Occultifur tropicalis f.a., sp. nov., a novel cystobasidiomycetous yeast species isolated from tropical regions

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Five strains representing a single novel anamorphic yeast species were isolated from sugar cane. Two strains were from tissue (DMKU-SE38, DMKU-SE59T) and two from the external surface (DMKU-SP385, DMKU-SP403) of leaves collected in Thailand and the fifth (IMUFRJ 52020) from the rhizoplane of sugar cane in an organically cultivated field in Brazil. On the basis of sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, they were classified as representing a single species of the genus Occultifur. The sequences of the D1/D2 region of the LSU rRNA genes and the ITS regions of the five strains were either identical or differed from each other by only one nucleotide substitution. The novel species was related most closely to Occultifur brasiliensis f.a. CBS 12687T but with 0.7–1.0 % nucleotide substitutions (4–6 nt) in the D1/D2 region of the LSU rRNA gene and 2.5–2.7 % nucleotide substitutions (14–15 nt) in the ITS region. The name Occultifur tropicalis f.a., sp. nov. is proposed. The type strain is DMKU-SE59T (= BCC 61184T = NBRC 109696T = CBS 13389T).

The genus Occultifur was proposed by Oberwinkler (1990) to accommodate Occultifur internus, a mycoparasitic, simple-pored, auricularioid and diomorphic basidiomycetous yeast (Sampaio & Oberwinkler, 2011). It is a teleomorphic genus and belongs to the subphylum Pucciniomycotina, the class Cystobasidiomycetes and the order Cystobasidiales. In the latest edition of The Yeasts: A Taxonomic Study there are only three species in this genus with validly published names: Occultifur internus, Occultifur externus and Occultifur corticorum (Sampaio & Oberwinkler, 2011). However, living cultures and molecular sequence data are available only for O. externus (Sampaio & Oberwinkler, 2011). An additional anamorphic species (O. brasiliensis f.a.) that was isolated from tanks of the bromeliad Vriesea minarum has recently been proposed (Gomes et al., 2015).

Plant leaves and soil are two important and interrelated habitats of yeasts, as microbes are expected to be washed off the leaves into the soil. Endophytic and epiphytic yeasts can colonize healthy plant leaf tissue and external leaf surfaces, respectively, and they belong to either the Ascomycota or the Basidiomycota although the most common species are members of the phylum Basidiomycota (Isaeva et al., 2010; Oliveira et al., 2012; Akhtyamova & Sattarova, 2013; Kaewwichian et al., 2013; Khunnamwong et al., 2014; Limtong et al., 2014). The presence of yeast in soil depends on many factors, e.g. type of soil, rainfall and climate (Sláviková and Vadkertiová, 2003). Soil yeasts can also be either ascomycetous or basidiomycetous species. (Sláviková & Vadkertiová, 2003; Connell et al., 2008; Yurkov et al., 2012; Polburee et al., 2014). In the course of the investigation of endophytic and epiphytic yeasts from sugar cane leaves in Thailand and yeast diversity associated with the soil, rhizoplane and phylloplane of sugar cane in Brazil, five strains representing a novel species of the genus Occultifur were
obtained. In this article, these five strains are described as *Occultifur tropicalis* f.a., sp. nov.

**Yeast isolation**

Strains DMKU-SE38, DMKU-SE59T, DMKU-SP385 and DMKU-SP403 were isolated from surface-sterilized sugar cane (*Saccharum officinarum L.*) leaves collected in Thailand (Khunnamwong et al., 2014). Strains DMKU-SE38 and DMKU-SE59 were isolated by plating of leaf washings from two samples of sugar cane leaf collected on 2 March 2012 from Kao Liao district, Nakhon Sawan province (15° 51’ 1” N 100° 4’ 43” E) and Chai Badan district, Lopburi province (15° 11’ 22 N 101° 7’ 37” E), respectively. Strains DMKU-SP385 and DMKU-SP403 were derived from two samples of sugar cane leaf collected on 19 April 2012 from Nong Muang district, Lopburi province (15° 14’ 50” N 100° 39’ 15” E). Strain IMUFRJ 52020 was isolated by spreading yeast cells from rhizoplane of sugar cane organically cultivated at an experimental farm [Sistema Integrado de Produção Agroecológica (Integrated System of Agroecological Production) (SIPA)], in Seropédica district, Rio de Janeiro, Brazil (22° 45’ 21” S 43° 40’ 38” W) in January 2008 (Ribeiro et al., 2011). The purified yeast strains were suspended in YM broth supplemented with 10 % (v/v) glycerol and maintained at −80 °C.

**DNA sequencing and phylogenetic analysis**

The sequences of the D1/D2 region of the LSU rRNA gene and the ITS (ITS1–5.8S rRNA–ITS2) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1and ITS4 (White et al., 1990), respectively. DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and the ITS region were performed as described previously (Limtong et al., 2007). The PCR product was checked by agarose gel electrophoresis and purified using a HiYield Gel/PCR DNA Fragments Extraction kit (RBC Bioscience), according to the manufacturer’s protocol. The purified product was submitted to Macrogen (Seoul, Republic of Korea) for sequencing with primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene and ITS1 and ITS4 for the ITS region. For strain IMUFRJ 52020, DNA was extracted and amplified as described previously (Ribeiro et al., 2011). Genomic DNA was amplified using ITS1f (Gardes & Bruns, 1993) and NL4 (O’Donnell, 1993) primers for the ITS region and the D1/D2 region of the LSU rRNA gene. Automated sequencing was performed using the MegaBACE system (GE Healthcare). The sequences were compared pairwise using the BLASTN search program (Altschul et al., 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 2.1 (Thompson et al., 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura’s two-parameter correction (Kimura, 1980), using the neighbour joining method (Saitou & Nei, 1987) and MEGA software version 6.06 (Tamura et al., 2013). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). *Erythrobasidium hasegawanum* CBS 8253 was the outgroup in the analysis.

**Phenotypic characterization**

Strains DMKU-SE38, DMKU-SE59T, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020 were characterized morphologically, biochemically and physiologically according to the standard methods described by Kurtzman et al. (2011). Formation of pseudohyphae and true hyphae was investigated by cultivation on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) and corn meal agar (2 % corn meal infusion and 1.5 % agar) in slide culture at 25 °C for up to 4 weeks. Ballistospore formation was investigated by the method described by Kurtzman et al. (2011) on inverted PDA and corn meal agar plates and incubated at 15 °C and 25 °C for up to 4 weeks. Sexual processes were investigated for individual strains or strain pairs on PDA, corn meal agar, 5 % malt extract agar (5 % malt extract and 1.5 % agar) and YM agar at 15 °C and 25 °C for up to 6 weeks. Carbon source and nitrogen source assimilation tests were conducted in liquid medium and starved inocula was used in nitrogen assimilation tests (Kurtzman et al., 2011). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlemeyer flasks containing 250 ml of yeast extract peptone glucose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % glucose) on a rotary shaker at 28 °C for 48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isoprennologues were identified by HPLC as described previously (Limtong et al., 2007).

**Novel species delineation and identification**

Analysis of the D1/D2 region of the LSU rRNA gene sequence and ITS region revealed that the five strains (DMKU-SE38, DMKU-SE59T, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020) represented a single novel species. The sequences of the D1/D2 region of the LSU rRNA genes of the four strains isolated in Thailand comprising the two endophytic strains (DMKU-SE59T and DMKU-SE38) and the two epiphytic strains (DMKU-SP385 and DMKU-SP403) were either identical or differed by one gap or one nucleotide substitution with one gap from the sequence of strain IMUFRJ 52020 from Brazil. In terms of pairwise sequence similarity of the D1/D2 region of the LSU rRNA gene the most closely related species to the five strains was *O. brasiliensis* f.a. CBS 12687T (KC698874). The sequence of the D1/D2 region of the most closely related species differed from those of the four strains from Thailand (DMKU-SE38, DMKU-SE59T, DMKU-SP385 and DMKU-SP403) by 1.0 % nucleotide substitutions (six nucleotides) and from strain IMUFRJ 52020 by 0.7 % nucleotide substitutions (four nucleotides and three gaps). The sequence of the D1/D2 region of the LSU rRNA gene of the novel species differed from that of *O. externus* CBS 8732T (AF189909) by 1.3–1.4 % nucleotide substitutions (seven to
eight nucleotides and zero to three gaps). The nucleotide sequences of the ITS region of three strains DMKU-SE59T, DMKU-SE38 and IMUFRJ 52020 were either identical or differed by only one gap or one nucleotide substitution with one gap from those of the other two strains (DMKU-SP385 and DMKU-SP403), which were identical to each other. The ITS sequences of the five strains differed by 2.5–2.7% nucleotide substitutions (14–15 nucleotides and 10–11 gaps) from the sequence of O. brasiiliensis f.a. CBS 12687T (KC698874) and 3.4–3.6% nucleotide substitutions (19–20 nucleotides and seven to eight gaps) from that of O. externus CBS 8732T (AF444567). A phylogenetic tree based on the sequence of the D1/D2 region of the LSU rRNA gene and ITS region showing positions of the five strains (DMKU-SE38, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020) were located in the same position, clustered with the sequences of the five strains differed by only one gap or one nucleotide substitution with one gap from those of the other two strains (DMKU-SP385 and DMKU-SP403) which were identical to each other. The novel species delineation was based primarily on the analysis of the combined sequences of the ITS region and the D1/D2 region of the LSU rRNA gene, which indicated the reciprocal monophyletic nature of the species in this clade; therefore, a phylogenetic species concept applies. Therefore, on this basis, we concluded that the five strains represent a novel species. Although sexual reproduction was not observed, according to the nomenclatural rules for fungi of the International Code of Nomenclature for algae, fungi and plants, the most important requirement is the adoption of ‘one fungus, one name’ (Miller et al., 2011). Consequently, the novel species was assigned to the genus Occultifur, and the designation forma asexualis (f.a.) was included following the recommendation of Lachance (2012). The name Occultifur tropicalis f.a., sp. nov. (MB 810993) is proposed.

**Description of Occultifur tropicalis**

Khunnamwong, Surussawadee, Jindamorakot, Ribeiro, Hagler & Limtong sp. nov.

*Occultifur tropicalis* (tro.pi.ca’lis. N.L. masc. adj. tropicalis of or belonging to tropical area where the type strain was isolated). MycoBank accession number is MB 810993.

Growth occurs in YM broth. After 3 days at 25 °C, cells are subglobose to ovoid (3–5 × 3.5–9 μm) and occur singly or in pairs (Fig. 2). Budding is polar. After 3 days of growth on YM agar at 25 °C, the streak culture is pink to orange in colour, soft with a smooth surface and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA and corn meal agar after 4 weeks at 25 °C. Ballistospores are not produced when cultured on PDA agar at 15 °C for up to 4 weeks. Sexual reproduction is not observed in any of the strains or when strains are

![Fig. 1. Phylogenetic tree based on the sequence of the D1/D2 region of the LSU rRNA gene and ITS region showing positions of Occultifur tropicalis f.a. sp. nov. (DMKU-SE59T, DMKU-SE38, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020) with respect to closely related species. The phylogenetic tree was reconstructed using the neighbour joining method by MEGA software version 6.0, and the nucleotide substitution rates (K_{nuc} values) were computed from evolutionary distance data using Kimura’s two-parameter model. The scale bar indicates an evolutionary distance of 0.01 K_{nuc}. Numbers at the nodes indicate percentages of bootstrap samples, derived from 1000 replicates; only values >50% are shown. The numbers in parentheses are GenBank accession numbers. *Erythrobasidium hasegawianum* CBS 8253T was the outgroup in the analysis.](image-url)
paired on PDA agar, corn meal agar, 5 % malt extract agar and YM agar at 15 °C and 25 °C for up to 6 weeks. Fermentation is absent. D-glucose, D-galactose, L-sorbose, N-acetylgucosamine, D-xylene, L-arabinose, D-arabinose, sucrose, maltose, α-α-trehalose, met-α-D-glucoside (weakly), cellobiose, salicin (slowly), melibiose (weakly), soluble starch (weakly), lactose (slowly), melezitose, glycerol, ribitol (weakly), D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-ketogluconic acid, 5-ketogluconic acid, D-glucurionate, D-glucuronate, D-galacturonic acid (slowly), DL-lactate, succinate, citrate (weakly) and ethanol are assimilated, but D-ribose, 1-rhamnose, raffinose, inulin, erythritol, galactitol, myo-inositol and methanol are not assimilated. Ammonium sulfate, ethylamine HCl, L-lysine and cadaverine are assimilated, but potassium nitrate and sodium nitrite are not assimilated. No growth occurs in vitamin-free medium or with 0.01 % cycloheximide or 0.1 % cycloheximide or on medium containing 15 % (w/v) sodium chloride/5 % (w/v) glucose. Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose is present. Growth is present at 15, 25, 30 and 35 °C, but absent at 37, 40, 42 and 45 °C. Acid formation is absent. Starch-like compounds are not produced. Diazonium blue B colour and urease reaction are positive. The major ubiquinone is Q-10.

The type strain, DMKU-SE59T, was isolated from the tissue of a sugar cane (Saccharum officinarum) leaf collected from Chai Badan district, Lopburi province, Thailand. This strain has been deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Phuthumthani, Thailand as BCC 61184T, the NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan as NBRC 109696T and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 13389T. The MycoBank registration number is MB 810993.

In practice, O. tropicalis f.a. can be distinguished from the most closely related species in terms of pairwise sequence similarity of the D1–D2 region, O. brasilienis f.a., not only by the analysis of the sequence of the D1/D2 region of the LSU rRNA gene and the ITS region, but also by some phenotypic characteristics. O. tropicalis f.a. sp. nov. assimilates D-arabinose (weakly), sucrose (delayed), maltose, melibiose (weakly), soluble starch (weakly), ribitol (weakly) and DL-lactate (slowly) but O. brasilienis f.a. does not. O. tropicalis f.a. does not assimilate D-ribose but O. brasilienis f.a. does. O. brasilienis f.a. grows (weak) at 37 °C but O. tropicalis f.a. does not. Growth on medium containing 50 % glucose and 10 % (w/v) sodium chloride/5 % (w/v) is present for O. tropicalis f.a. sp. nov. but absent for the type strain of O. brasilienis f.a. O. brasilienis f.a. grows on medium containing 0.01 % cycloheximide but O. tropicalis f.a. sp. nov. does not. The phenotypic characteristics of the novel species reveal few differences from O. externus. These differences include that O. tropicalis f.a. sp. nov. assimilates N-acetylgucosamine and creatine but O. externus does not, growth on medium containing 50 % glucose is present for O. tropicalis f.a. sp. nov. but absent for O. externus and that no growth occurs with 0.01 % cycloheximide and 0.1 % cycloheximide for O. tropicalis f.a. sp. nov., but growth occurs for O. externus.

Two species of yeast of the genus Occultifur have been reported to be mycoparasites, i.e. O. internus, which is a parasite of dacrymycetaceous fungi, and O. corticorum, which is a parasite of Hyphoderma praeternissum, whereas one species of the genus, namely O. externus, may be as saprophyte because it has been reported to have been isolated from plant litter (Sampaio & Oberwinkler, 2011). All 10 strains of O. brasilienis f.a. were isolated from water tanks of Vriesea minarum, indicating that this is an important habitat for it. In this study, two strains (DMKU-SE38 and DMKU-SE59) of the novel species were isolated from sugar cane leaf tissue, two strains (DMKU-SP385 and DMKU-SE403) were isolated from the surfaces of sugar cane leaves collected in Thailand and one strain was isolated from rhizoplane of sugar cane collected in Brazil. However, it was difficult to conclude that the novel species is associated with sugar cane because sugar cane is a cultivated plant so the novel species might be an exotic microbe. It should be noted that both Thailand and Brazil are in the tropics, but they are on opposite sides of the planet.

Acknowledgements

This work was supported by the Thailand Research Fund (TRF) through the TRF Research-Team Promotion Grant (RTA5480009), the Royal Golden Jubilee PhD program grant no. PHD/0128/2554 and the Higher Education Research Promotion and National Research University Project of Thailand. The Brazilian authors thank the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and the Programa de Apoio a Núcleos de Excelência/Ministerio da Ciência, Tecnologia e Inovação (PRONEX/MCT) for financial support and Embrapa Agrobiologia, Pesagro/RIO and Universidade Federal do Rio de Janeiro (UFRJ) for permission to collect IMUFRJ strains at SIPA.

Fig. 2. Occultifur tropicalis f.a. sp. nov. (DMKU-SE59T) grown in YM broth for 3 days at 25 °C. Bar, 10 µm.
References


