Metschnikowia cerradonensis sp. nov., a yeast species isolated from ephemeral flowers and their nitidulid beetles in Brazil

Carlos A. Rosa,¹ Marc-André Lachance,² Lia C. R. S. Teixeira,¹ Raphael S. Pimenta³ and Paula B. Morais³

Correspondence
Marc-André Lachance
lachance@uwo.ca

¹Departamento de Microbiologia, ICB, C.P. 486, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil

²Department of Biology, University of Western Ontario, London, Ontario N6A 5B7, Canada

³Laboratório de Microbiologia Ambiental e Biologia, Campus Universitário de Palmas, Fundação Universidade Federal do Tocantins, Palmas, Tocantins, 77010-154, Brazil

A novel yeast species, *Metschnikowia cerradonensis* sp. nov., is described from 12 strains isolated from flowers of *Ipomoea carnea* and from beetles of the genus *Conotelus* in the Cerrado ecosystem in the region of Jalapão, Tocantins State, Brazil. Analysis of the sequences of the rRNA gene cluster suggested that *M. cerradonensis* is closely related to *Metschnikowia santaceciliae*, *Metschnikowia continentalis* and an undescribed species represented by strain UWOPS 00-154.1. These species mate together but ascospores are very rarely formed, showing that they represent distinct biological species. *M. cerradonensis* is apparently endemic to the Cerrado ecosystem of the Jalapão area. The type strain of *M. cerradonensis* is UFMG 03-T67.1^T (h⁺) (= CBS 10409^T = NRRL Y-48067^T) and the designated allotype is UFMG 03-T68.1 (h⁻) (= CBS 10410 = NRRL Y-48068).

The large-spored group of the genus *Metschnikowia* forms a subclade with eight recognized teleomorphic species and the anamorphic species *Candida ipomoeae* (Lachance *et al.*, 2005). The distribution of most of the taxa is regional, suggesting that they arose in time through allopatric speciation (Marinoni & Lachance, 2004). *Metschnikowia hawaiiensis* is associated with the nitidulid beetle *Prosopius subaeneus*, which is endemic to the island of Hawai'i, supporting the view that this yeast has speciated in that region (Lachance *et al.*, 1990, 2005). Similarly, *Metschnikowia hamakuensis*, *Metschnikowia kamakouana* and *Metschnikowia mauinuiana* are associated with endemic nitidulid beetles living on various endemic plants on three Hawaiian islands (Lachance *et al.*, 2005). The habitat of *Metschnikowia lochheadii* and *Metschnikowia santaceciliae* is in beetles of the genus *Conotelus* and other insects associated with flowers of *Ipomoea* (morning glory) species in north-western Guanacaste Province, Costa Rica (Lachance *et al.*, 2001, 2003). *M. lochheadii* is also present in insects found in various flowers on the Hawaiian Islands, where it appears to have been introduced. *Metschnikowia borealis* is restricted to eastern North America, where it is also associated with

Conotelus species and Convolvulaceae flowers (Lachance *et al.*, 1998; Marinoni & Lachance, 2004). *Metschnikowia continentalis* was isolated from *Ipomoea* flowers and species of the genus *Conotelus* in Brazil (Lachance *et al.*, 1998), where it has a widespread distribution (C. A. Rosa, unpublished results). *Candida ipomoeae* is an asexual relative of all these species associated with *Conotelus* beetles and is found in abundance in all regions where *Conotelus* species occur, except for localities near the Great Lakes area and northward (Marinoni & Lachance, 2004).

During a study of the yeasts associated with ephemeral flowers in northern Brazil, several strains of a yeast producing large acicular ascospores were isolated from a site in the Cerrado ecosystem. Mating experiments showed that this yeast was capable of mating with *M. continentalis*. However, ascospores were rarely formed. This and other mating results, in combination with an analysis of rDNA sequences, demonstrated that these isolates represent a novel species in the large-spored group of the genus *Metschnikowia*. The name proposed for this species is *Metschnikowia cerradonensis* sp. nov.

Strain collection and characterization

Details of the strains considered in this study are given in Table 1. Collections were made in 2003 and 2005. Flowers of *Ipomoea carnea* and associated beetles were collected from

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ641236–DQ641243.

The Mycobank accession number for *Metschnikowia cerradonensis* sp. nov. is MB510051 (<http://www.mycobank.org>).

Table 1. Details of strains of *Metschnikowia cerradonensis* sp. nov. isolated in this study

UFMG, Universidade Federal de Minas Gerais, Brazil.

Strain	Mating type	Source
UFMG 03-T67.1 ^T	h ⁺	<i>Conotelus</i> beetle
UFMG 03-T68.1 (allotype)	h ⁻	<i>Conotelus</i> beetle
UFMG 03-T62.1	h ⁺	<i>Ipomoea carnea</i> flower
UFMG 03-T64.1	h ⁻	<i>Ipomoea carnea</i> flower
UFMG 03-T64.2	h ⁺	<i>Ipomoea carnea</i> flower
UFMG 03-T215.2	h ⁺	<i>Conotelus</i> beetle
UFMG 03-T216.2	h ⁺	<i>Conotelus</i> beetle
UFMG 05-T332.2	h ⁻	<i>Conotelus</i> beetle
UFMG 05-T333	h ⁻	<i>Conotelus</i> beetle
UFMG 05-T334.1	h ⁺	<i>Conotelus</i> beetle
UFMG 05-T338.1	h ⁺	<i>Ipomoea carnea</i> flower
UFMG 05-T339.1	h ⁻	<i>Ipomoea carnea</i> flower

the margins of a small lake ('fervedouro') in the city of São Felix do Tocantins, Jalapão, Brazil, and from several streets and public gardens in the city. Other isolates were retrieved from flowers of *Ipomoea* species in two forest fragments ('Ipucas') of the Lago Verde estate, in the flooded plains of the Araguaia River, as well as at the entrance of the Ecological Reserve of Canguçu, near the Javaés River. These areas are all found within the Brazilian Cerrado ecosystem of Tocantins State. The nectary region of the *Ipomoea* flowers was scraped gently with a sterile loop and streak-inoculated onto YM agar (1.0 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3 % yeast extract, 2.0 % agar) containing 100 mg chloramphenicol l⁻¹. *Conotelus* beetles visiting the flowers were placed on YM agar plates and kept for 10–15 min before being removed. Material deposited by the insects was spread evenly with a sterile loop. Representative yeast colonies were purified and maintained on YM slants or in liquid nitrogen in the yeast collections of the Universidade Federal de Minas Gerais (UFMG) or the University of Western Ontario (UWOPS). The yeasts were characterized using standard methods (Yarrow, 1998). Identities were verified using the keys of Kurtzman & Fell (1998).

Delineation and identification of heterothallic, haplontic *Metschnikowia* species are derived from mating reactions. Active cultures were mixed in pairs on yeast carbon base (Difco) supplemented with 0.01 % ammonium sulfate, incubated at 22 °C, and examined periodically for the formation of zygotes, asci or ascospores.

The sequence of the complete rDNA repeating unit was determined by PCR amplification using the following forward (f) and reverse (r) primers, listed in order of their occurrence in the sequence: MetETS2r (5'ACCTC-CGGGTCCCCAAATTTGCAT3'), IG2r (5'GGCTTAATC-TTTGAGACAAGC3'), MetETS3f (5'ATTTATCGCCCA-AGTCTCGGGATG3'), SSU1f (5'CTGGTTGATCCTGC-CAGTAGTCATA3'), SSU6r (5'AGCTGGAATTACCGCG-GCTGCT3'), SSU3f (5'TGGAGGGCAAGTCTGGTGCCA3'),

SSU4r (5'AACTAAGAACGGCCATGCACCA3'), SSU2r (5'ATGATCCTTCCGCAGGTTACAC3'), IT1f (5'TAGGT-GAACCTGCGGAAGGATCAT3'), IT2r (5'CTTTTCCTC-CGCTTATTGATATGC3'), NL1f (5'GCATATCAATAAG-CGGAGGAAAAG3'), NL4r (5'GGTCCGTGTTTCAAGA-CGG3'), LSU3f (5'CGTCTTGAAACACGGACCAAG3'), LSU5f (5'TAGCCCTGAAAATGGATGGCGCT3'), LSU4r (5'CTCCCACTTATTCTACACCC3'), LSU2r (5'GATCGT-AACAACAAGGCTACTC3'), IG1f (5'CGTGAGACAGG-TTAGTTTTACCC3') and MetIGS1f (5'CCAATGTTTCG-AAGATTTGCTTTGGC3'). Sequencing templates were amplified directly from whole cells as described by Marinoni & Lachance (2004). Amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced in an ABI sequencer at the John P. Robarts Research Institute (London, Ontario, Canada). The sequences were assembled, edited and aligned with the program DNAMAN version 4.1 (Lynnon BioSoft), which makes use of the CLUSTAL algorithm (Thompson *et al.*, 1994). The program SPLITSTREE 4 (version 4.3; Huson & Bryant, 2006) was used to generate a split decomposition tree. A satisfactory alignment of a large portion of the intergenic spacer region was not possible owing to the abundance of internal repeats of various sizes. Therefore, gapped positions were excluded, such that 6076 nucleotide positions out of 7447 were retained.

Two authentic strains of *M. continentalis* and five strains of *M. cerradonensis* sp. nov. were used for PCR fingerprinting. DNA templates were prepared as described by de Barros Lopes *et al.* (1998). The primer EI1 (5'CTGGCTTGG-TGTATGT3') targets intron-splicing sites in hypermutable regions of the *Saccharomyces* genome. PCR assays were performed as described by the authors. PCR products were analysed by 1 % agarose gel electrophoresis.

Ecology and species relationships

Strains of *M. cerradonensis* sp. nov. were isolated from flowers of *I. carnea* and from *Conotelus* species collected on the margins of a small lake in São Felix do Tocantins. Morning glory flowers sampled in public gardens and streets of São Felix yielded only basidiomycetous yeasts and *Candida azyma*. Samples taken in two other areas of the Cerrado, located approximately 300 km from Jalapão, yielded only *M. continentalis*, suggesting that *M. cerradonensis* might be endemic to the Jalapão region.

The phylogenetic relationships among *M. cerradonensis* and its relatives are shown in Fig. 1. The use of split decomposition instead of the more usual neighbour-joining or other phylogenetic analysis methods was dictated by the fact that different parts of the rDNA cluster would lead to somewhat contradictory phylogenies. Although such inconsistencies would be flagged by low bootstrap values, alternative branching orders would remain unknown. Split decomposition (Huson & Bryant, 2006) displays simultaneously the major trends suggested by the sequence data as parallelograms in which the longer sides join the

Fig. 1. Split decomposition network of the rDNA sequences of *Metschnikowia cerradonensis* sp. nov. and related species. Only ungapped positions were used in the analysis. Bar, 1% sequence divergence.

better supported hierarchy. In the present case (Fig. 1), the clear interpretation is that *M. cerradonensis*, *M. santaceciliae* and *M. continentalis* are sister species, but that several alternative branching orders are possible among the remaining species. For example, the split decomposition network suggests that *Metschnikowia* sp. UWOPS 00-154.1 and *Candida ipomoeae* are almost as likely to be basal members of the subclade containing the novel species as they are to be sister species with *M. borealis* and *M. hawaiiensis*, respectively. Nonetheless, an affinity of the novel species with *M. lochheadii* and *Metschnikowia* sp. UWOPS 00-154.1 is evident.

Analysis of shorter regions of the rDNA unit, for example the popular D1/D2 LSU domains, has led to somewhat different conclusions about some of these species, including the original belief that *M. continentalis* and *M. borealis* should be viewed as varieties of a single species (Lachance *et al.*, 1998), or that *M. lochheadii* and *Candida ipomoeae* might represent a teleomorph–anamorph pair (Lachance *et al.*, 2001). Both suggestions were later disproved (Marinoni & Lachance, 2004). *M. cerradonensis* differs from *M. santaceciliae* and *M. continentalis* only by two substitutions and 1–2 indels in the D2 domain and from *M. santaceciliae* by one substitution in the D1 domain. The internal transcribed spacer region of *M. cerradonensis* contains several substitutions and is 21 and 30 nt longer than that in *M. santaceciliae* and *M. continentalis*, respectively. Differences from *M. lochheadii* are slightly greater. The species boundaries were established by mixing strains of the above four species plus *Metschnikowia* sp. UWOPS 00-154.1 in every possible combination. Rare single-spored asci were observed on occasion in crosses involving *M. cerradonensis*, *M. santaceciliae* and *M. continentalis*. By contrast, intraspecific crosses gave rise to abundant two-spored asci. Sterile asci are formed in extraspecific mixtures of complementary mating types of all members of the large-spored subclade.

M. cerradonensis and its most closely related species were indistinguishable based on growth test profiles. For this reason, PCR amplification with the intron-splicing-site primer was useful in distinguishing *M. continentalis* from *M.*

cerradonensis, as both species might possibly occur in the same samples. The two species showed distinct PCR fingerprint profiles (Fig. 2). Conspecific isolates generally produce similar banding profiles (de Barros Lopes *et al.*, 1998; Carreiro *et al.*, 2004; Pimenta *et al.*, 2005). This was the case here, although some variation was detectable within the two species. Ascospore formation in mixtures with the mating types of authentic strains or rDNA sequencing are recommended for definitive identification.

Latin diagnosis of *Metschnikowia cerradonensis* Rosa, Lachance et Morais sp. nov.

In medio YM post dies tres cellulae singulae, binae, aut in catenis brevis, globosae aut ovoidae (2–4 × 3–6 µm), aliquando cum tubis longibus. Cultura in agar malti post dies 14 infimoconvexa, glabra, candida et butyrosa aut coriacea. In agar farinae Zea mays post dies 14 pseudomycelium formatur. Post dies unus, cellulae stirpium interfertiliium mixtarum in agar carbonis fundamento tubi junctionis formantur. Post dies tres, magni asci cylindrati possunt videri. Asci stabiles sunt. Ascosporae aculeatae

Fig. 2. PCR fingerprints of *Metschnikowia continentalis* and *Metschnikowia cerradonensis* sp. nov. strains obtained using the primer El1. Lanes: 1, 1 kb Plus DNA ladder; 2, *M. continentalis* UFMG 05-T65 (h⁺); 3, *M. continentalis* UFMG 05-T13 (h⁻); 4, *M. cerradonensis* UFMG 03-T68.1 (h⁻); 5, *M. cerradonensis* UFMG 03-T67.1^T (h⁺); 6, *M. cerradonensis* UFMG 05-T338.1 (h⁺); 7, *M. cerradonensis* UFMG 05-T332.2 (h⁻); 8, *M. cerradonensis* UFMG 05-T334.1 (h⁺).

(1.3–1.6 × 60–150 µm). *Glucosum fermentatur*. *Glucosum*, *sucrosus*, *galactosum*, *trehalosum*, *maltosum*, *melezitosum*, *cellobiosum*, *salicinum* (exigue), *L-sorbosum*, *D-xylosum*, *glycerolum* (lente et exigue), *ribitolum* (lente), *xylitolum*, *mannitolum*, *glucitolum*, *acidum succinicum*, *acidum citricum* (lente), *acidum gluconicum*, *N-acetylglucosaminum*, *ethylacetat* (lente) et *hexadecanum* assimilantur, at non *inulinum*, *raffinoseum*, *melibiosum*, *lactosum*, *methyl-5-D-glucosidum*, *amylum solubile*, *L-rhamnosum*, *L-arabinosum*, *D-arabinosum*, *D-ribosum*, *methanolum*, *2-propanolum*, *ethanolum*, *erythritolum*, *galactitolum*, *meso-inositolum*, *acidum lacticum*, *glucosaminum* nec *acetoneum*. *Ethylaminum*, *lysinum* et *cadaverinum* assimilantur at non *natrium nitricum* nec *natrium nitrosum*. Ad crescentiam *vitamina externa necessaria sunt*. *Augmentum* in 34 °C, at non 35 °C.

Habitat *Conotelus* sp. et *Ipomoea carnea* in Brazil. *Typus*: UFMG 03-T67.1^T (h⁺). *Allotypus*: UFMG 03-T68.1 (h⁻). In *collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum*, sub no. CBS 10409 et CBS 10410 *depositae sunt*.

Description of *Metschnikowia cerradonensis* Rosa, Lachance & Morais sp. nov.

Metschnikowia cerradonensis (cer.ra.do.nen'sis. L. nom. sing. m. adj. *cerradonensis* of Cerrado, referring to the Cerrado ecosystem, where all known strains of the species were recovered).

On YM agar after 3 days at 25 °C, cells are spherical to ovoidal, occur singly, in parent–bud pairs or in short chains, and measure 2–4 × 3–6 µm. Highly refringent cells as well as very long germ tubes are observed on occasion. After 2 weeks, colonies are of low convexity or are convex, glossy, white, small to medium sized, butyrous or leathery owing to intertwined tubes. In Dalmau plate cultures on cornmeal agar after 2 weeks, abundant pseudohyphae are formed in some, but not all, cultures. On yeast carbon base agar at 22 °C, mixtures of cells of complementary mating types give rise to zygotes and asci after 12–24 h. After 3 days, mature asci containing two acicular ascospores (1.3–1.6 × 40–150 µm) are formed in a persistent ascus that retains more or less conspicuous vestiges of the conjugated parent cells (Fig. 3). Gas production from glucose begins after 1–2 days. Galactose, sucrose, maltose and trehalose are not fermented. Glucose, sucrose, galactose, trehalose, maltose, melezitose, cellobiose, salicin (weak), *L-sorbose*, *D-xylose*, glycerol (slow and weak), ribitol (slow), xylitol, mannitol, glucitol, succinic acid, citric acid (slow), gluconic acid, *N-acetylglucosamine*, ethyl acetate and hexadecane are assimilated. No growth occurs on inulin, raffinose, melibiose, lactose, soluble starch, *L-rhamnose*, *L-arabinose*, *D-arabinose*, *D-ribose*, methanol, 2-propanol, ethanol, erythritol, galactitol, *myo-inositol*, lactic acid, *D-glucosamine* or acetone. Assimilates the following nitrogen compounds: lysine, ethylamine-HCl and cadaverine; but negative in tests for nitrate and nitrite. No growth in

Fig. 3. Phase-contrast micrograph of a mature ascus with two acicular ascospores of *Metschnikowia cerradonensis* sp. nov. on yeast carbon base agar with 0.01 % ammonium sulfate after 2 weeks at 17 °C. Bar, 10 µm.

vitamin-free medium, but growth is observed in amino-acid-free medium. Grows at 34 °C, but not at 35 °C. Growth on YM agar with 10 % sodium chloride is slow. No growth in 50 % glucose/yeast extract (0.5 %). Starch-like compounds are not produced. No growth in 100 µg cycloheximide ml⁻¹. Urease-negative. Diazonium blue B reaction is negative.

The type strain, UFMG 03-T67.1^T (h⁺) (=CBS 10409^T=NRRL Y-48067^T), was recovered from *Conotelus* beetles (Coleoptera: Nitidulidae) associated with flowers of *Ipomoea carnea* growing in the margins of a small lake of the Jalapão area in the city of São Felix do Tocantins, Tocantins State, Brazil. The designated allotype, UFMG 03-T68.1 (h⁻) (=CBS 10410=NRRL Y-48068), was recovered from *Conotelus* beetles.

Acknowledgements

This work was funded by Programa Norte de Pesquisas e Pós-Graduação do Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brazil (PNOPG-CNPq), CNPq – Brazil (process no. 477528/03-1), Fundação do Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, process no. CBB – 378/04) and the Natural Science and Engineering Research Council of Canada (M.-A. L.).

References

- Carreiro, S. C., Pagnocca, F. C., Bacci, M., Jr, Lachance, M. A., Bueno, O. C., Hebling, M. J., Ruivo, C. C. & Rosa, C. A. (2004). *Sympodiomyces attinorum* sp. nov., a yeast species associated with nests of the leaf-cutting ant *Atta sexdens*. *Int J Syst Evol Microbiol* **54**, 1891–1894.
- de Barros Lopes, M., Soden, A., Martens, A. L., Henschke, P. A. & Langridge, P. (1998). Differentiation and species identification of yeasts using PCR. *Int J Syst Bacteriol* **48**, 279–286.
- Huson, D. H. & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* **23**, 254–267.
- Kurtzman, C. P. & Fell, J. W. (1998). *The Yeasts, a Taxonomic Study*. Amsterdam: Elsevier.
- Lachance, M. A., Starmer, W. T. & Phaff, H. J. (1990). *Metschnikowia hawaiiensis* sp. nov., a heterothallic haploid yeast from Hawaiian morning glory and associated drosophilids. *Int J Syst Bacteriol* **40**, 415–420.

- Lachance, M. A., Rosa, C. A., Starmer, W. T., Schlag-Edler, B., Barker, J. S. F. & Bowles, J. M. (1998). *Metschnikowia continentalis* var. *continentalis*, *Metschnikowia continentalis* var. *borealis*, and *Metschnikowia hibisci*, new heterothallic haploid yeasts from ephemeral flowers and associated insects. *Can J Microbiol* **44**, 279–288.
- Lachance, M. A., Bowles, J. M., Kwon, S., Marinoni, G., Starmer, W. T. & Janzen, D. H. (2001). *Metschnikowia lochheadii* and *Metschnikowia drosophilae*, two new yeast species isolated from insects associated with flowers. *Can J Microbiol* **47**, 103–109.
- Lachance, M. A., Bowles, J. M. & Starmer, W. T. (2003). *Metschnikowia santaceciliae*, *Candida hawaiiiana*, and *Candida kipukae*, three new yeast species associated with insects of tropical morning glory. *FEMS Yeast Res* **3**, 97–103.
- Lachance, M. A., Ewing, C. P., Bowles, J. M. & Starmer, W. T. (2005). *Metschnikowia hamakuensis* sp. nov., *Metschnikowia kamakouna* sp. nov. and *Metschnikowia mauinuiana* sp. nov., three endemic yeasts from Hawaiian nitidulid beetles. *Int J Syst Evol Microbiol* **55**, 1369–1377.
- Marinoni, G. & Lachance, M. A. (2004). Speciation in the large-spored *Metschnikowia* clade and establishment of a new species, *Metschnikowia borealis* comb. nov. *FEMS Yeast Res* **4**, 587–596.
- Pimenta, R. S., Alves, P. D. D., Corrêa, A., Jr, Lachance, M. A., Prasad, G. S., Rajaram, Sinha, B. R. R. P. & Rosa, C. A. (2005). *Geotrichum silvicola* sp. nov., a novel asexual arthroconidial yeast species related to the genus *Galactomyces*. *Int J Syst Evol Microbiol* **55**, 497–501.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Yarrow, D. (1998). Methods for the isolation and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.