Cryptotrichosporon anacardii gen. nov., sp. nov., a new trichosporonoid capsulate basidiomycetous yeast from Nigeria that is able to form melanin on niger seed agar

Ikechukwu Okoli[1], Christie A. Oyeka[1], Kyung J. Kwon-Chung[2], Bart Theelen[3], Vincent Robert[3], Johannes Z. Groenewald[3], Diane C. McFadden[4], Arturo Casadevall[4] & Teun Boekhout[3]

[1]Department of Applied Microbiology, Nnamdi Azikiwe University, Anambra State, Nigeria; [2]Laboratory of Clinical Infectious Diseases/NIAID, National Institutes of Health, Bethesda, MD, USA; [3]Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; and [4]Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Correspondence: Teun Boekhout, Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. Tel.: +31 30 2122671; fax: +31 30 2512097; e-mail: boekhout@cbs.knaw.nl

Received 20 April 2006; revised 12 July 2006; accepted 18 July 2006.
First published online 27 September 2006.

DOI:10.1111/j.1567-1364.2006.00164.x

Editor: Cletus Kurtzman

Keywords
Cryptotrichosporon anacardii; *Cryptococcus neoformans*; *Trichosporonales*; basidiomycetous yeast; taxonomic conflict; monophyly.

Abstract

Five yeast isolates obtained from cashew tree flowers in Nigeria resembled *Cryptococcus neoformans* phenotypically by producing brown pigmented colonies on niger seed agar, expressing a capsule, and being able to grow at 37 °C. However, rRNA gene sequences, including the 18S rRNA gene, the D1/D2 domains of the 26S rRNA gene and the ITS1+2 regions, suggested that these yeasts form a basal lineage within the *Trichosporonales* (*Tremellomycetidae*, *Hymenomycetes*, *Basidiomycota*, *Fungi*). Since the isolates could not be identified with any known genus and species within the *Trichosporonales*, we describe them as *Cryptotrichosporon anacardii* gen. et sp. nov. with CBS 9551^{T} (=NRRL Y-27671) as the type strain. The taxonomic conflict between phenetic and molecular classification schemes within this group of fungi is discussed, and is resolved in favor of the latter.

Introduction

The human pathogenic yeast *Cryptococcus neoformans* is known to be associated with various trees, in particular *Eucalyptus* spp. (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992). More recently, a wide variety of trees has been found to harbor this species, mainly on bark, decaying wood or wood debris, e.g. *Syzygium jambolana* (Java plum tree), *Syzygium cucumi* (Indian black berry tree), *Cassia grandis* (pink shower tree), *Ficus microcarpa* (curtain fig tree), *F. religiosa* (peepul tree), *Senna multijuga* (November shower tree), *Moquilea tomentosa* (pottery tree) and *Terminalia catappa* (Indian almond tree) (Lazera *et al.*, 1996, 1998; Callejas *et al.*, 1998; Randhawa *et al.*, 2003). Because we were interested in finding the environmental sources of *Cryptococcus neoformans* in Nigeria, we started an ecological investigation in June 2002.

The cashew tree (*Anacardium occidentale*) is a tropical species found in most countries in West Africa, including Nigeria, where it grows primarily in the southeastern part of the country. Flowering of the cashew tree starts in December/January, and early fruiting occurs in February. Between the months of March and April, matured yellow or red succulent fruits are produced.

During our investigations into the ecology of *Cryptococcus neoformans* in Nigeria, we obtained five yeast isolates, which phenotypically resembled *Cryptococcus neoformans* in forming capsules and producing brown colonies on niger seed agar. However, sequencing of the ITS1 and ITS2 regions and the D1/D2 domains of the 26S rRNA gene as well as the 18S rRNA gene suggested a distant phylogenetic position from *Cryptococcus neoformans*, the type species of the genus *Cryptococcus*, which belongs to the *Tremellales* (*Tremellomycetidae*, *Hymenomycetes*, *Basidiomycota*, *Fungi*). In contrast, our yeast species formed a basal lineage within the *Trichosporonales*, and not within the *Tremellales*, based on all three rRNA gene regions analyzed. The Nigerian yeasts are only distantly related to the generic type species of *Cryptococcus*, based on three different domains of the rRNA gene operon. Hence, the question remains of whether to place them in the genus *Cryptococcus* using the phenotypic characters, or to consider the taxonomic consequences of our molecular

analyses. We favor the latter option, and consequently propose a new genus and species, *Cryptotrichosporon anacardii* gen. nov. et sp. nov., to accommodate the Nigerian isolates within the *Trichosporonales*.

Materials and methods

Collection of samples

One hundred and forty-one samples of fresh and dead flowers of the cashew tree were collected during June 2002 at three different locations in Nnobi in Idemili South Local Government Area of Anambra State, Nigeria (latitude 6°3′N, longitude 6°57′E, altitude 213 m). Annual rainfall in this region is about 2000 mm, mean annual maximum temperatures vary between 25 and 36 °C, and mean annual minimum temperatures vary between 14 and 23 °C. Relative humidity varies between 20% in the morning and 30% in the afternoon in the dry season (November–April), and from 50% in the morning to 90% in the afternoon in the rainy season (May–October). Isolates that formed melanin-like pigments were selected as follows. Duplicate sets of flowers were pulverized with a sterile pestle and mortar, and aseptically transferred into a 250-mL Erlenmeyer flask. About 50 mL of sterile distilled water (SDW) was added, and the suspension was shaken vigorously for 10–15 min and allowed to settle for 5 min. Five milliliters of the supernatant was mixed with an equal amount of SDW containing chloramphenicol (0.05 mg mL^{-1}) and allowed to stand at room temperature (25–36 °C) for 1 h. Half-milliliter volumes were then plated on niger seed (*Guizotia abyssinica*) agar plates (50 g of niger seed, 10 g of sucrose, 1 g of creatinine, 1 g of potassium dihydrogen orthophosphate, 20 g of agar, 1000 mL of distilled water) containing 0.05 mg mL^{-1} chloramphenicol. The plates were incubated at 25 and 37 °C for 72 h.

The yeast isolates were subjected to standard morphologic and microscopic examination, and those forming colonies with a light brown color (see below) were maintained on Sabouraud dextrose agar (SDA) slants containing 0.05 mg mL^{-1} chloramphenicol.

Morphology, mating, growth tests and serotyping

The morphology of the isolates was investigated using line inoculations on the following media: YPGA (1% yeast extract, 0.5% peptone, 4% glucose) agar, yeast malt extract agar (YMA, Difco, Detroit, MI), yeast morphology agar (YMoA, Difco), malt extract agar (MEA, Oxoid, Detroit, MI), SDA (Difco) and potato dextrose agar (PDA, Difco). Formation of ballistoconidia was also investigated using these media. Nuclear staining was performed by suspending yeast cells in a drop of 10% picogreen (Molecular Probes, Leiden, The Netherlands) in phosphate-buffered saline (PBS), storing in the dark for 2 h, and viewing under a fluorescence microscope (Zeiss Axioskop, Zeiss, Weesp, The Netherlands) using filter 05 (excitation wavelength 395–440 nm, emission wavelength 460 nm).

Mating experiments were performed on corn meal agar (CMA, Difco) at room temperature. Plates were incubated for several weeks at 25 °C and checked microscopically at regular intervals.

Growth at different temperatures (20, 25, 30, 35, 37 and 40 °C) was evaluated using inoculated YPGA slants placed in incubators at the appropriate temperature. In addition, growth at 37 °C was confirmed on SDA plates sealed with parafilm to reduce the rate of desiccation. Needle point holes were made in the parafilm to permit aeration, and the plates were incubated for 10 days at 37 °C.

The nutritional requirements of the yeast strains were investigated using the microtiter plate method as described by Kurtzman *et al.* (2003). The results were compared with the CBS database as described by Robert (2003). Fermentation, diazonium Blue B (DBB) reaction and urease activities were assessed as described by Yarrow (1998). Serotyping of each isolate was performed using the Crypto-Check Iatron Kit (Iatron Laboratories, Tokyo, Japan).

Formation of melanin-like pigments

Formation of melanin-like pigments was investigated using minimal media (4 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 0.975 g of glycine, 3 g of glucose, 51 μL of thiamine, and 1 L of sterile distilled water) containing 1 or 2 mM of phenolic compound, and incubation at 30 °C for up to 14 days. The phenolic compounds investigated were L-3,4-dihydroxyphenylalanine (L-dopa), methyl-dihydroxyphenylalanine (methyl-dopa), epinephrine, norepinephrine and tyrosine. Cells of the Nigerian yeast strains (i.e. CBS 9549, CBS 9550, CBS 9551, CBS 9552, and CBS 9553) were patched on separate agar plates of the minimal media. Production of pale to very dark brown pigment was regarded as positive for laccase activity.

Capsular characteristics

The presence of a capsule was investigated on YPGA using negative staining with India ink. To estimate the size of the capsule, cells were suspended in India ink preparation, and pictures were taken with an Olympus AX70 microscope (Olympus Optical Co., Tokyo, Japan), photographed with a QImaging Retiga 1300 digital camera using the QCAPTURE SUITE V2.46 software (QImaging, Burnaby, BC, Canada), and processed with ADOBE PHOTOSHOP 7.0 for Windows (San Jose, CA). The cell diameters, including and excluding the

capsule, were measured. Owing to the morphology of the cell, which is mostly ellipsoidal, measurements were taken for both the long and the short axis. Thus, four values were obtained. The capsule thickness of each cell was given by [(length with capsule − length without capsule) + (width with capsule − width without capsule)] divided by four. Thirty cells were measured for each strain and the values were averaged.

The Hestrin assay was used to give an indication of the level of acetylation of the capsule. This assay is based on the reaction of acetyl groups of polysaccharides with hydroxylamine in alkali to form hydroxamic acids, and was performed as described by Hestrin (1949) using 48-h-old cultures of the five strains grown in Sabouraud dextrose broth (SDB). The cultures were washed five times with SDW and resuspended at a density of 5×10^8 cells mL^{-1}. Capsules were further biochemically characterized using the methods of Goren & Milddlebrook (1967) and Dubois *et al.* (1956).

Capsular charge was measured as the ζ potential. Cells of the five strains were harvested from 48-h SDB cultures grown at 30 °C, washed twice with PBS and once with 1 mM KCl solution, and resuspended in 1 mM KCl solution to 1×10^7 cells mL^{-1}. The ζ potentials of the five strains were measured using the Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) according to the manufacturer's instructions.

rRNA gene sequencing

gDNA was isolated as described by O'Donnell *et al.* (1997). The ITS region and D1/D2 domains of the 26S rRNA gene were amplified using the primers V9 (5′-TGC GTT GAT TAC GTC CCT GC) and RLR3R (5′-GGT CCG TGT TTC AAG AC) according to standard DNA-sequencing protocols (Fell *et al.*, 2000; Boekhout *et al.*, 2003). Sequencing primers used for the ITS1, 5.8S rRNA gene and ITS2 regions were ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC), and those used for the LSU rRNA gene region were NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG) and RLR3R. The 18S rRNA gene was amplified using the NS1 and NS24 primers described by White *et al.* (1990), using standard PCR protocols. For sequencing the primers NS1, oli1, oli10, oli9, oli11, oli 14, oli 3, and oli13 standard PCR conditions were used (de Hoog *et al.*, 2005).

The sequences were size fractionated on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA) and analyzed using the LASERGENE software package (DNA STAR Inc., Madison, WI). Genbank accession numbers of the D1/D2 domains of the LSU are AY158665–AY158670, those for the ITS region are AY158671–AY158676, and those for the 18S rRNA gene are DQ242635 and DQ242636. For a complete analysis, we sequenced the ITS region and the D1/D2 domains of *Bullera formosensis* (accession numbers AY787859 and AY787858) and the ITS region of *Cryptococcus haglerorum* (accession number AY787857). The corrected sequences for both ITS and LSU were compared with those present in the GenBank/NCBI database using the BLAST 2.0 program (Altschul *et al.*, 1990). The closest matches were selected to make an alignment in MEGALIGN (DNASTAR Inc., Madison, WI) using the CLUSTAL W method, including a gap penalty of 10.0 and a gap length penalty of 0.10. Phylogenetic trees were generated using PAUP* version 4.0b10 for Macintosh (Swofford, 2002). Neighbor-joining analysis was performed with the uncorrected ('p') substitution model, alignment gaps were treated as missing data, and all characters were unordered and of equal weight. For parsimony analysis, gaps were treated as missing data and all characters were unordered and of equal weight. The heuristic search was performed with 1000 random taxa additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed, and all equally parsimonious trees were saved. The robustness of the obtained trees was evaluated by 1000 bootstrap replications. Other statistical measures included were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC).

Results

Phenetic characteristics

Yeast isolates becoming light brown on niger seed agar plates were found for 15 of the 141 fresh flower samples, but for none of the dead flower samples. The light brown colonies were produced after 48 h of incubation at 25–36 °C. Four of the strains (CBS 9549, CBS 9550, CBS 9551, and CBS 9552) produced dark brown pigment with four phenolic compounds (L-dopa, methyl-dopa, epinephrine, and norepinephrine) (Table 1). Also, the intensity of the brown pigment increased with higher concentrations of the phenolic compounds in the minimal media, except for strain CBS 9553 (Table 1). None of the five strains of the Nigerian yeast isolates produced brown pigment with tyrosine. Furthermore, the strains showed evidence of secretion of laccase into the medium and formation of melanin, as indicated by the brownish halo surrounding the cell patches in the medium (Fig. 1).

After incubation for 10 days, mucoid to slimy, pale yellowish brown (isabella) colonies were apparent on YMoA, YMA, and YPGA. The morphology of the cells was usually ellipsoidal to limoniform, and the cells measured 4.5–8.5 × 2.5–4.5 μm (Fig. 2c), but inflated cells, measuring 8–11 × 7–9 μm, were also observed (Fig. 2b). Formation of ballistoconidia was not observed.

Table 1. Pigmentation by five strains of *Cryptotrichosporon anacardii* with different phenolic substrates

	L-dopa		Methyl-dopa		Epinephrine		Norepinephrine		Tyrosine	
Strain (CBS)	1 mM	2 mM	1 mM	2 mM	1 mM	2 mM	1 mM	2 mM	1 mM	2 mM
9549	+	++	+	++	f	++	+	++	–	–
9550	+	++	+	++	f	++	+	++	–	–
9551	+	++	+	++	f	++	+	++	–	–
9552	+	++	+	++	f	++	+	++	–	–
9553	f	vf	vf	–	f	vf	vf	vf	–	–

+, dark brown; ++, very dark brown; f, light brown; vf, very light brown; –, no brown pigmentation.

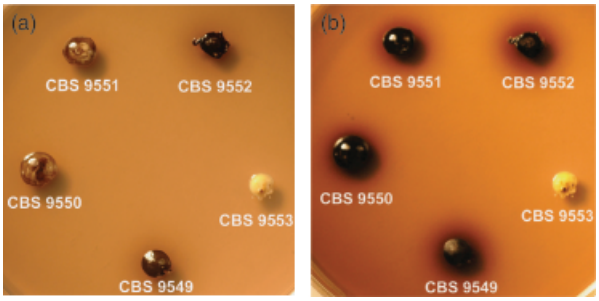

Fig. 1. Melanin-like pigment formation by *Cryptotrichosporon anacardii* on phenolic compound. Patches of five strains after 6 (a) and 12 (b) days of incubation on minimal medium with 2 mM methyl-dihydroxyphenylalanine.

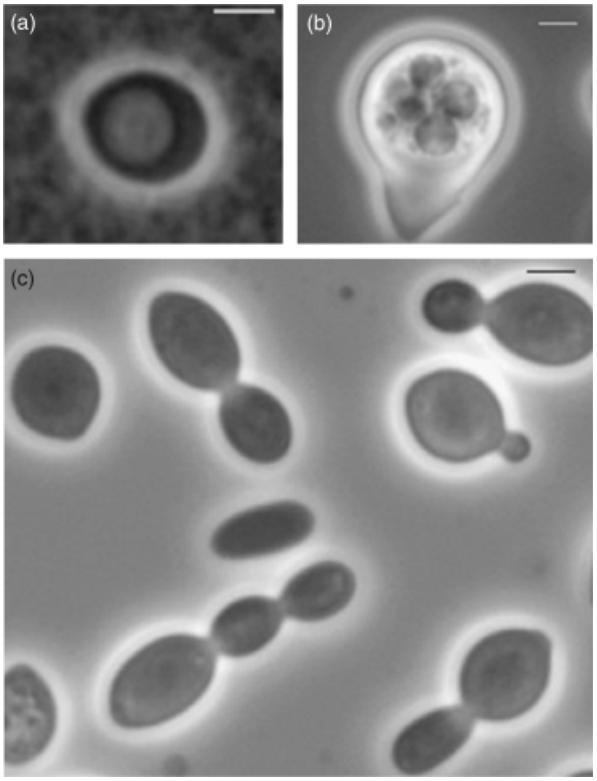

Fig. 2. Morphology of *Cryptotrichosporon anacardii*. (a) Presence of a capsule in CBS 9553 after 4 weeks of growth on YPGA upon negative staining with India ink. (b) Inflated cells of CBS 9553 after 10 days of growth on MEA observed using phase contrast optics. (c) Morphology of budding cells of CBS 9553 after 10 days of growth on YMoA observed using phase contrast optics. Scale bar = 5 μm.

In liquid medium with minimal shaking, sediment, some floating flocks and a (partial) ring were formed. Staining with picogreen demonstrated the presence of a single nucleus per cell. Crossings on CMA did not result in any mating reactions.

The five strains investigated showed nearly identical biochemical profiles. Fermentation of D-glucose was absent. Soluble extracellular carbohydrates were present after addition of Lugol's solution to a culture grown in 2% glucose in yeast nitrogen base (Difco). Assimilation of the carbon compounds D-glucose, D-galactose, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, maltose, α,α-trehalose, cellobiose, salicin, arbutin, melezitose, starch, xylitol, 5-keto-D-gluconate, D-glucurate, palatinose, galactaric acid, and gentobiose was positive. L-Sorbose, D-glucosamine, methyl-α-D-glucoside, melibiose, lactose, raffinose, inulin, glycerol, erythritol, L-arabinitol, D-mannitol, galactitol, D-galacturonate, DL-lactate, succinate, citrate, propane-1,2-diol, butane-2,3-diol, quinic acid, D-galactonate, levulinate, D-tartaric acid, meso-tartaric acid, uric acid, ethylene, glycol, Tween-40, Tween-60 and Tween-80 were not assimilated. Growth was variable, weak or absent on sucrose, ribitol, D-glucitol, myo-inositol, D-glucono-1,5-lactone, D-glucuronate and L-tartaric acid. Assimilation of D-gluconate and L-malic acid was weak to positive. Assimilation of nitrogen compounds was usually absent or weak. Only L-lysine was clearly assimilated. Nitrate, cadaverine, creatine, creatinine, D-proline and putrescine were not assimilated, and assimilation of nitrite, ethylamine, glucosamine, imidazole and D-tryptophan was weak to absent. Growth was absent without vitamins. Growth was inhibited by 0.01% cycloheximide, by 1% acetic acid, and after addition of 10% and 16% NaCl. Growth occurred at pH 9.5, but was variable at pH 3. The DBB reaction and urease activity were positive. Growth was absent at 10 °C, but occurred readily between 17 and 33 °C. Growth was weak at 36 °C and delayed at 37 °C. At this latter temperature, colonies appeared only after about 8–10 days,

and colonies of strains CBS 9549, CBS 9550 and CBS 9551 appeared before those of CBS 9552 and CBS 9553.

Capsules were visible under the microscope with India ink staining (Fig. 2a). The strains varied in capsule size with CBS 9552 ($1.83 \pm 0.64\,\mu m$, $n = 5$) > CBS 9549 ($1.33 \pm 0.44\,\mu m$, $n = 5$) > CBS 9551 ($0.82 \pm 0.21\,\mu m$, $n = 5$) > CBS 9550 ($0.65 \pm 0.21\,\mu m$, $n = 5$) > CBS 9553 ($0.55 \pm 0.20\,\mu m$, $n = 5$). Serotyping results with polyclonal as well as monoclonal factor sera were all negative. There was no agglutination reaction with factor sera, indicating that the chemical structure of the capsule is antigenically different to that of glucuronoxylomannan produced by *Cryptococcus neoformans*. All five strains had large negative charges from the ζ potential readings, as follows ($n = 5$): CBS 9549, -34.86 (mV) ± 0.77; CBS 9550, -34.97 (mV) ± 0.80; CBS 9551, -33.17 (mV) ± 1.36; CBS 9552, -36.95 (mV) ± 1.34; and CBS 9553, -22.20 (mV) ± 1.09 (average of three readings). These values are similar to those previously reported for *Cryptococcus neoformans* (Nosanchuk & Casadevall, 1997), and suggest that the capsule is anionic in nature.

The Hestrin assay was positive for all isolates, implying the presence of acetyl groups in their capsules. The capsule from one of the Nigerian isolates (CBS 9551) was chemically removed by briefly suspending the cells in dimethylsulfoxide (DMSO). This yielded a water-insoluble material that was positive by the phenol–sulfuric acid method for carbohydrate detection, suggesting that the capsule is composed of a polysaccharide. Although seemingly similar to the capsules of *Cryptococcus neoformans* in charge and acetylation, the DMSO-extracted capsular material of CBS 9551 differed from the water-soluble capsule of *Cryptococcus neoformans*. Further investigation of the solubility of the capsular material from CBS 9551 showed that it was soluble in 100% DMSO, but insoluble in 100% ethanol, 100% methanol, 0.15 M NaCl, and 200 mM NH_4HCO_3.

Molecular systematics

Sequence analysis of the 18S rRNA gene, the D1/D2 domains of the 26S rRNA gene and the ITS1+2 regions demonstrated that the isolates clustered within the *Hymenomycetes*, and more specifically within the *Tremellomycetidae*, where they formed a basal lineage within the *Trichosporonales* (Figs 3, 4; see 18S rRNA gene tree at www.cbs.knaw.nl/publications/cryptotrichosporon). With neighbor-joining (NJ) analysis, the 18S rRNA gene, D1/D2 and ITS trees placed the Nigerian isolates within the *Trichosporonales*, with moderate bootstrap support of 80%, 61% and 80%, respectively (data not shown). The NJ 18S rRNA gene tree placed our isolates next to a more basal cluster of three species positioned by phenetic criteria in the genus *Bullera*, namely *B. formosensus*, the not yet described *B. nakhonratchasimensis* and *Bullera* species. The remainder of the *Trichosporonales* cluster was composed mainly of species classified in the genus *Trichosporon*, some unidentified basidiomycetous yeasts, and some *Cryptococcus* species, such as *Cryptococcus fragicola*, *Cryptococcus daszewskae*, *Cryptococcus curvatus*, *Cryptococcus musci*, *Cryptococcus ramirezgomezianus*, *Cryptococcus humicola*, *Cryptococcus pseudolongus* and *Cryptococcus longus*. Also, *Asterotremella parasitica* clustered in the *Trichosporonales* clade. Bootstrap support for the *Trichosporonales* clade was 87%, and the placement of the Nigerian isolates as a basal lineage within this clade was supported by 80%. The *Trichosporonales* yielded seven subclades, which agreed with those obtained in the ITS and D1/D2 analysis (Figs 3, 4; www.cbs.knaw.nl/publications/cryptotrichosporon).

Parsimony analysis of the ITS resulted in five equally parsimonious trees, all with the Nigerian isolates clustering basally within the *Trichosporonales* (data not shown). The parsimony consensus tree showed the inclusion of the Nigerian yeast isolates as a basal lineage within the *Trichosporonales* with only 63% bootstrap support. Parsimony analysis of the D1/D2 domains resulted in a single most parsimonious tree with the Nigerian isolates occurring as a basal lineage within the *Trichosporonales*, which, however, was hardly supported by a bootstrap value of 57%. After concatenation of the ITS and D1/D2 domains, 12 equally parsimonious trees were generated. One of these trees is presented in Fig. 3, indicating the bootstrap values based on 1000 bootstrap replicates. It can be seen that the Nigerian yeast isolates formed a well-supported basal lineage within the *Trichosporonales*, as did the *Bullera formosensis* cluster. Within *Trichosporon*, seven distinct clusters could be discerned, namely the *ovoides*-, *gracile*-, *Hyalodendron*-, *cutaneum*-, *Trichosporon*-like *Cryptococcus* spp., *B. formosensis*, and the Nigerian yeast clusters. The clustering of the Nigerian yeast isolates and the *B. formosensis* cluster as basal lineages within the *Trichosporonales* is supported by a 96% bootstrap value. Unlike with the 18S rRNA gene analysis, the exact relationship between the Nigerian yeast isolates and the *B. formosensis* cluster was not resolved. Probably, *Cryptococcus fragicola* represents an individual cluster as well. The *Hyalodendron* cluster contained some undescribed basidiomycetous yeasts, mainly isolated from beetles (Suh *et al.*, 2005).

Discussion

Phenetic characteristics

We isolated an encapsulated yeast species from fresh flowers of the cashew tree in Nigeria, which formed pale brown colonies on niger seed agar. Therefore, we initially suspected these yeasts to be *Cryptococcus neoformans*. The ecological association of these *Cryptococcus neoformans*-like yeasts with

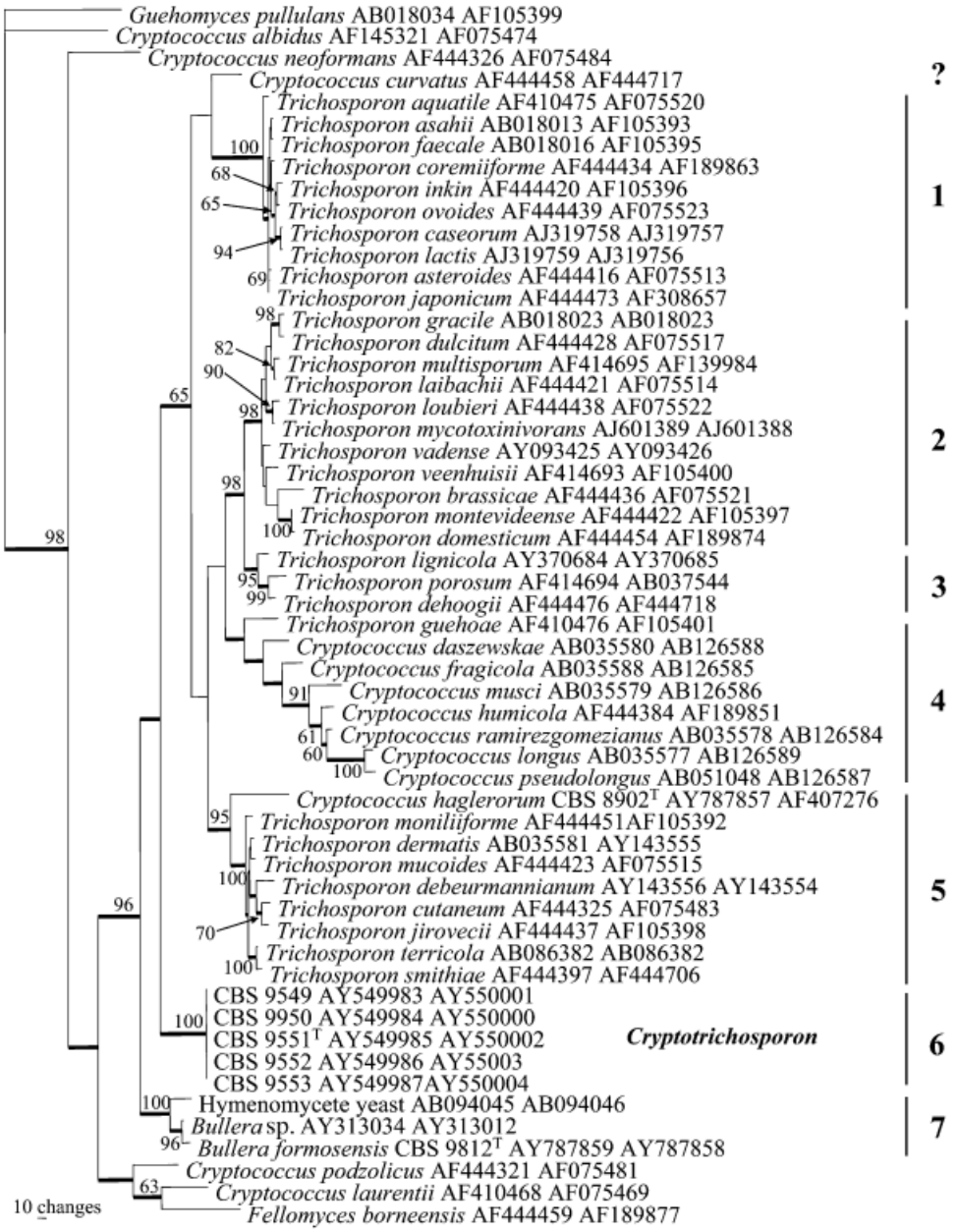

Fig. 3. One of 12 equally parsimonious trees showing the phylogenetic position of the *Cryptotrichosporon anacardii* cluster based on combined ITS and D1/D2 nucleotide datasets using 1000 random taxon additions (CI = 0.442, RI = 0.722, RC = 0.319, HI = 0.558). The numbers indicated on the branches represent bootstrap values of 1000 replicates. Strict consensus branches are thickened. Note the basal position of *Cryptotrichosporon anacardii* and the *Bullera formosensis* cluster within the *Trichosporonales*. As in the 18S rRNA gene tree, seven distinct clusters occur, which may represent individual genera. *Guehomyces pullulans* and *Cryptoccus albidus* were used as outgroups. Question mark indicates taxa of uncertain affinity.

cashew nut tree interested us, because this might represent a possible new host tree for this fungus in Africa. However, attempts to serotype the isolates using polyclonal or monoclonal factors specific for *Cryptococcus neoformans* were unsuccessful, suggesting that the capsules of these yeasts were antigenically different, thus questioning the initial presumptive identification as *Cryptococcus neoformans*.

Capsules are present in many basidiomycetous yeasts, most of which belong to the *Tremellales* and *Filobasidiales*, and the genes involved in capsule biosynthesis may differ considerably among the various species (Petter *et al*., 2001). The presence of an anionic capsule in the Nigerian yeast isolates was strongly suggested by the high negative charges detected in the ζ potential readings. Prior studies comparing

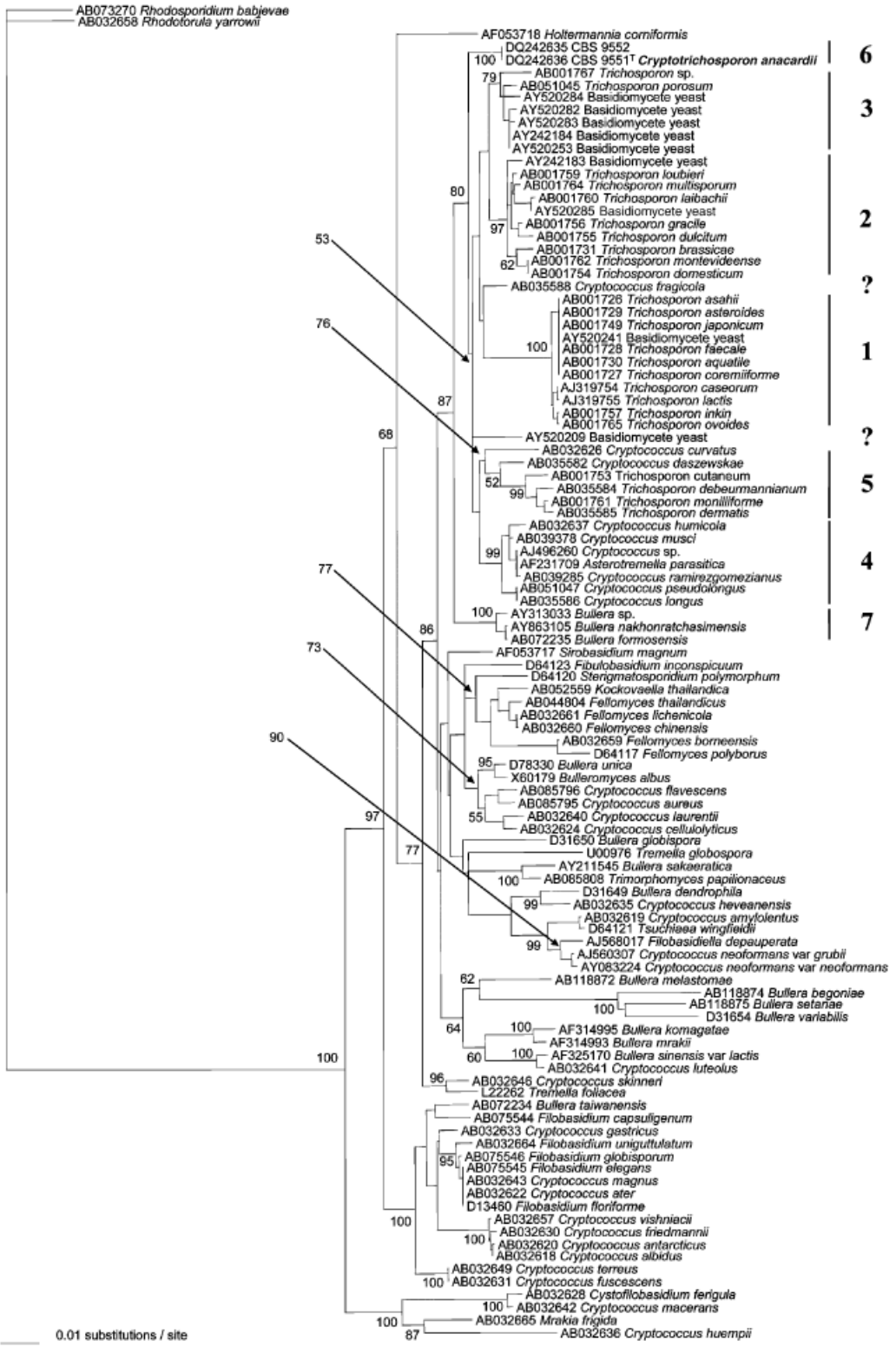

Fig. 4. Bootstrapped neighbor-joining tree of the 18S rRNA gene of *Cryptotrichosporon anacardii* (DQ242635/6) indicating a basal position within the *Trichosporonales* (www.cbs.knaw.nl/publications/cryptotrichosporon). *Rhodosporidium babjevae* and *Rhodotorula yarrowii* were used as outgroups. Note the seven subclusters within the *Trichosporonales*. Question marks indicate taxa of uncertain affinity.

ζ potentials of encapsulated and nonencapsulated yeast cells have shown that the capsule is responsible for the high negative values associated with encapsulated cryptococcal strains (Nosanchuk & Casadevall, 1997). The positive Hestrin assays implied the presence of acetyl groups, indicating a similarity between this polysaccharide and that of *Cryptococcus neoformans* (Cherniak *et al.*, 1980). However, the insolubility of the extracted capsular material demonstrated that the capsule of the Nigerian isolates has at least one distinct chemical property different from those of *Cryptococcus neoformans*. One potential structural explanation for the solubility difference is that the capsule of the Nigerian isolates may contain a highly branched glucuronoxylomannan. Such a structure would account for the negative charge, the presence of acetylation, and the failure of antibodies to the glucuronoxylomannan of *Cryptococcus neoformans* to recognize the Nigerian isolates. This would also be consistent with the isolated taxonomic position near *Trichosporon* spp., as these are known to contain glucuronoxylomannans on their surface. Further chemical and structural evaluation is required to explore this possibility.

The formation of brown colonies by the Nigerian isolates on niger seed agar or minimal medium supplemented with various phenolic compounds indicates a capability to synthesize melanin-like pigments, which is a rare feature among basidiomycetous yeasts in general (Petter *et al.*, 2001). Interestingly, the *CNLAC1* gene, involved in laccase biosynthesis in *Cryptococcus neoformans*, was demonstrated to be present in another melanin-like pigment-forming yeast species, namely *Cryptococcus podzolicus* (Petter *et al.*, 2001). Oxidation by laccase has been observed in isolates of *Cryptococcus albidus*, *Cryptococcus laurentii* and *Cryptococcus curvatus*, but with a lower activity than that of the enzyme produced by *Cryptococcus neoformans* (Ikeda *et al.*, 2002). None of the five strains of the Nigerian yeast isolates produced brown pigment with tyrosine, which is a feature shared with *Cryptococcus neoformans* (Polacheck *et al.*, 1982). The secretion of laccase or melanin into the growth medium by the Nigerian yeast isolates was suggested by a halo of pigmentation surrounding the colonies. Whereas this phenomenon readily occurs with the Nigerian isolates, it has been described only once, to our knowledge, in the literature and was detected in a *Cryptococcus neoformans* mutant (Idnurm *et al.*, 2004).

The presence of a capsule and melanin formation are prime diagnostic characteristics for *Cryptococcus neoformans*, and this may imply that either *Cryptococcus podzolicus* or the Nigerian yeast species can be easily misidentified as *Cryptococcus neoformans*, if the presence of a capsule and melanin formation are used as the only diagnostic criteria. Furthermore, it can be speculated that the Nigerian yeast species may be able to grow in immunodeficient warm-blooded animals, as it can grow slowly at 37 °C and express a polysaccharide capsule, and has the ability to form melanin-like pigments, characteristics that are all important virulence factors of *Cryptococcus neoformans* (Rhodes *et al.*, 1982; Buchanan & Murphy, 1998; Perfect *et al.*, 1998).

When the physiological profile of the Nigerian isolates was compared with the CBS database using the BIOLOMICS software, we found that it matched best the species description of *Cryptococcus humicola*. However, there are numerous physiological variables among the strains of *Cryptococcus humicola*, and the type strain of *Cryptococcus humicola* (CBS 571) exhibited more than 20 differences from the Nigerian isolates. Several other species, such as *Rhodotorula glutinis*, *Metschnikowia koreensis*, *Cryptococcus laurentii*, and *Sporobolomyces roseus*, were reported as close (two to three physiologic differences), but this also seems to be due to the variability of their reported physiological profiles.

The life cycle of the cashew tree flower-associated yeasts is not known. Mass mating experiments did not result in the production of a sexual stage. Therefore, two alternatives seem possible. First, they represent the anamorph of a sexually dimorphic trichosporonalean fungus, in which the relationship between the anamorph and teleomorph has not been established. Second, they reproduce only asexually. As none of the yeasts currently classified in the *Trichosporonales* have a known sexual state, it is possible that this lineage comprises mainly asexual fungi.

Phenetic vs. molecular classification

According to sequence analysis of the 18S rRNA gene, the D1/D2 domains of the 26S rRNA gene and the ITS1+2 regions, the Nigerian isolates formed a basal lineage within the *Trichosporonales*, and not within the *Tremellales* or *Filobasidiales*. The maximum bootstrap support for this inclusion was 96% using the combined ITS and D1/D2 datasets, and 87% in the 18S rRNA gene analysis. All three domains analyzed supported the relatedness of our yeast isolates with the *Trichosporonales*, but they did not match any known species and genus within the order. In principle, two options are possible in this case. The first option is to describe the isolates as a new species within an existing genus belonging to the *Trichosporonales*, i.e. as a new species of *Trichosporon*; the second option is to describe a new anamorphic genus to accommodate the yeast isolates. The main disadvantage of the first option is that the genus *Trichosporon*, which already seems polyphyletic, and may comprise various genera (Takashima *et al.*, 2001), will become even more polyphyletic and heterogeneous in phenotypic characteristics.

The order *Trichosporonales* (Fell *et al.*, 2000) accommodated initially only *Trichosporon* species. Recently, some *Cryptococcus*, *Hyalodendron* and *Bullera* species, e.g. *Cryptococcus curvatus*, *Cryptococcus humicola*, *Cryptococcus musea*,

Cryptococcus ramirezgomezianus, *Cryptococcus longus*, *Cryptococcus pseudolongus*, *Cryptococcus haglerorum*, *Cryptococcus daszewskae*, *B. formosensis*, and *Hyalodendron lignicola*, have been placed in or transferred to this order (Takashima *et al*., 2001; Nakase *et al*., 2002; Scorzetti *et al*., 2002; Middelhoven *et al*., 2003). We do not fully understand why in recent years new basidiomycetous yeast species, which phylogenetically belong to the *Trichosporonales*, have been placed in phylogenetically nonrelated genera such as *Cryptococcus* and *Bullera*. It seems that the use of traditional phenotypic characteristics as a guide for the classification of anamorphic basidiomycetous yeasts is still favored by various investigators, even when in conflict with results obtained by molecular phylogenetic studies. We think this is unfortunate, as some phenotypically defined genera of anamorphic basidiomycetous yeast species are highly polyphyletic (see above), and, hence, phylogenetically related species occur scattered among different genera. Good examples in this respect are the current concepts of the genera *Bullera* and *Cryptococcus*, which are characterized by the presence or absence of ballistoconidia, respectively. Species of these two genera occur mixed among each other in the tree of life, as they do not form genus-specific clades based on rRNA gene sequences, and both are highly polyphyletic (Fell *et al*., 2000; Scorzetti *et al*., 2002). Moreover, the main diagnostic phenotypic character, namely the formation of ballistoconidia, is not very stable (Nakase *et al*., 1995). This implies that their phenotypic classification may also be governed by trivial characteristics such as growth conditions and developmental stage. As a consequence of all this, cryptococcal species occur in various phylogenetic lineages of the hymenomycetous fungi, and occur intermingled with species classified in *Bullera*, *Dioszegia*, *Trichosporon*, *Tsuchiyaea*, and *Udeniomyces*. To further complicate the situation, various teleomorphic genera occur in these lineages as well, such as *Auriculibuller*, *Bulleribasidium*, *Cystofilobasidium*, *Fibulobasidium*, *Filobasidium*, *Filobasidiella*, *Mrakia*, and *Tremella* (Fell *et al*., 2000; Sampaio *et al*., 2002, 2004; Scorzetti *et al*., 2002).

One further aspect that needs to be considered relates to the comparison of phenetic and molecular (i.e. genomics) evolution. Comparative genomics analysis of a number of hemiascomycetous yeasts (Dujon *et al*., 2004; Dujon, 2006) revealed that phenetic divergence and evolution (i.e. morphologic characteristics and biological lifestyles) is limited in comparison to genomic diversification. For instance, the evolutionary divergence of the genomes of five hemiascomycetous species, *Saccharomyces cerevisiae*, *Candida glabrata*, *Kluyveromyces lactis*, *Debaryomyces hansenii* and *Yarrowia lipolytica*, is as great as that of the entire phylum Chordata (Dujon *et al*., 2004). The genomic divergence of two hemiascomycetous yeast species, *Saccharomyces cerevisiae* and *Candida glabrata*, which are considered to be rather closely related (Kurtzman, 2003; Kuramae *et al*., 2006), is as great as that of humans and fish (Dujon, 2006). Probably, the basidiomycetous yeasts show the same amount of genomic divergence, and, if this is true, phenetic criteria should be used with care in classification proposals.

With the aim of obtaining a reliable phylogenomics classification of yeasts (and filamentous fungi) (Kuramae *et al*., 2006) in the future, a holomorphic taxonomy is within reach, thus allowing us to abandon the artificial difference between anamorphic and teleomorphic classification schemes. In order to reduce the number of taxonomic changes required, we favor the use of conservative types, implying that a validly described generic type species occurring in a certain clade dictates the name of that clade, if justified at the generic level and taking into account the common priority rules.

Recently, some taxonomic changes have been proposed that are mainly or entirely based on molecular data. For instance, *Trichosporon pullulans*, which phenotypically resembles other species in the genus *Trichosporon*, was reclassified as *Guehomyces pullulans* (Fell & Scorzetti, 2004), and *Hyalodendron lignicola* was reclassified as *Trichosporon lignicola* (Fell & Scorzetti, 2004). A number of mite-associated yeast-like fungi, which morphologically resembled representatives of the ustilaginomycetous genus *Pseudozyma* and even some ascomycetes (i.e. *Fusidium*-like anamorphs), were classified in two new genera, namely *Acaromyces* and *Meira* (Boekhout *et al*., 2003).

The question to be answered here is how to interpret the molecular phylogenetic data of our Nigerian isolates in comparison with traditional phenotypic characteristics. First, it is clear that the isolates, although phenotypically resembling cryptococcal species, are only distantly related to this genus on the basis of rRNA gene analyses. One may, of course, favor the phenotypic resemblances, ignore the molecular relationships, and place the species in the genus *Cryptococcus* or one of the other phenotypically defined genera. In this scenario, the concept of highly polyphyletic anamorphic basidiomycetous genera is taken for granted. In our opinion, this is not an optimal solution, as such an artificial classification does not follow the principle of an evolution-based classification using monophyly as the leading concept. Therefore, we prefer to follow the molecular phylogenetic data, which place our fungi within the *Trichosporonales*. Hence, classification of our species within *Trichosporon* seems a realistic option. However, it is most likely that the seven recognized clades within the *Trichosporonales* will be reclassified in appropriate anamorphic genera (as long as their teleomorph connections are unknown) (Takashima *et al*., 2001). For some of these putative genera, names are already available; for example, the *ovoides* cluster forms the core of *Trichosporon*, the *Hyalodendron* cluster could be named *Hyalodendron*, and the *Trichosporon*-like

Cryptococcus spp. clade could be named *Vanrijia*, as the type species of the latter genus, *Cryptococcus humicola* (Moore, 1980), clustered here. For the other clusters, namely the *gracile* cluster, the *cutaneum* cluster, the *B. formosensis* cluster, and our Nigerian fungi, novel anamorphic genera are needed that reflect their phylogenetic relationships. It cannot be ruled out completely that the cluster with the Nigerian isolates, as well as that containing *B. formosensis*, may turn out to be a sister group to the *Trichosporonales*, if more species belonging to these clades are found or when more molecular markers are included in the analysis. Therefore, additional sampling is needed to determine the exact relationships within the *Trichosporonales*, as well as most, if not all, other clades belonging to the basidomycetous yeasts.

In short: (1) the Nigerian yeast isolates comprise a well-supported clade on the basis of three different parts of the nuclear rRNA gene operon; (2) the closest related generic type, *Trichosporon ovoides* Behrend, typifies a genus that is polyphyletic on the basis of molecular data and morphologically different; and (3) our new clade does not contain a generic type species. Hence, we propose to describe a new species and genus, *Cryptotrichosporon anacardii* gen. nov. et sp. nov., to accommodate the Nigerian yeast strains that form a basal lineage within the *Trichosporonales*.

Descriptions of proposed new taxa

Cryptotrichosporon Okoli & Boekhout, gen. nov.

Genus basale Trichosporonalium. Cellulae polariter ex basi lata gemmantes. Capsula adest. Pigmentum dilute brunneum in agaro seminibus confecto. Typus *Cryptotrichosporon anacardii*.

Etymology: *Cryptotrichosporon* refers to the intermediate position of the genus between *Cryptococcus* and *Trichosporon*, based on phenetic amd molecular systematics data, respectively. In addition, it refers to the cryptic nature of this trichosporonalean yeast, as it does not form hyphae or arthroconidia.

Cryptotrichosporon Okoli & Boekhout, gen. nov.

Yeast reproducing by budding, which forms a basal lineage within the *Trichosporonales*. Cells produce buds by polar budding on a broad base. A capsule is present, and the colonies form a pale brown pigment on niger seed agar. The type species is *Cryptotrichosporon anacardii*.

Cryptotrichosporon anacardii Okoli & Boekhout sp. nov.

Coloniae in agaro YPGA ad 15 mm diam post 7 dies temperatura 25 °C, modice convexae, mucidae vel mucidissimae, lucidae, dilute flavo-brunneae, reverso concolori, margine integra. Superficies marginem vs. modice radiatim striata. Pigmentum dilute brunneum in agaro seminibus confecto formatur. Cellulae ellipsoideae vel limoniformes, singulae vel breviter catenatae, 4.5–8.5 × 2.5–4.5 μm, post 20 dies filamenta brevia et catenae acropetales conidiorum, modice ramosae apparentes, conidiis fusiformibus. Cellulae utrinque e basi lata enteroblastice gemmantes, nonnumquam collare relinquentes. Cellulae giganteae, 8–11 × 7–9 μm, protuberantiis digitiformibus praeditae. Capsula gelatinosa formatur.

Typus CBS 9551 (= NRRL Y-27671), isolatus e flore *Anacardii occidentalis,* Nnobi in Area Gubernimentali Meridionali Idemili, Anambra in Nigeria.

Cryptotrichosporon anacardii Okoli & Boekhout sp. nov.

Colonies are up to 15 mm in diameter. On YPGA after 7 days at 25 °C, colonies are slightly convex to convex, slimy to very slimy, shiny, pale yellowish brown (isabella) with a concolorous reverse, and with an entire margin. The surface towards the margin may show a slight transverse striation. On niger seed agar, colonies are pale brown. Cells measure 4.5–8.5 × 2.5–4.5 μm, and are ellipsoidal to limoniform, and single or in short chains; after 3 weeks, short filaments and, acropetally, somewhat branched chains of fusiform blastoconidia may develop. Budding is enteroblastic, and occurs polarly on a broad base; a collarette may be visible. Inflated cells, measuring 8–11 × 7–9 μm, with finger-like protuberances occur. A capsule is visible in India ink preparations. Type specimen: CBS 9551 (= NRRL Y-27671), isolated from a flower of the cashew tree from Nnobi in Idemili South Local Government Area of Anambra State, Nigeria.

Etymology: The epithet *anacardii* refers to the habitat of the yeast in a flower of the Cashew tree (*Anacardium occidentale*).

Additional strains: CBS 9549, CBS 9550, CBS 9552 and CBS 9553, all from Nnobi, Idemili South Local Government Area of Anambra State, Nigeria.

Acknowledgements

Ikechukwu Okoli was supported partly by the International Society for Infectious Diseases (ISID), as a scientific exchange fellow at Albert Einstein College of Medicine, New York, USA. Christie Amechi Oyeka was supported by a Nnamdi Azikiwe University Senate Research Grant. We are grateful to Dr Oscar Zaragoza for technical assistance. Kyung J. Kwon-Chung's work was supported by funds from the intramural program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health,

Bethesda, MD. The 18S rRNA gene sequence was kindly provided by Kasper Luijsterburg (CBS).

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Boekhout T, Theelen B, Houbraken J, Robert R, Scorzetti G, Gerson U & Sztejnberg A (2003) New anamorphic acaropathogenic fungi belonging to the Ustilaginomycetes: *Meira geulakonigii* Boekhout, Gerson & Sztejnberg, Gen. et Spec. Nov., *Meira argovae* Boekhout, Gerson & Sztejnberg Spec. Nov. and *Acaromyces ingoldii* Boekhout, Gerson & Sztejnberg Gen. et Spec. Nov. *Int J Syst Evol Microbiol* **53**: 1655–1664.

Buchanan KL & Murphy JW (1998) What makes *Cryptococcus neoformans* a pathogen? *Emerg Infect Dis* **4**: 71–83.

Callejas A, Ordonez N, Rodriguez MC & Castaneda E (1998) First isolation of *Cryptococcus neoformans* var. *gattii*, serotype C, from the environment in Colombia. *Med Mycol* **36**: 341–344.

Cherniak R, Reiss E, Slodki ME, Plattner RD & Blumer SO (1980) Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Mol Immunol* **17**: 1025–1032.

de Hoog GS, Göttlich E, Platas G, Genilloud O, Leotta G & van Brummelen J (2005) Evolution, taxonomy and ecology of the genus *Thelebolus* in Antarctica. *Stud Mycol* **51**: 33–76.

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**: 350–356.

Dujon B (2006) Yeasts illustrate the molecular mechanisms of eukaryote genome evolution. *Trends Genet* **22**: 375–387.

Dujon B, Sherman D, Fischer G *et al.* (2004) Genome evolution in yeasts. *Nature* **430**: 35–44.

Ellis DH & Pfeiffer TJ (1990) Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* **28**: 1642–1644.

Fell JW & Scorzetti G (2004) Reassignment of the basidiomycetous yeasts *Trichosporon pullulans* to *Guehomyces pullulans* gen. nov., comb. nov. and *Hyalodendron lignicola* to *Trichosporon lignicola* comb. nov. *Int J Syst Evol Microbiol* **54**: 995–998.

Fell JW, Boekhout T, Fonseca A, Scorzetti G & Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**: 1351–1271.

Goren MB & Milddlebrook GM (1967) Protein conjugates of polysaccharide from *Cryptococcus neoformans*. *J Immunol* **98**: 901–913.

Hestrin S (1949) The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J Biol Chem* **180**: 249–261.

Idnurm A, Reedy JL, Nussbaum JC & Heitman J (2004) *Cryptococcus neoformans* virulence gene discovery through insertion mutagenesis. *Eukaryot Cell* **3**: 420–429.

Ikeda R, Sugita T, Jacobson ES & Shinoda T (2002) Laccase and melanization in clinically important *Cryptococcus* species other than *Cryptococcus neoformans*. *J Clin Microbiol* **40**: 1214–1218.

Kuramae E, Robert V, Snel B, Weiss M & Boekhout T (2006) Phylogenomics reveal a robust fungal tree of life. *FEMS Yeast Res*, in press. doi:10.1111/j.1567-1364.2006.00119.x

Kurtzman CP (2003) Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. *FEMS Yeast Res* **4**: 233–245.

Kurtzman CP, Boekhout T, Robert V, Fell JW & Deak T (2003) Methods to identify yeasts. *Yeasts in Food. Beneficial and Detrimental Aspects* (Boekhout T & Robert V, eds), pp. 69–121. Behr's Verlag, Hamburg.

Lazera MS, Pires FD, Camillo-Coura L, Nishikawa MM, Bezerra CC, Trilles L & Wanke B (1996) Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J Med Vet Mycol* **34**: 127–131.

Lazera MS, Cavalcanti MA, Trilles L, Nishikawa MM & Wanke B (1998) *Cryptococcus neoformans* var. *gattii* – evidence for a natural habitat related to decaying wood in a pottery tree hollow. *Med Mycol* **36**: 119–122.

Middelhoven WJ, Fonseca A, Carreiro SC, Pagnocca FC & Bueno OC (2003) *Cryptococcus haglerorum* sp. nov., an anamorphic basidiomycetous yeast isolated from nests of the leaf-cutting ant *Atta sexdens*. *Antonie van Leeuwenhoek* **83**: 167–174.

Moore RT (1980) Taxonomic proposals for the classification of marine yeasts and other yeast-like fungi including the smuts. *Bot Mar* **23**: 361–373.

Nakase T, Suh S-O & Hamamoto M (1995) Molecular systematics of ballistoconidium-forming yeasts. *Stud Mycol* **38**: 163–173.

Nakase T, Tsuzuki S & Takashima M (2002) *Bullera taiwanensis* sp. nov. and *Bullera formosensis* sp nov, two new ballistoconidium-forming yeast species isolated from plant leaves in Taiwan. *J Gen Appl Microbiol* **48**: 345–355.

Nosanchuk JD & Casadevall A (1997) Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. *Infect Immun* **65**: 1836–1841.

O'Donnell K, Cigelnik E, Weber NS & Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S rDNA sequence analysis. *Mycologia* **89**: 48–65.

Perfect JR, Wong B, Chang YC, Kwon-Chung KJ & Williamson PR (1998) *Cryptococcus neoformans* virulence and host defences. *Med Mycol* **36**: 79–86.

Petter R, Kang BS, Boekhout T, Davis BJ & Kwon-Chung KJ (2001) A survey of heterobasidiomycetous yeasts for the presence of the genes homologous to virulence factors of *Filobasidiella neoformans*, *CNLAC1* and *CAP59*. *Microbiology* **147**: 2029–2036.

Pfeiffer TJ & Ellis DH (1992) Environmental isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus tereticornis*. *J Med Vet Mycol* **30**: 407–408.

Polacheck I, Hearing VJ & Kwon-Chung KJ (1982) Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *J Bacteriol* **150**: 1212–1220.

Randhawa HS, Kowshik T & Khan ZU (2003) Decayed wood of *Syzygium cumini* and *Ficus religiosa* living trees in Delhi/New Delhi metropolitan area as natural habitat of *Cryptococcus neoformans*. *Med Mycol* **41**: 199–209.

Rhodes JC, Polacheck I & Kwon-Chung KJ (1982) Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. *Infect Immun* **36**: 1175–1184.

Robert V (2003) Data processing. *Yeasts in Food. Beneficial and Detrimental Aspects* (Boekhout T & Robert V, eds), pp. 139–169. Behr's Verlag, Hamburg.

Sampaio JP, Weiss M, Gadanho M & Bauer R (2002) New taxa in the Tremellales: *Bulleribasidium oberjochense* gen. et sp. nov., *Papiliotrema bandonii* gen. et sp. nov., and *Fibulobasidium murrhardtense* sp. nov.. *Mycologia* **94**: 873–887.

Sampaio JP, Inácio J, Fonseca A, Gadanho M, Spencer-Martins I, Scorzetti G & Fell JW (2004) *Auriculibuller fuscus* gen. nov., sp. nov. and *Bullera japonica* sp. nov., novel taxa in the Tremellales. *Int J Syst Evol Microbiol* **54**: 987–993.

Scorzetti G, Fell JW, Fonseca A & Statzell-Tallman A (2002) Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res* **2**: 495–517.

Suh S-O, McHugh JV, Pollock DD & Blackwell M (2005) The beetle gut: a hyperdiverse source of novel yeasts. *Mycol Res* **109**: 261–265.

Swofford DL (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sinauer Associates, Sunderland, MA.

Takashima M, Sugita T & Shinoda T (2001) Reclassification of the *Cryptococcus humicola* complex. *Int J Syst Evol Microbiol* **51**: 2199–2210.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MS & Gelfand DH, eds), pp. 315–322. Academic Press, Orlando, FL.

Yarrow D (1998) Methods. *The Yeasts, A Taxonomic Study*, 4th edn (Kurtzman CP & Fell JW, eds), pp. 77–100. Elsevier, Amsterdam.