

Cryptococcus randhawai sp. nov., a novel anamorphic basidiomycetous yeast isolated from tree trunk hollow of *Ficus religiosa* (peepal tree) from New Delhi, India

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Abstract A novel anamorphic *Cryptococcus* species is described, which was isolated in New Delhi (India) from decaying wood of a tree trunk hollow of *Ficus religiosa*. On the basis of sequence analysis of the D1/D2 domains of the 26S rRNA gene and the internally transcribed spacer (ITS)-1 and ITS-2 region sequences, the isolate belonged to the *Cryptococcus albidus* cluster (Filobasidiales, Tremellomycetes) and was closely related to *Cryptococcus saitoi*, *Cryptococcus cerealis* and *Cryptococcus friedmannii* with 98% sequence identity. Phenotypically, the species differed from *C. saitoi* with respect to growth temperature (up to 37°C), presence of a thin capsule, ability to grow in the absence of vitamins, and inability to assimilate citrate and

ethylamine. With respect to *C. friedmannii*, it differed in growth temperature, ability to assimilate lactose, raffinose, L-rhamnose, *myo*-inositol, and inability to utilize citrate. Furthermore, our isolate also differed from *C. cerealis* in growth temperature, presence of capsule and inability to assimilate L-sorbose. In view of the above phenotypic differences and unique rDNA sequences, we consider that our isolate represents a new species of *Cryptococcus*, and therefore, a new species, *Cryptococcus randhawai* is proposed for this taxon. The type strain J11/2002 has been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS10160) and CABI Biosciences (IMI 393306).

Keywords *Cryptococcus randhawai* sp. nov. · Tree trunk hollow · *Ficus religiosa* · New Delhi

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Introduction

The members of the polyphyletic anamorphic basidiomycetous yeast genus *Cryptococcus* are generally characterized by the ability to assimilate D-glucuronate, absence of fermentative ability, presence of xylose in cell hydrolysates, positive urease and diazonium blue B reactions, and the presence of coenzymes Q-9 and Q-10 (Fell and Statzel-Tallman 1998). Although *Cryptococcus neoformans* was first

isolated from peach juice (Sanfelice 1894), it was not until 1972 when Staib and coworkers showed that a wide variety of plant substrates supported *in vitro* colonization by this fungus (Staib et al. 1972). Subsequently, the association of *Cryptococcus gattii* with *Eucalyptus camaldulensis* and *Eucalyptus tereticornis* was discovered by investigators from Australia (Pfeiffer and Ellis 1992). More recently, *C. gattii* as well as *C. neoformans* have been isolated from a variety of tree species particularly from the decaying wood of trunk hollows (Lazera et al. 1998; Randhawa et al. 2001, 2003; Kidd et al. 2004; Granados and Castañeda 2005). Plant and plant-related substrates have also been the source of several other *Cryptococcus* species (Herzberg et al. 2002; Hong et al. 2002).

During our ecological studies aimed at investigating trees as a natural habitat of *C. neoformans* and *C. gattii* in North-western India (Randhawa et al. 2001, 2003), we isolated three types of yeast colonies from the decaying wood samples using simplified Staib's niger seed medium (Paliwal and Randhawa 1978). The sample was collected from a trunk hollow of a *Ficus religiosa* tree (Bo tree, which is also known as the Peepal or Religious Fig tree, family *Moraceae*) located in Mandir Marg area, New Delhi (India) on December 12, 2002. Using the Vitek 2 Yeast ID system, these three types of colonies were identified as *C. neoformans*, *C. albidus*, and an unidentified yeast species (J11/2002), which is now described here as a new species under the proposed name *Cryptococcus randhawai* sp. nov.

Materials and methods

Isolation and phenotypic characterization

The decaying wood material from tree trunk hollows was collected and nearly 1 g of each sample was suspended in 10 ml of sterile saline solution (0.9% NaCl) containing gentamycin (25 mg/l). The sample was mixed thoroughly and then allowed to settle for 30 min. Aliquots of supernatant were plated in duplicate on simplified Staib's niger seed medium, prepared as described previously (Randhawa et al. 2003). The inoculated plates were incubated at 28°C and examined every 24 h up to 10 days. Yeast-like colonies were examined microscopically and isolated

in pure culture by dilution plating. The colony and microscopic characteristics of the isolate were further studied on Sabouraud dextrose agar and malt extract agar. Physiological tests for the assimilation of various compounds were performed in duplicate according to recommended procedures (Barnett et al. 2000). The minimum inhibitory concentrations of our isolate for different antifungal agents were determined by Etest (AB BioDisk, Solna, Sweden) (Asadzadeh et al. 2008).

Genotypic characterization

The genomic DNA from the isolate was prepared as described previously and used as template for PCR amplification (Ahmad et al. 2002). The divergent domains (D1/D2) at the 5' end of the large subunit (26S) rRNA gene were amplified with primers NL-1 (5'-GCATATCAATAAGCGGAGGA AAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGA CCG-3') (Kurtzman and Robnet 1998). The ITS region of rDNA containing the ITS-1, 5.8S rRNA gene and ITS-2 was amplified by using ITS1 and ITS4 primers (Ahmad et al. 2004, 2005). The amplification of the genomic DNA for D1/D2 and ITS regions and detection and sequencing of the amplicons were carried out as described previously (Ahmad et al. 2004, 2005). The sequencing primers, in addition to the amplification primers, included NL-2A (5'-CTTGTTTCGCTATCGGTCTC-3'), NL-3A (5'-GAGACCGATAGCGAACAAG-3') and NLR3R (5'-GGTCCGTGTTTCAAGAC-3') for the sequencing of the D1/D2 region of 26S rRNA gene (Khan et al. 2008) and ITS1FS (5'-ACCTGCGGAAGGAT CATT-3'), ITS2 (5'-TCGCTGCGTTCTTCATCGAT GC-3'), ITS3 (Ahmad et al. 2005) and ITS4RS (5'-GATATGCTTAAGTTCAGCG-3') for the sequencing of the ITS region. Alignments of sequences were created and ambiguous sites were manually corrected using Seqman version 8 (Lasergene, DNASTar, Madison, WI, USA). GenBank basic local alignment search tool (BLAST) searches were performed for preliminary phylogenetic placement of the isolate. The DNA sequences for the type strains of several *Cryptococcus* species related to *C. albidus* available in GenBank (Fonseca et al. 2000) were retrieved. Pair-wise comparisons of sequences were carried out with MEGA version 4 (Kumar et al. 2008). The phylogenetic trees for the D1/D2 region of 26S rRNA gene and

ITS-1 and -2 regions of rDNA were constructed with Maximum Parsimony method with the Close-Neighbor-Interchange algorithm in which the initial trees were obtained with the random addition of sequences (10 replicates) (Nei and Kumar 2000) and gaps were eliminated from the data set with the complete deletion of gaps option. The robustness of the branches was assessed by bootstrap analysis with 1,000 replicates.

Results and discussion

Colony and microscopic characteristics

On Sabouraud dextrose agar and malt extract agar at 28°C, the isolate produced cream-colored, smooth, shiny colonies which on aging became slightly tan. No pigment was produced on simplified Staib’s niger seed agar. The growth was good up to 35°C and only scanty at 37°C. No growth was observed at 40°C (Table 1). Microscopically, the yeast cells were globose to ovoid measuring (4–6.2) × (4.5–7.0) µm and showed polar budding (Fig. 1a). India ink examination revealed a thin capsule (Fig. 1b). Dalmau culture on cornmeal agar did not produce any

hyphae or pseudohyphae when examined up to 3 weeks and no sexual reproduction was observed either on cornmeal agar or on 5% malt extract agar (Oxoid, Basingstroke, UK).

Antifungal susceptibility

The minimum inhibitory concentrations of our isolate for different antifungal agents were as follows: amphotericin B, 0.125 µg/ml; fluconazole, 3 µg/ml; itraconazole, 0.125 µg/ml; voriconazole, 0.064 µg/ml; caspofungin >32 µg/ml; and 5-flucytosine, >32 µg/ml.

Phylogenetic analysis based on 26S rRNA and ITS region sequences

An amplicon of ~ 650 bp obtained from the D1/D2 region of *C. randhawai* was sequenced and the BLAST search revealed maximum identity (~98%) with the corresponding sequences available in the databanks from several strains of *Cryptococcus saitoi*. Although the D1 region sequences were identical, they differed at 10 nucleotide positions within the D2 region. The D1/D2 region sequences

Table 1 Comparative morphological and physiological characteristics of *C. randhawai* and strains of other genetically-related species

Characteristic/Test	<i>C. randhawai</i>	<i>C. saitoi</i>	<i>C. friedmannii</i>	<i>C. cerealis</i>	<i>C. bhutanensis</i>	<i>C. antarcticus</i>
Assimilation of						
Lactose	+	±	–	+(D)	±	–
Raffinose	+	+	–	W	±	±
myo-inositol	+	+	–	+	–	+(D)
L-sorbose	–	–	–	+(D)	+(D)	–
L-rhamnose	+	+	–	+	±	+(D)
Citrate	–	+	+(D)	–	+(D)	±
Ethylamine	–	+	–	±	+	±
Without vitamins	+	–	+(D)	ND	–	+
Growth at						
30°C	+	W	–	+	–	–
35°C	+	–	–	–	–	–
37°C	W	–	–	–	–	–
Capsule formation	+	–	±	–	–	–

The data for *C. saitoi* CBS 1975^T are taken from Fonseca et al. (2000), data for *C. friedmannii* CBS 7160^T, *C. bhutanensis* CBS 6924^T and *C. antarcticus* CBS 7687^T are taken from Barnett et al. (2000) and Fell and Statzel-Tallman (1998) and the data for *C. cerealis* are taken from Passoth et al. (2009)

+ present/positive, – absent/negative, ± variable, W weak, D delayed growth after 7 days, ND not done

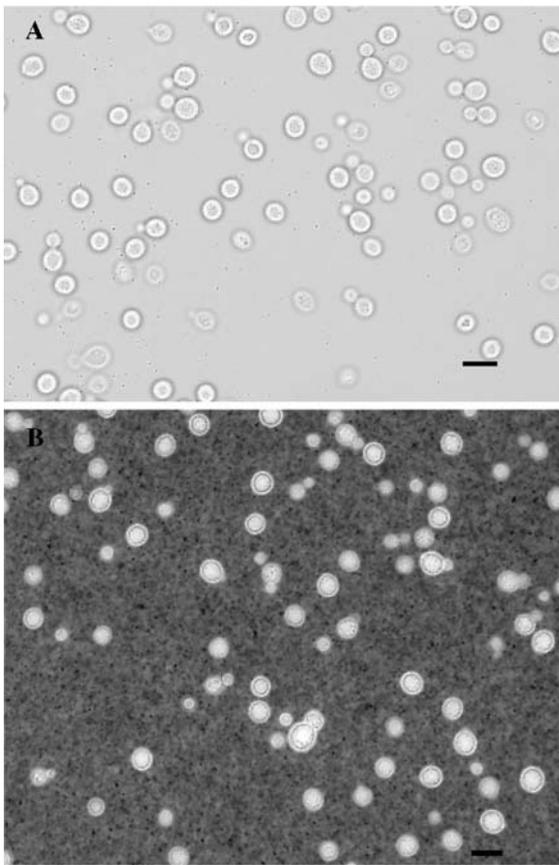


Fig. 1 **a** The microscopic morphology of *C. randhawai* grown in malt extract agar for 48 h at 25°C; **b** India ink mount showing the presence of a thin capsule (bar = 10 µm)

from other *Cryptococcus* species were found to be more divergent. The bootstrapped Maximum Parsimony phylogenetic tree obtained from the D1/D2 region sequences from other closely related *Cryptococcus* species is shown in Fig. 2. Our isolate J11/2002 (CBS10160) formed a distinct and separate lineage within the *Cryptococcus saitoi*/*Cryptococcus cerealis*/*Cryptococcus friedmannii* cluster (Fig. 2). Subsequent to our isolation, Espino del Castillo and co-workers isolated a yeast strain from leaf litter in Mexico in 2008 (unpublished). The D1/D2 region sequence of 26S rRNA gene (GenBank Accession No. EU833230) of this isolate was 100% identical with the sequence of our isolate J11/2002. The recovery of the second strain of *C. randhawai* from Mexico suggests that this taxon has a much wider geographic distribution and decaying plant material may serve as its natural habitat.

The ITS sequence of our isolate was determined and the corresponding sequences from closely related *Cryptococcus* species (based on D1/D2 sequences) available from GenBank were retrieved. The bootstrapped Maximum Parsimony phylogenetic tree based on ITS region sequences was also constructed (Fig. 3). Similar to the D1/D2-based phylogenetic tree, our isolate J11/2002 (CBS10160) again formed a distinct and separate lineage within the *C. saitoi*/*C. cerealis*/*C. friedmannii* cluster (Fig. 3).

In the phylogenetic trees derived from the D1/D2 domains sequences and ITS-1 and -2 regions, our isolate J11/2002 (CBS10160) formed a distinct and separate lineage within the *C. saitoi*/*C. cerealis*/*C. friedmannii* cluster. Phenotypically, isolate J11/2002 (CBS10160) differed from *C. saitoi* with respect to growth temperature (up to 37°C), presence of a thin capsule, ability to grow in absence of vitamins, inability to assimilate, citrate, and ethylamine (Table 1). Likewise, it differed from *C. friedmannii* with respect to growth temperature, assimilation of lactose, raffinose, L-rhamnose and *myo*-inositol, and from *C. cerealis* in growth temperature, presence of capsule and inability to assimilate L-sorbose (Table 1). Additionally, our isolate also differed phenotypically and genotypically with two other closely related *Cryptococcus* species viz. *Cryptococcus bhutanensis* and *Cryptococcus antarcticus* (Table 1).

Considering the above difference of >1% in D1/D2 domains of the 26S rRNA gene (Kurtzman and Robnett 1998; Fell et al. 2000), the novel ITS region sequences and unique phenotypic characteristics, isolate J11/2002 (CBS10160) represents a new species closely related to the *C. saitoi*/*C. cerealis*/*C. friedmannii* cluster. A new species, *Cryptococcus randhawai* sp. nov. is hereby proposed and the isolate CBS 10160 is designated as the type strain for this new species.

Latin diagnosis of *Cryptococcus randhawai*
CBS10160^T Khan, Ahmad, Hagen, Fell, Kowshik,
Chandy et Boekhout sp. nov

In medio agar Sabouraud *dextrosico* et malti extracto agar confecto 28°C *coloniae cremae, laevigatae, micantes, vetustiores bubalinae*. Incrementum bonum usque 35°C, parcum 37°C. In agar farina *maydis confecto* in Dalmau intra tres hebdomadas nullae

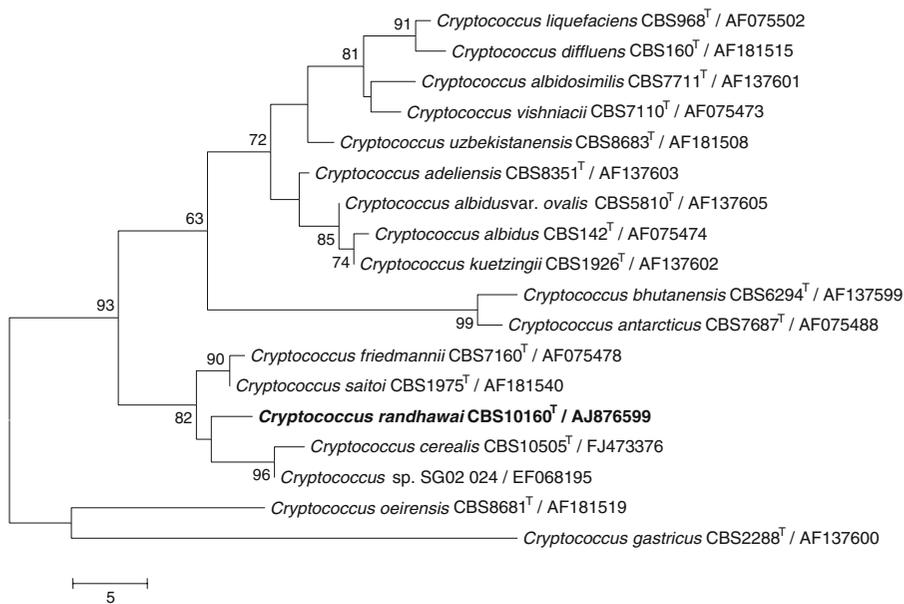


Fig. 2 Phylogenetic tree showing the placement of *C. randhawai* sp. nov. (marked in bold) and closely related *Cryptococcus* species based on the analysis of the D1/D2 domains of the large subunit (26S) rRNA. Type strains are indicated by superscript T. Sequences not generated during this

study were obtained from GenBank (accession numbers are shown in parentheses). The tree was constructed by bootstrap Maximum Parsimony analysis of aligned sequences and the numbers at nodes indicate percentage of bootstrap sampling from 1,000 replicates. The bar represents 5 nucleotide changes

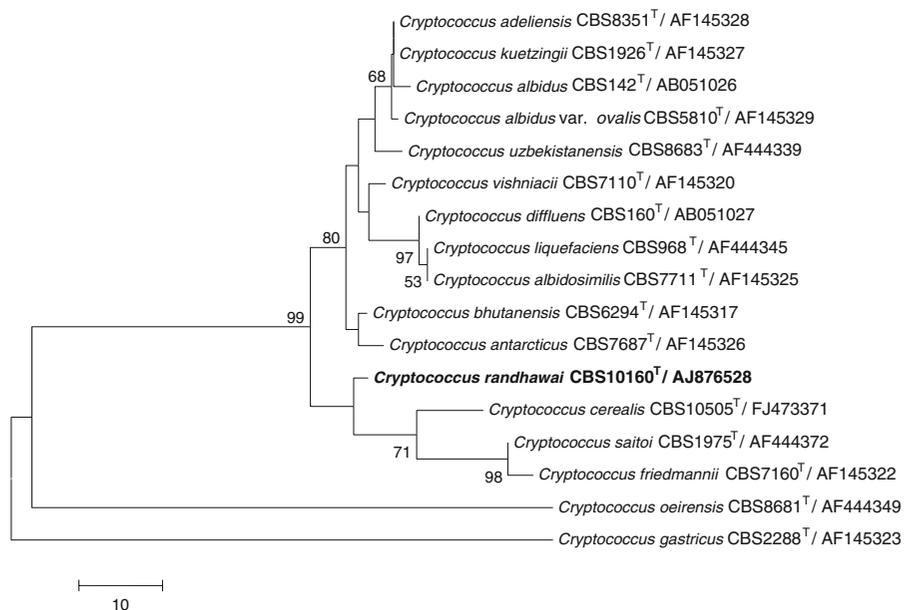


Fig. 3 Phylogenetic tree showing the placement of *C. randhawai* sp. nov. (marked in bold) and some closely related species based on ITS region (ITS-1-5.8S rRNA gene-ITS-2) of rDNA. The tree was constructed by bootstrap

Maximum Parsimony analysis of aligned sequences and the numbers at nodes indicate percentage of bootstrap sampling from 1,000 replicates. The bar represents 10 nucleotide changes

hyphae vel pseudohyphae *neque* structurae sexuales in farina maydis neque 2% malti extracto. *Substrata assimilata*: D-glucosum, D-galactosum, D-xylosum, L-arabinosum, sucrosus, maltosus, α -trehalosus, methylum α -glucosidum, cellobiosum, salicinum, arbutinum, lactosum, L-rhamnosum, raffinimum, melezitium, *amylum solubile*, xylytolium, L-arabinitolum, D-glucitolium, D-mannitolium, myo-inositolum, 2-keto-D-gluconatum, D-gluconatum, D-glucuronatum, succinatum, saccharatum, cadaverinum, D-tryptophanum et nitratum; *parce assimilata*: D-arabinosum, ethanolium et methanolium; *non assimilata*: L-sorbosum, D-glucosaminum, D-ribosum, melibiosum, inulinum, glycerolum, meso-erythritolum, ribitolium, galactitolium, gluconum- δ -lactonum, D-galacturonatum, DL-lactatum, citratum, *propene-1,2-diolum*, butan-2,3-diolum, acidum quinicum, *acidum* galactonicum, ethylaminum, L-lysinum, imidazolium. Ureaso et diazonio caeruleo B reagit. Typus CBS 10160 *lyophilus*.

Description of *Cryptococcus randhawai* Khan, Ahmad, Hagen, Fell, Kowshik, Chandy, Boekhout sp. nov

Etymology: *Cryptococcus randhawai*, named after Prof. Harbans Singh Randhawa, in recognition of his invaluable contribution to the development of medical mycology in India.

On Sabouraud dextrose agar and malt extract agar at 28°C, the isolate produced cream-colored, smooth, shiny colonies which on aging became slightly tan. The growth was good up to 35°C and only scanty at 37°C. Dalmau culture on cornmeal agar did not produce any hyphae or pseudohyphae when examined up to 3 weeks and no sexual structures were observed either on cornmeal or 2% malt agar. The assimilation profile for different compounds was as follows: D-glucose, D-galactose, D-xylose, L-arabinose, sucrose, maltose, α -trehalose, methyl α -glucoside, cellobiose, salicin, arbutin, lactose, L-rhamnose, raffinose, melezitose, soluble starch, xylytol, L-arabinitol, D-glucitol, D-mannitol, myo-inositol, 2-keto-D-gluconate, D-gluconate, D-glucuronate, succinate, saccharate, cadaverine, D-tryptophan, and nitrate were assimilated; D-arabinose, ethanol and methanol showed weak/delayed assimilation, and L-sorbose, D-glucosamine, D-ribose, melibiose, inulin, glycerol, meso-erythritol, ribitol, galactitol, glucono- δ -lactone,

D-galacturonate, DL-lactate, citrate, propane 1, 2 diol, butane 2,3 diol, quinic acid, galactonic acid, ethylamine, and L-lysine, were not assimilated. The isolate showed positive urease and diazonium blue B reaction. Type strain is CBS10160.

The DNA sequences of the D1/D2 domains and the ITS-1 and -2 loci have been submitted to EMBL under accession nos. AJ876528 and AJ876599, respectively. The isolate has been deposited in CABI biosciences and Centraalbureau voor Schimmelcultures under the accession numbers. IMI 393306 and CBS10160, respectively.

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