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

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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 anacardiigen. gen. et sp. nov. with CBS 9551T (= 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 cucum (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). Since the isolates could not be identified with any known genus and species within the Trichosporonales, we describe them as Cryptotrichosporon anacardiigen. gen. et sp. nov. with CBS 9551T (= 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.
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 were 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 morphological 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 KH2PO4, 2.5 g of MgSO4·7H2O, 0.975 g of glycine, 3 g of glucose, 51 mL 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 \([\text{length with capsule} - \text{length without capsule} + (\text{width with capsule} - \text{width without capsule})]/4\). 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 \(\times 10^8\) cells mL\(^{-1}\). Capsules were further biochemically characterized using the methods of Goren & Mildelebrook (1967) and Dubois et al. (1956).

Capsular charge was measured as the \(\zeta\) 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 \(\times 10^7\) cells mL\(^{-1}\). The \(\zeta\) 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 GAT 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 MEGAALIGN (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 consistency 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 \(\times\) 2.5–4.5 μm (Fig. 2c), but inflated cells, measuring 8–11 \(\times\) 7–9 μm, were also observed (Fig. 2b). Formation of ballistoconidia was not observed.
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,β-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-pro-

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<th>Strain (CBS)</th>
<th>l-dopa</th>
<th>Methyl-dopa</th>
<th>Epinephrine</th>
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+, 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-dihydroxypheny-

line and putrescine were not assimilated, and assimilation of nitrite, ethylamine, glucosamine, imidazole and D-trypto-

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

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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.
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 ± 0.64 μm, n = 5) > CBS 9549 (1.33 ± 0.44 μm, n = 5) > CBS 9551 (0.82 ± 0.21 μm, n = 5) > CBS 9550 (0.65 ± 0.21 μm, n = 5) > CBS 9553 (0.55 ± 0.20 μm, 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 capsule 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 NH4HCO3.

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. formosensis, 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 fragiocola, Cryptococcus daszewskae, Cryptococcus curvatus, Cryptococcus musci, Cryptococcus raimirezgomeznans, 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 fragiocola 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
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 z potential readings. Prior studies comparing

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**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.
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.
 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 Hes-trin 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,
Cryptotrichosporon anacardii gen. nov., sp. nov.

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., 2002; 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, Tsuchiyae, and Udeniomycetes. To further complicate the situation, various teleomorphic genera occur in these lineages as well, such as Auriculibuller, Bullerisbasidium, Cystofibobasidium, Filobasidium, Filobasiella, 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 Trichosphorinales, 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 Trichosphorinales, as well as most, if not all, other clades belonging to the basidomycoceous 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, Trichosphoron 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, Cryptotrichosphoron anacardii gen. nov. et sp. nov., to accommodate the Nigerian yeast strains that form a basal lineage within the Trichosphorinales.

Descriptions of proposed new taxa

Cryptotrichosphoron Okoli & Boekhout, gen. nov.


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

Cryptotrichosphoron Okoli & Boekhout, gen. nov.

Yeast reproducing by budding, which forms a basal lineage within the Trichosphorinales. 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 Cryptotrichosphoron anacardii.

Cryptotrichosphoron anacardii Okoli & Boekhout sp. nov.


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

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**References**


