Candida leandrae sp. nov., an asexual ascomycetous yeast species isolated from tropical plants

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The novel yeast species *Candida leandrae* is described based on eight isolates from decaying fruits of *Leandra reversa* Cogn. (Melastomataceae) in an Atlantic rainforest site in Brazil, one from a Convolvulaceae flower in Costa Rica and one from a drosophilid in Hawai'i. The strains differed in their colony morphology, one being butyrous and smooth and the other being filamentous and rugose. Sequences of the D1/D2 domains of the large-subunit rRNA gene from both morphotypes were identical. *C. leandrae* belongs to the *Kodamaea* clade and is closely related to *Candida restingae*. The two species can be separated on the basis of growth at 37 °C and the assimilation of melezitose, negative in the novel species. The type culture of *C. leandrae* is strain UNESP 00-64R^T (=CBS 9735^T=NRRL Y-27757^T).

The *Kodamaea* clade (Rosa *et al.*, 1999; Lachance *et al.*, 1999) contains a growing number of interesting yeast species associated with plants (Lachance *et al.*, 2001), mushrooms (Nakase *et al.*, 1999) and associated insects. During a screening of yeasts associated with Atlantic rainforest fruits, an asporogenous species physiologically similar to *Candida restingae* was isolated from fruits of *Leandra reversa* Cogn. (Melastomataceae). The yeast presented two different colonial morphotypes, one being butyrous and smooth and the other being filamentous and rugose. Sequencing of the D1/D2 domains of the large-subunit rRNA gene indicated that the morphotypes are conspecific and confirmed a close relationship with *C. restingae*. In an independent study of the yeast community of Convolvulaceae flowers and associated insects in Hawai'i and Costa Rica, two additional strains with similar growth characteristics and D1/D2 sequences were recovered. We now describe the novel species as *Candida leandrae*.

Yeast isolation and characterization

C. leandrae strains were isolated independently from three localities. Eight strains were recovered from fruits of *L. reversa* (Melastomataceae). Ten fruits were collected in Picinguaba area, an Atlantic Rain Forest site at the 'Serra do Mar' State Park in São Paulo state, Brazil, during the

summer of 2000. The fruits were individually blended in 2 ml sterilized distilled water and 0.1 ml of the suspension was spread on YM agar (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 2 % agar) containing 100 mg chloramphenicol l⁻¹ (Trindade *et al.*, 2002). Plates were incubated at 25 °C for 3–5 days. Strain UWOPS 00-612b2 was recovered from *Drosophila floricola* collected in a flower of *Ipomoea indica* (Convolvulaceae) on the Island of Oahu, Hawai'i. The fly was captured aseptically and allowed to deposit yeast cells on the agar medium. Strain UWOPS 01-664c3 was isolated from a flower of *Merremia tuberosa* (Convolvulaceae), near Dos Rios, Guanacaste Province, Costa Rica. Flower scrapings were streak-inoculated on the agar medium. The plates were kept at room temperature until colonies were sufficiently differentiated. Representative yeast colonies were purified and maintained in YM slants or in liquid nitrogen. Yeasts were characterized by standard methods (Yarrow, 1998) and their identification was attempted using the keys of Kurtzman & Fell (1998) and the CD-ROM *Yeasts of the World* (Boekhout *et al.*, 2002).

DNA sequence analysis

Yeast DNA was extracted and purified according to a protocol recommended for the Genomic Prep. Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech). The divergent D1/D2 domain (nucleotides 63–642 for *Saccharomyces cerevisiae*) at the 5' end of the large-subunit rRNA gene was symmetrically amplified with primers

The GenBank/EMBL/DDBJ accession number for the large-subunit rRNA sequence of strain UNESP 00-64R^T is AY449659.

NL-1 and NL-4 (O'Donnell, 1993). Each PCR was performed with the Ready-To-Go kit (Amersham Pharmacia Biotech) adding 1.5 µl solution containing approximately 100 ng DNA; 1.6 µl NL-1 primer; 1.1 µl NL-4 primer (6 pmol each) and 17.8 µl Milli-Q water. The sequence products were resolved in an ABI Prism 377 DNA sequencer (Applied Biosystems) at the Center for the Study of Social Insects – UNESP, Rio Claro, São Paulo, Brazil. Alternatively, the DNA was amplified directly from whole cells and sequenced as described by Lachance *et al.* (1999). The sequences were edited and aligned using CLUSTAL W 1.4 (Thompson *et al.*, 1994) provided in the program DNAMAN 4.1 (Lynnon Biosoft, Vaudreuil, Québec, Canada), which was used also for construction of the neighbour-joining tree.

Species delineation, classification and ecology

The novel species *C. leandrae* formed two distinct colony morphotypes (Fig. 1). Six strains (UNESP 00-63L, 00-64L, 00-65L and 00-66L; UWOPS 00-612b2 and 01-664c3) formed butyrous and smooth colonies and four (UNESP 00-63R, 00-64R^T, 00-65R and 00-66R) formed rugose and membranous colonies. The colony phenotypes were stable within each strain. The strains had nearly identical physiological profiles and the sequences of their D1/D2 large-subunit rRNA gene regions were identical. Sequence analysis further demonstrated that the species is related to *C. restingae* and belongs to the *Kodamaea* clade (Fig. 2). Extensive variability in colony morphology has been noted for *Kodamaea kakaduensis* (Lachance *et al.*, 1999), *Kodamaea anthophila* and *Kodamaea nitidulidarum*, but not for *C. restingae* (Rosa *et al.*, 1999). The D1/D2 sequences of *C. leandrae* and *C. restingae* differed by nine substitutions and three gaps in 467 bases, indicating that the two taxa represent phylogenetically distinct species. The isolates were examined after growth on most common sporulation media (5% malt extract agar, cornmeal agar, Fowell acetate agar and dilute V8 agar), but asci were not formed. Mixed pairs showed no signs of conjugation, suggesting that *C. leandrae* occurs in nature in the asexual form.

Species in the *Kodamaea* clade have been isolated mostly

Fig. 1. Colonies of *C. leandrae* after 3 days on yeast extract/malt extract agar. (Left) Rugose colony, strain UNESP 00-64R^T, and (right) smooth colony, strain UNESP 00-64L. Bar, 0.5 mm.

Fig. 2. Neighbour-joining phylogram based on the D1/D2 divergent domains of the large-subunit rRNA gene of *C. leandrae* and related species. Percentage bootstrap values were obtained from 1000 replicates. Only values above 50% are shown. Bar, 5% sequence divergence.

from flowers and associated insects (Lachance *et al.*, 2001). *Kodamaea ohmeri* has been recovered sporadically from a variety of substrates including flowers, insects or clinical specimens (Kurtzman, 1998; Rosa *et al.*, 2003). Other species in the clade are associated with flowers and insects in tropical ecosystems (Rosa *et al.*, 1999; Lachance *et al.*, 1999, 2001), or with mushrooms (Nakase *et al.*, 1999), where they are presumably vectored by fungivorous insects. *C. restingae* was isolated from cactus flowers in Brazil and from insects collected in a cactus flower in Costa Rica (Lachance *et al.*, 2001). One strain was isolated from an unidentified fruit in an Atlantic Rain Forest site in Minas Gerais, Brazil. *C. leandrae* is probably associated with insects that visit decaying fruits and flowers in tropical ecosystems. Among several fruits collected in Picinguaba area, only decaying fruits of *L. reversa* yielded *C. leandrae*, suggesting a possible association with insects that exhibit a preference for that tree species. The very low frequency of recovery in hundreds of Convolvulaceae flowers or associated insects examined in Hawai'i and Costa Rica would suggest that the flowers and their insects are not a primary habitat of the species, but demonstrate its broad geographical distribution in the neotropics.

Identification

C. leandrae is easily distinguished from *C. restingae* based on the assimilation of inulin, growth on 50% glucose, and the absence of growth on melezitose, at 37 °C, or in the presence of 10% NaCl. These characters combined with the utilization of succinic acid and the lack of growth on melibiose allow separation from all other similar species. Confirmation of identity by D1/D2 sequencing is prudent.

Latin diagnosis of *Candida leandrae* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25 °C, cellulae ellipsoideae aut elongatae, singulae aut in catenis brevis (2–4 × 3–6 µm).

Cultura in agar extracta malti et levidinis continente post 4 dies ad 25 °C, candida, aliquando cum lineis radiabus, glabra aut rugosa, butyrosa aut firma. In agar farinae *Zea mays* post dies 14 pseudomycelium formatur. Asci non formantur. Glucosum fermentatur. Glucosum, inulinum (interdum exigue), sucrosus, raffinosis (interdum exigue), galactosus, trehalosus, maltosus, methyl α -D-glucosidus, L-sorbosus, D-xylosus (interdum lente), D-ribosus (variabile), ethanolus, glycerolus, ribitolus, xylitolus, mannitolus, glucitolus, acidum succinicum et acidum citricum (interdum exigue), acidum gluconicum (variabile), glucono- δ -lactonus, 2-ketogluconatus, N-acetylglucosaminus, et n-hexadecanus (exigues) assimilantur. Non assimilantur melibiosus, lactosus, melezitosis, amylo solubilis, cellobiosus (interdum exigue), salicinus (variabile), L-rhamnosus, L-arabinosus, D-arabinosus, methanolus, erythritolus, galactitolus, L-arabinitolus, meso-inositolus, acidum glucuronicum, acidum lacticum, 5-keto gluconatus et glucosaminus (variabile). Lysinus, ethylaminus et cadaverinus assimilantur at non natrium nitrosus nec natrium nitricus. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 35 °C, at non 37 °C. Materia amyloidea non formantur. Crescit in agar extrato fermenti confecto 50 partes glucosi per centum. Non crescit in medio 100 $\mu\text{g ml}^{-1}$ cycloeximido addito. Ureum non finditur. Diazonium caeruleum B negativum.

Habitat fructus *Leandra reversa* Cogn. (Melastomataceae) flores Convolvulacearum et insecta juncta. Typus stirpis UNESP 00-64R^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9735^T, typus stirpis deposita est.

Description of *Candida leandrae* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

Candida leandrae (le.an.d'rae. N.L. gen. n. *leandrae* of *Leandra reversa*).

In yeast extract (0.5 %) glucose (2 %) broth after 3 days at 25 °C, the cells are ellipsoidal to elongate (2–4 $\times$ 3–6 μm), occur singly, in budding pairs or in short chains (Fig. 3). On YM agar after 4 days at 25 °C, the colonies are white, convex, sometimes fringed, glabrous or membranous, smooth or rugose, butyrous to tough due to filamentous growth. After 2 weeks in Dalmau plate culture on cornmeal agar, a rudimentary pseudomycelium is formed. Asci are not formed on common sporulation media. Glucose fermentation is complete after 2 days to 2 weeks. Assimilation of carbon compounds: glucose, inulin (sometimes weak), sucrose, raffinose (sometimes weak), galactose, trehalose, maltose, methyl α -D-glucoside, L-sorbose, D-xylose (sometimes slow), D-ribose (variable), ethanol, glycerol, ribitol, xylitol, D-mannitol, D-glucitol, succinic

Fig. 3. Photomicrographs of cells of *C. leandrae* strain UNESP 00-64R^T (rugose colony) after (A) 3 days on 5 % malt extract at 25 °C and (B) 2 weeks on cornmeal agar at 25 °C. Cells of *C. leandrae* strain UNESP 00-64L (smooth colony) after (C) 3 days on 5 % malt extract at 25 °C and (D) 2 weeks on cornmeal agar at 25 °C. Bars, 10 μm .

acid, citric acid (sometimes weak), D-gluconic acid (variable), glucono- δ -lactone, 2-ketogluconic acid, N-acetyl-D-glucosamine and hexadecane (sometimes weak) are assimilated; no growth occurs on melibiose, lactose, melezitose, starch, cellobiose (occasionally weak), salicin (variable), L-rhamnose, L-arabinose, D-arabinose, methanol, erythritol, L-arabinitol, galactitol, myo-inositol, D-glucuronic acid, DL-lactic acid, 5-ketogluconic acid or D-glucosamine (variable). Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth in vitamin-free medium is negative. Growth in amino acid-free medium is positive. Growth at 35 °C is positive and at 37 °C negative. Acid formation on chalk agar is slow, weak or absent. Urease activity and diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50 % glucose/yeast extract agar is positive. Growth on YM agar with 5 % NaCl is positive; 10 % NaCl negative. Growth in the presence of 0.01 % cycloheximide is negative. Growth in the presence of 1 % of acetic acid is negative.

The type culture is strain UNESP 00-64R^T. It was isolated from a decaying fruit of *L. reversa* in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 9735^T (=NRRL Y-27757^T).

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