they are strains of the same species. Sequences of related species were retrieved from the nucleic acid databases and phylogenetic trees were constructed separately for the ITS region (data not shown), the D1/D2 domain of the LSU rDNA (see Supplementary Fig. S1 available in IJSEM Online) and also the combination of the ITS and D1/D2 domain (Fig. 1). Phylogenetic analysis showed that strain S-15L^T belongs to the *Bulleromyces* clade

Table 1. Strains used in this study

Strain	Source and date of isolation	Location
S-15L ^T (=MTCC 7075 ^T =CBS 10406 ^T)	Inflorescence of <i>Digera</i> sp. (October 2004)	Sunderpura, Rajasthan, India (29° 55' 18" N 73° 51' 39" E)
3-C1 (=MTCC 7847)	Inflorescence of <i>A. echinoides</i> (September 2005)	Rawatbhata, Rajasthan, India (25° 10' 34" N 75° 49' 51" E)
<i>C. laurentii</i> CBS 139 ^T (=MTCC 2898 ^T)	Palm wine (not known)	The Congo

Table 2. Physiological characteristics that differentiate strains S-15L^T and 3-C1 from closely related strains

Strains: 1, *C. rajasthanensis* sp. nov. S-15L^T (strain 3-C1 gave identical results); 2, *C. laurentii* CBS 139^T; 3, *Cryptococcus* sp. CBS 7140; 4, *C. aureus* CBS 318^T; 5, *Bullera japonica* CBS 2013^T; 6, *C. nemorosus* VKM Y-2906^T. Symbols: +, positive; −, negative; w, weak; d, delayed; s, slow.

Characteristic	1	2	3	4	5	6
Assimilation of:						
L-Sorbose	w	−	−	−	+	w
Glycerol	−	−	+	w	d	s
Ethanol	w	w	+	−	−	+
Nitrite	+	−	−	−	−	−
Citrate	w	−	+	−	+	+
Erythritol	w	+	+	−	+	−
Growth at:						
35 °C	−	+	+	−	−	−
37 °C	−	−	+	−	−	−
Starch formation	w	+	+	+	+	+

in the Tremellales *sensu* Scorzetti *et al.* (2002), clustering with *C. laurentii* as its closest relative (Fig. 1). The topologies of both the D1/D2 and combined ITS and D1/D2 trees were similar, showing only minor differences. In the D1/D2 tree, *Cryptococcus aureus* and *Cryptococcus flavescentis* appeared in a cluster along with *Cryptococcus nemorosus*, *Cryptococcus perniciosus*, *Cryptococcus taeanensis* and *Bullera japonica*, whereas they appear to be basal to all related species in the combined tree. This clade (demarcated by *Cryptococcus nemorosus* and *Cryptococcus flavescentis*) is supported by 100 % bootstrap values in this tree. Sequence analysis showed that strain S-15L^T differs from *C. laurentii* by 1.6 % divergence (9 substitutions) in the D1/D2 domain of the LSU rDNA, and by 7.5 % divergence in the combined ITS1 and ITS2 sequences (10 substitutions and 2 indels in ITS1; 8 substitutions and 2 indels in ITS2). On the basis of the D1/D2-domain sequence analysis, other species related to the novel strains are *C. aureus* (2.9 % divergence), *Bullera japonica* (3.1 %), *C. nemorosus* (3.2 %), *C. perniciosus* (3.6 %), *C. flavescentis* (3.6 %), *Cryptococcus cellulolyticus* (4.4 %) and *Bullera pseudoalba* (4.4 %). Variation in the combined sequences of ITS1 and ITS2 (excluding 5.8S rDNA) was more than 9 % with respect to all other related species.

An understanding of the role and diversity of basidiomycetous yeast species in natural habitats has been slow to develop because of difficulties associated with identification procedures (Fell *et al.*, 2000). As a consequence, some yeasts, such as *C. laurentii*, are considered to be ubiquitous (de Azeredo *et al.*, 1998; Saldanha-da-Gama *et al.*, 1997; Slavikova & Vadkertiova, 2000); this could be due to incorrect delineation of this species, as several tests used for its identification give variable results (Fell &

Fig. 1. Neighbour-joining tree of strains S-15L^T and 3-C1 and related species, based on sequences of the ITS region of the rDNA cluster and the D1/D2 domain of LSU rDNA. Evolutionary distances were calculated according to Kimura (1980). Numbers at nodes are bootstrap values from 100 replicate samplings; values less than 70 are not shown. *Fellomyces polyborus* CBS 6072^T was used as the outgroup. Bar, 2 % variation.

Statzell-Tallman, 1998; Barnett *et al.*, 2000). Interestingly, it is also difficult to distinguish *C. laurentii* from *C. cellulolyticus*, *Cryptococcus flavus*, *Cryptococcus humicola* and *Cryptococcus hungaricus* on the basis of physiological characteristics (Barnett *et al.*, 2000). After a comprehensive study of all basidiomycetous yeasts, Fell *et al.* (2000) suggested that strains that differ in two or more nucleotides in the D1/D2 domain of the LSU rDNA may represent different taxa. These authors also showed that biologically distinct species that could not be separated using D1/D2 analysis could be differentiated using ITS sequence analysis. Scorzetti *et al.* (2002) recommended a combined sequence analysis (the D1/D2 domain and the ITS region) for species identification of basidiomycetous yeasts. Sugita *et al.* (2000) reported genetic diversity in the ITS and D1/D2 regions among clinical isolates of *C. laurentii*: ten isolates examined in that study were found to belong to seven different species. Three novel *Cryptococcus* species that were previously considered as synonyms of *C. laurentii* were reinstated on the basis of sequencing of the 18S rDNA and the D1/D2 region of the LSU rDNA (Takashima *et al.*, 2003). Similarly, strains CBS 9604^T and CBS 7140, which could not be distinguished from *C. laurentii* on the basis of phenotypic characteristics (Kurtzman & Fell, 1998; Barnett *et al.*, 2000), were shown to differ from *C. laurentii* in terms of ITS and D1/D2 sequences. Strain CBS 9604^T was recently described as *Cryptococcus allantoinivorans* (Middelhoven, 2005) and CBS 7140 also appears to represent a novel species, as it differs from the type strain of *C. laurentii* (CBS 139^T) by one base substitution in the D1/D2 domain and ten base

substitutions in ITS region. Strain S-15L^T differs from CBS 7140 by eight substitutions in the D1/D2 domain, by eight substitutions and one indel in ITS1, and by seven substitutions and one indel in ITS2. Comparisons with all of the described species of the *C. laurentii* complex show that the two novel yeast strains (S-15L^T and 3-C1) reported in this investigation are the closest relatives of *C. laurentii*; however, they differ from *C. laurentii* in six physiological tests, show more than 1 % divergence in the D1/D2 domain and show 7.5 % divergence in combined ITS1 and ITS2 regions. These data strongly support the assignment of S-15L^T and 3-C1 to a novel species of the genus *Cryptococcus*, for which we propose the name *Cryptococcus rajasthanensis* sp. nov.

Latin description of *Cryptococcus rajasthanensis* P. Saluja et G. S. Prasad sp. nov.

In liquido YEPD post 2 dies 25 °C cellulae ovaes, 3.5–6.5 × 3.5–5.5 µm. Coloniae dilute flavae, viscosae, margine integra; post 20 dies luteo-bubalinae. In agaro farinae zae mycelium non formantur. Teleomorphe ignota. Substrata carbonica assimilata: D-galactosum, D-glucosaminum, L-arabinosum, D-arabinosum, D-ribosum, D-xylosum, L-rhamnosum, sucrosum, maltosum, methyl α-D-glucosidum, melibiosum, cellobiosum, salicinum, arbutinum, raffinose, lactosum, melezitose, erythritolum (parce), ribitolum, L-arabinitolum, xylitolum, D-glucitolum, D-mannitolum, myo-inositolum, galactitolum, N-acetylglucosaminum, 2-keto-D-gluconatum, D-glucuronatum, acidum D-gluconicum (Na⁺), D-glucono-1,5-lactonum, trehalosum, citratum (parce), L-sorbosum (parce), ethanolum (parce), inulinum (parce), succinatolum (parce), neque amyllum, methanolum, DL-lactatum, glycerolum. Substrata nitrogenica assimilata: nitritum, cadaverinum, lysinum, creatinum (parce), ethylaminum; neque nitratum. Non fermentat neque crescit 0.01 vel 0.1 % cycloheximido addito. Acidum aceticum haud formatum. 50 vel 60 % glucosii addito haud crescit. Substantia amyli similis parce formatur. Reactiones Diazonium caeruleum B et ureasii positivae. 25 et 30 °C crescit, neque 35 °C. Isolatus ex flores, in Rajasthan, India. Typus S-15L^T (=MTCC 7075^T=CBS 10406^T). Conservatur in Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

Description of *Cryptococcus rajasthanensis* P. Saluja & G. S. Prasad sp. nov.

Cryptococcus rajasthanensis (ra.jas.than.en'sis. N.L. nom. masc. adj. *rajasthanensis* referring to Rajasthan State in India, from which the first strains were isolated).

After 2 days growth in YEPD broth at 25 °C, cells are oval, 3.5–6.5 × 3.5–5.5 µm (Fig. 2). Aerobic growth results in pale-yellow, circular colonies with entire margins. Colonies are viscous in consistency and turn yellow–tan after 20 days incubation at 25 °C. Neither mycelium nor pseudomycelium is observed in Dalmau plate culture on cornmeal agar or on PDA, even after 30 days. No sexual structures are

Fig. 2. Cell morphology of strain S-15L^T after 48 h in YEPD broth. Bar, 5 µm.

observed in mixed or pure cultures plated on cornmeal agar, 2 % malt agar, 5 % malt agar (Fig. 2) and PDA. Assimilation of carbon compounds is as follows: D-galactose, D-glucosamine, L-arabinose, D-arabinose, D-ribose, D-xylose, L-rhamnose, sucrose, maltose, methyl α-D-glucoside, melibiose, cellobiose, salicin, arbutin, raffinose, lactose, melezitose, erythritol (weakly), ribitol, L-arabinitol, xylitol, D-glucitol, D-mannitol, myo-inositol, galactitol, N-acetylglucosamine, 2-keto-D-gluconate, D-glucuronate, D-gluconic acid sodium salt, D-glucono-1,5 lactone, α,α-trehalose, citrate (weakly), L-sorbose (weakly) and succinate (weakly), inulin (weakly), ethanol (weakly) are assimilated, but starch, methanol, DL-lactate and glycerol are not assimilated. Nitrite, cadaverine, lysine, creatine (weakly) and ethylamine are assimilated. Nitrate is not assimilated. Fermentation is absent, and no growth occurs in the presence of 0.01 or 0.1 % cycloheximide. Acetic acid production is absent. No growth occurs on 50 or 60 % glucose. Production of starch-like compounds is weakly positive. Reactions with diazonium blue B and for urease are positive. Grows at 25 and 30 °C, but not at 35 °C.

The type strain, S-15L^T (deposited in the Microbial Type Culture Collection and Gene Bank, Chandigarh, India, as MTCC 7075^T and in the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 10406^T), and strain 3-C1 were isolated from inflorescences of plants from Rajasthan State in India.

Acknowledgements

This study was supported by the Council of Scientific and Industrial Research (India) and the Department of Biotechnology, Government of India, in the form of a grant to the Microbial Type Culture Collection and Gene Bank. P.S. is a recipient of a senior research fellowship from the Council of Scientific and Industrial Research. The authors are grateful to Mr Gurdeep, Punjab University (Chandigarh, India) for his help in identifying the plants and to Mr Paramjit for his help with the physiological characterization of the strains.

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