Lachancea mirantina sp. nov., an ascomycetous yeast isolated from the cachaça fermentation process

Luciana Filgueira Pereira,¹ César Raimundo Lima Costa, Jr,²
Bereneuza Tavares Ramos Valente Brasileiro³
and Marcos Antonio de Moraes, Jr^{1,2}

Correspondence

Marcos Antonio de Moraes, Jr
marcos.moraes@pesquisador.
cnpq.br

¹Grupo Interdepartamental de Engenharia Metabólica, CCB, Universidade Federal de Pernambuco, Recife, PE 50760-901, Brazil

²Departamento de Genética, CCB, Universidade Federal de Pernambuco, Recife, PE 50760-901, Brazil

³Centro de Biologia e Saúde, Universidade Católica de Pernambuco, Recife, PE 50050-900, Brazil

In the present work, a novel ascomycete species, *Lachancea mirantina* sp. nov., isolated from the fermentation process that produces cachaça, a Brazilian spirit, is proposed. Nucleotide sequence analysis of the 26S D1/D2 rDNA locus showed that *L. mirantina* sp. nov. was genetically related to *Lachancea cidri* and *Lachancea fermentati*, although some physiological traits showed remarkable differences. Analysis of the D1/D2 large-subunit rDNA molecular marker showed a clear distinction among all three species, confirming that *L. mirantina* sp. nov. belongs to a separate taxonomic species in the *Lachancea* clade. The type strain of *Lachancea mirantina* sp. nov. is URM 5925^T (=CLIB 1160^T =CBS 11717^T).

Industrial alcohol fermentation using sugar cane juice or molasses is a complex environment in which many yeast species are found, some as contaminants and others just as adventitious organisms (da Silva-Filho *et al.*, 2005; Basílio *et al.*, 2008). The influence of each species on the alcohol fermentation process is still unknown. Cachaça is a typical Brazilian beverage that is produced from sugar cane juice fermentation. The fermented must from this process is then distilled to produce a spirit with about 40° GL that can be consumed directly or after ageing in oak barrels (Oliveira *et al.*, 2005). A complex succession of yeast species has been observed during the course of this type of fermentation (Pataro *et al.*, 2000; Gomes *et al.*, 2007; Vila Nova *et al.*, 2009). Previously, we reported the differences in yeast populations associated with the technological levels in the cachaça distilleries, with the highest number of species being observed for the less controlled processes (Vila Nova *et al.*, 2009). One of those yeasts, isolate SPC2(7)^T, was genetically related to *Zygosaccharomyces* sp. IFO 11066 isolated from sake fermentation in Japan (Vila Nova *et al.*, 2009). In the present work, we have extended the identification of that isolate using conventional biochemical and molecular taxonomic approaches by sequencing of the D1/D2 region of the large subunit of the rRNA gene (Kurtzman & Robnett, 1998). Other genetic loci of isolate SPC2(7)^T (ITS1-5.8S-ITS2 and

EFAl gene) were also sequenced for confirmatory multigene analysis (Kurtzman & Robnett, 2003). Using the phylogenetic species concept, a novel species, *Lachancea mirantina* sp. nov., isolated from the cachaça fermentation process, is proposed.

Yeast isolation and characterization

The yeast isolate SPC2(7)^T (=URM 5925^T =CLIB 1160^T =CBS 11717^T) was isolated from the fermentation process for cachaça production in the distillery *Mirante da Cachaça*, located in the city of Paudalho (07° 53' 48" S 35° 10' 47" W, 69 m above sea-level), Pernambuco State, Brazil. The isolation procedure and fermentation conditions have been reported by Vila Nova *et al.* (2009) and the strain was characterized by using standard methods (Yarrow, 1998).

DNA sequencing and cladistic analysis

The variable domain D1/D2 of the 26S rRNA gene of strain URM 5925^T was amplified using primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GTCCGTGTTTCAAGACGG-3'), according to de Souza Liberal *et al.* (2007). The *EFAl* gene was amplified using primers yEF1-for (5'-GTAAGGGTTCTTTCAAGTACGC-3') and yEF1-rev (5'-GGAGTCACCAAAGACGTTACC-3') designed from the conserved 5'-terminal and 3'-terminal regions of the *Zygosaccharomyces* gene deposited at the NCBI database (GenBank accession nos AF402076.1,

The GenBank/EMBL/DDJB accession number for the 26S D1/D2 rDNA sequence of *Lachancea mirantina* URM 5925^T is FJ666084.

AF402075.1, AF402059.1, AF402058.1 and AF402055.1). Sequences were analysed by CLUSTAL X multiple alignment in the BioEdit sequence alignment editor 7.0.5.3v (Hall, 1999). The completely conserved sequences were used for primer design with the same editor. The amplification products of ITS, D1/D2 and *EFA1* loci were purified by using the Wizard PCR Preps DNA purification system (Promega), checked for purity by agarose gel electrophoresis and for concentration by comparing with λ -HindIII marker (Invitrogen), and submitted to DNA sequencing after being labelled using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Labelled products were analysed in an ABI Prism 3100 DNA sequencer (Applied Biosystems). Nucleotide sequences were analysed in BioEdit and submitted to BLASTN at the NCBI. The most similar sequences were downloaded in FASTA format and aligned with CLUSTAL W of the BioEdit program. Aligned regions were analysed for the phylogenetic signal using Tree-puzzle v. 5.2 software. A cladistic tree was reconstructed using PAUP 4.0 (Swofford, 2002) by the neighbour-joining method (Saitou & Nei, 1987) with the Kimura-2 parameter nucleotide substitution model (Kimura, 1980) and by the maximum-likelihood method (Felsenstein, 1985). Results were checked for the best evolution model by the program MODELTEST 3.7 (Posada & Crandall, 1998). Reliability was tested by bootstrap analysis with 1000 pseudo-replicates.

Molecular identification of strain URM 5925^T

Previous cladistic analysis with 26S D1/D2 nucleotide sequences was performed with yeasts isolated from different cachaça fermentation processes. In those analyses, strain URM 5925^T was identified and the D1/D2 region of this isolate showed 100 % sequence similarity with that of a yeast designated *Zygosaccharomyces* sp. strain IFO 11066 (Vila Nova *et al.*, 2009), which had been isolated in Japan from sake fermentation (data not published). Both URM 5925^T and *Zygosaccharomyces* sp. IFO 11066 clustered within the *Zygosaccharomyces* clade together with *Zygosaccharomyces fermentati* and *Zygosaccharomyces cidri* and were originally considered to be species of the genus *Zygosaccharomyces* (Vila Nova *et al.*, 2009). However, those two species were recently reclassified within a new genus, *Lachancea*, that was separated from the genus *Zygosaccharomyces* by phylogenetic analysis (Kurtzman, 2003). Although they were closely related, strains URM 5925^T and IFO 11066 defined a distinct branch in the cluster of what is now designated *Lachancea cidri* and *Lachancea fermentati*; this analysis was heavily supported by all clustering methods tested (Vila Nova *et al.*, 2009). Therefore, it is suggested that these isolates represent a novel species of the genus *Lachancea*.

In view of this possibility, the molecular analysis of strain URM 5925^T was extended to include other molecular markers. D1/D2 nucleotide sequence analysis was repeated for strain URM 5925^T (GenBank accession no. FJ666084)

and 100 % similarity to the sequence of strain IFO 11066 was confirmed; compared with the *L. fermentati* sequence, 17 nt substitutions were observed, which represents 3.5 % (17/493) nucleotide divergence. The limit of 1 % nucleotide divergence is now widely accepted as a guideline for the delimitation of species for the ascomycetous yeasts (Kurtzman & Robnett, 1998), although some exceptions have been reported (Lachance *et al.*, 2003). In view of this assumption, strains URM 5925^T and IFO 11066 belong to a novel species in the genus *Lachancea*. A similar analysis was performed by sequencing the ITS and EF-1 α loci. The ITS locus of strain URM 5925^T (accession no. FJ666085.1) showed 94 % similarity (4 % gaps) to that of *L. fermentati* (AY046206), 93 % similarity (2 % gaps) to that of *L. cidri* (AY046205) and 93 % similarity (2 % gaps) to that of *Lachancea thermotolerans* (FJ153217). In the case of the EF-1 α locus of strain URM 5925^T (GenBank accession no. GQ292877), BLAST results showed 94 % similarity (no gaps) to *L. fermentati* (AF402076). The cladistic analysis tree of 26S D1/D2 nucleotide sequences included the eight other recently described species of the genus. Recently, three novel species in this genus have been proposed: *Lachancea meyersii* was isolated from mangrove habitats in the northern Bahamas Islands and can be distinguished from other members of the genus *Lachancea* by the combined characteristics of lack of growth on galactose and growth on maltose (Fell *et al.*, 2004); *Lachancea dasiensis* was isolated from leaves and soil in Thailand and was closely related physiologically and phylogenetically to *L. thermotolerans* and *Lachancea waltii* (Lee *et al.*, 2009); and *Lachancea nothofagi* was isolated from trees in native forests in Patagonia, Argentina, and belongs to the *L. thermotolerans* clade (Mestre *et al.*, 2010). Branch separation was highly supported by the cladistic analysis and showed that *L. mirantina* sp. nov. now represents the ninth species of this genus (Fig. 1). According to the phylogenetic species concept and the place of isolation, a novel species of the genus *Lachancea*, *Lachancea mirantina* sp. nov., is proposed.

Latin diagnosis of *Lachancea mirantina* Pereira, Costa, Brasileiro, Rosa *et* Moraes sp. nov.

In medio liquido post dies 3 cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–4 × 3–5 µm). Cultura in agar malti post dies 5 (28 °C) parva, convexa, glabra et candida. In agar farinae Zea mays post dies 14, mycelium nec pseudomycelium non formantur. Species homothallica. Asci continentes 1–4 ascoporas globosae. Glucosum, sucrosum et maltosum fermentatur. Glucosum, galactosum (lente), sucrosum, maltosum (lente), trehalosum (lente), raffinoseum (lente), inulinum (lente), glycerolum, acidum lacticum (lente), glucitolum, mannitolum, salicinum et xylitolum (lente) assimilantur, at non L-sorboseum, cellobiosum, lactosum, melibiosum, melezitosum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, ethanolum, erythritolum, ribitolum, galactitolum, acidum succinicum, acidum citricum, myo-inositolum,

Fig. 1. Maximum-likelihood tree recovered in a neighbour-joining framework with a GTR + I and Distance corrected with Kimura two-parameter model of sequence evolution, with all characters within the D1/D2 26S dataset in the clade *Lachancea*. Values above the branches represent support values from Distance analysis with 1000 bootstrap replicates. Values below the branches represent maximum-likelihood (1000 replicates) bootstrap percentages. Two species of the genus *Kluyveromyces* were used as the outgroup. Bar, 0.05 substitutions per site.

methanolum, hexadecanum, acetona, ethyl acetat, 2-propanolum nec acidum gluconicum. N-Acetylglucosaminum, ethylaminum et cadaverinum assimilantur, at non lysinum, natrium nitricum nec natrium nitrosum. Ad crescentiam vitamina externa necessaria sunt. Augmentum in 30 °C, at non 37 °C. Habitat fermentatus cachaça in Brazil. Typus URM 5925^T. In collectione zymotica, Centre International de Ressources Microbiennes, Grignon (sub no. CLIB 1160^T) et Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum (sub no. CBS 11717^T) typus stirps deposita est.

Description of *Lachancea mirantina* Pereira, Costa, Brasileiro, Rosa & Morais sp. nov.

Lachancea mirantina (mi.ran.ti'na. N.L. fem. adj. *mirantina* pertaining to the place of isolation, the distillery *Mirante da Cachaça*).

In yeast extract (0.5 %)-glucose (2 %) broth after 3 days at 28 °C (± 2 °C), cells are ovoid to ellipsoidal (2–4 $\times$ 3–5 μ m), with simple budding. On yeast extract-malt extract (YM) agar after 5 days at 28 °C (± 2 °C), yeast colonies are white, convex, smooth and opalescent. After 14 days on cornmeal agar in Dalmau plates, neither pseudomycelium nor true mycelium are produced. After 2 days on agar media with low nitrogen/carbon ratio (e.g. yeast carbon base with 0.01 % ammonium sulfate), conjugated pairs of cells gave rise to asci containing one to four spherical ascospores (Fig. 2), from which single spores were not liberated. The species is homothallic, as conjugation takes place between cells of a single haploid population. Glucose, sucrose and maltose are fermented weakly. D-Glucose, D-galactose (slow), sucrose, maltose (slow), trehalose (slow), raffinose (slow), inulin (slow), glycerol, DL-lactate (slow), D-sorbitol (D-glucitol), D-mannitol, salicin and xylitol (slow) are assimilated. No growth occurs on L-sorbose, cellobiose, lactose, melibiose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, erythritol, ribitol, galactitol, succinate, citrate, myo-inositol, methanol, hexadecane, acetone, ethyl acetate,

2-propanol or gluconate. Positive for assimilation of the nitrogen compounds *N*-acetylglucosamine, ethylamine.HCl and cadaverine. Unable to assimilate lysine, like *L. cidri* (Yarrow, 1998) and *L. nothofagi* (Mestre *et al.*, 2010). Like *L. nothofagi*, it cannot assimilate nitrate (Mestre *et al.*, 2010). Growth in amino acid-free medium is positive. Grows at 30 °C, but not at 37 °C. Does not grow on YM agar with 10 % sodium chloride or on 50 % glucose (50 %)-yeast extract (0.5 %) medium. Starch-like compounds are not produced. Negative for growth in 10 mg cycloheximide ml⁻¹, urease activity and the diazonium Blue B reaction.

The type strain is URM 5925^T (=CLIB 1160^T =CBS 11717^T), isolated from the cachaça fermentation process.

Fig. 2. Vegetative cells and asci containing one to four spherical ascospores of *Lachancea mirantina* URM 5925^T after 3 days of incubation on yeast carbon base medium supplemented with 100 mg ammonium sulfate l⁻¹ at 28 °C (± 2 °C). Arrow indicates the halter structure typical of members of the *Zygosaccharomyces*/*Lachancea* clade. Bar, 10 μ m.

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