Candida heliconiae sp. nov., *Candida picinguabensis* sp. nov. and *Candida saopaulonensis* sp. nov., three ascomycetous yeasts from *Heliconia velloziana* (Heliconiaceae)

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Strains belonging to three novel yeast species, *Candida heliconiae* (four isolates), *Candida picinguabensis* (three isolates) and *Candida saopaulonensis* (two isolates), were recovered in the year 2000 from water of flower bracts of *Heliconia velloziana* L. Emig. (Heliconiaceae) found in a forest ecosystem site in an Atlantic rainforest of south-eastern Brazil. *C. picinguabensis* and *C. saopaulonensis* were nearly identical in morphology and physiology, but sequence divergence in the D1/D2 domain of the large-subunit rDNA indicated that they should be regarded as different species. They belong to the Metschnikowiaceae clade. *C. heliconiae* had affinities to *Pichia mexicana* and related species, but was genetically isolated from all currently accepted species in that group. The type strains are *C. heliconiae* UNESP 00-91C1^T (= CBS 10000^T=NRRL Y-27813^T), *C. picinguabensis* UNESP 00-89^T (= CBS 9999^T=NRRL Y-27814^T) and *C. saopaulonensis* UNESP 00-99^T (= CBS 10001^T=NRRL Y-27815^T).

The family Heliconiaceae contains a single genus, *Heliconia* L., with approximately 250 species dispersed in neotropical areas from the north of Mexico to the south of Brazil (Santos, 1978; Dahlgren *et al.*, 1985; Kress, 1990). A small paleotropical group, with eight species, occurs in islands of the South Pacific (Tomlinson, 1969; Kress, 1985). Approximately 40 Brazilian species are known. They occur mostly in the Amazon basin and in the Atlantic coastal forest (Kress, 1990). *Heliconia velloziana* is an endemic species from the Atlantic Forest and occurs from the south-east to the south of Brazil (Mello-Filho, 1975; Santos, 1978; Citadini-Zanette & Baptista, 1989). One characteristic of the genus *Heliconia* is rapidly decaying floral parts enclosed by massive, surviving bracts. The nectar in the bracts is thought to be the site of development of communities of yeasts and bacteria (Schnittler & Stephenson, 2002). In this paper, we describe the occurrence of the novel yeast species *Candida heliconiae*, *Candida picinguabensis* and *Candida saopaulonensis* in *Heliconia velloziana*.

The GenBank/EMBL/DBJ accession numbers for the large-subunit rRNA gene sequences of strains UNESP 00-91C1^T, UNESP 00-89^T and UNESP 00-99^T are AY566406, AY566407 and AY695398, respectively.

Yeast isolation and characterization

Four strains of *C. heliconiae*, three of *C. picinguabensis* and two of *C. saopaulonensis* were isolated from the water of flower bracts of *Heliconia velloziana* collected in the Picinguaba area, an Atlantic rainforest site in the 'Serra do Mar' State Park in São Paulo State, Brazil (23° 22' S 44° 48' W). This State Park contains one of the largest continuous areas of the remaining Brazilian Atlantic Forest in eastern São Paulo State, and is located 230 km from the city of São Paulo. Collections were made from 14 plants during spring (September) 2000. The water accumulated in the bract was stirred with a sterile loop that was then used to streak-inoculate three plates of YM agar (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 2 % agar) containing 100 mg chloramphenicol l⁻¹ (Trindade *et al.*, 2002). The plates were incubated at 25 °C for 5 days. Selected representative colonies were purified and maintained on YM agar slants at 4 °C and at –80 °C. The yeasts were characterized by using standard methods (Yarrow, 1998) and their identification was carried out 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 domains of the large-subunit rDNA were symmetrically amplified with primers NL-1 and NL-4 (O'Donnell, 1993). Each PCR was performed with the Ready-To-Go kit (Amersham Pharmacia Biotech), according to the manufacturer's recommendations.

The sequence products were resolved in an ABI Prism 377 DNA sequencer (Applied Biosystems) at the Centro de Estudos de Insetos Sociais – UNESP, Rio Claro, São Paulo, Brazil. Alternatively, the DNA was amplified directly from whole cells and sequenced as described by Lachance *et al.* (1999). Sequence alignment and tree construction were done with the program DNAMAN 4.1 (Lynnon Biosoft).

Species delineation, classification and ecology

All strains were examined after growth on common sporulation media, either alone or in pairwise mixtures. Conjugation or ascus formation was not observed. In the absence of a sexual cycle, species delineation relied on sequence divergence. Based on the analysis of the large-subunit rDNA D1/D2 domains, *C. pinguabensis* and *C. saopaulonensis* represent sister species with affinities to the Metschnikowiaceae clade. The sequences of the two taxa differed from each other by 18 substitutions and three gaps, which supports the hypothesis that they represent separate species (Kurtzman & Robnett, 1998). Physiologically, the two species differed only in the assimilation of galactose and the ability to grow in the presence of 10 µg cycloheximide ml⁻¹. The species shown in Fig. 1(a) are representatives of neighbouring clades, chosen to identify the approximate phylogenetic position of the novel species. A reliable connection with any known species within the Metschnikowiaceae clade could not be established, although a weak link with *Metschnikowia* and related *Candida* species found in beetles and other insects of morning glories was apparent. *C. heliconiae* has no clearly identifiable sister species and occupies a basal position in a clade that contains *Pichia mexicana* and related *Pichia* or *Candida* species. The species in Fig. 1(b) were selected to assist in localizing *C. heliconiae* phylogenetically. A weak connection was found with *Candida sinolaborantium* and other species known to be associated with the plant–insect interface.

Although the newly described species were isolated from water accumulated in bracts of *Heliconia velloziana*, it cannot be assumed that they are associated only with this plant, as *Heliconia* species are visited by hummingbirds attracted by the flower's nectar (Stiles, 1975). In addition to the novel species described herein, other yeasts isolated from the same substrates included *Candida azyma*, *Candida boidinii*, *Candida pseudointermedia*, *Candida restingae*, *Candida silvae*, *Debaryomyces* spp., *Hanseniaspora uvarum*, *Kluyveromyces* sp., *Kodamaea* sp., *Metschnikowia koreensis* and *Metschnikowia* sp., which are often found in flowers (Rosa *et al.*, 1999; Hong *et al.*, 2001). As *C. pinguabensis* and *C. saopaulonensis* have similar physiologies and morphologies, they are expected to occur in similar microhabitats. Our results suggest that these novel species are nectar-inhabiting yeasts.

Fig. 1. Neighbour-joining dendrograms, based on sequences of the D1/D2 domains of the large-subunit rDNA, depicting the approximate phylogenetic positions of *C. pinguabensis* and *C. saopaulonensis* (a) and *C. heliconiae* (b). Bootstrap values greater than 75%, determined from 1000 interactions, are shown. Bars, sequence divergence with Kimura's two-parameter correction. Root placement was based on the inclusion of *Schizosaccharomyces pombe* (NRRL Y-12796^T, GenBank accession no. U40085; not shown) as an outgroup.

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

In medio liquido post dies tres ad 25 °C, cellulae globosae aut ovoidae, singulae aut binae (3–5.5 × 3–5 µm). Cultura in agar extracta malti et levidinis continente post 4 dies ad 25 °C, albida cremae et butyrosa. In agar farinae Zea mays post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, galactosum, L-sorboseum, sucrosum, maltosum, cellobiosum, melezitium, D-xylosum, L-arabiosum (variabile), D-arabiosum, D-glucosaminum (variabile), N-acetyl-D-glucosaminum, ethanolum, glycerolum, ribitolum, mannitolum, glucitolum, methyl α-D-glucosidum, acidum gluconicum (lente), salicinum (exigue), et glucono-δ-lactonum assimulantur. Non assimilantur trehalosum, lactosum, melibiosum, raffinoseum, inulinum, amyllum solubile, D-ribosum, L-rhamnosum, methanolum, erythritolum, galactitolum, acidum lacticum, acidum succinicum, acidum citricum, myo-inositolum, n-hexadecanum (lente), 2-keto-D-gluconatum, 5-keto-D-gluconatum et xylitolum. L-Lysinum, ethylaminum et cada-verinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35 °C, at non 37 °C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Non crescit agar extrato fermenti

confecto 50 partes glucosi per centum. Habitat aqua et Heliconia velloziana L. Emigd. (Heliconiaceae). *Typus stirps* UNESP 00-91C1^T. *In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum, sub no.* CBS 10000^T, *typus stirps deposita est.*

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

Candida heliconiae (he.li.co'ni.ae. N.L. gen. n. *heliconiae* of *Heliconia velloziana*, referring to the plant from which the species was isolated).

In yeast extract (0.5 %) glucose (2 %) broth after 3 days at 25 °C, cells occur singly or in budding pairs. Cells are spheroidal to ovoid (3–5.5 × 3–5 µm). Buds are produced multilaterally (Fig. 2a). On YM agar after 4 days at 25 °C, colonies are cream-coloured or white, low-convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on cornmeal agar, pseudomycelium or true mycelium is not formed. Glucose fermentation is complete after 2–5 days. The carbon compounds glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, melezitose, D-xylose, L-arabinose (variable), D-arabinose, D-glucosamine (variable), N-acetyl-D-glucosamine, ethanol, glycerol, ribitol, mannitol, glucitol, methyl α-D-glucoside, D-gluconic acid (slow), salicin (weak) and glucono-δ-lactone are assimilated. No growth occurs on trehalose, lactose, melibiose, raffinose, inulin, starch, D-ribose, L-rhamnose, methanol, erythritol, galactitol, DL-lactic acid, succinic acid, citric acid, *myo*-inositol, n-hexadecane, 2-keto-D-gluconate, 5-keto-D-gluconate or xylitol. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35 °C is positive and negative at 37 °C. Acid formation on chalk agar is 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 negative. Growth on YM agar with 10 % NaCl is negative. Growth in the presence of 10 and 100 µg cycloheximide

ml⁻¹ is positive. Growth in the presence of 1 % acetic acid is negative.

The type strain, UNESP 00-91C1^T, was isolated from water accumulated in flower bracts of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 10000^T (= NRRL Y-27813^T).

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

In medio liquido post dies tres ad 25 °C, cellulae globosae, singulae aut binae (3–7 × 4–8 µm). Cultura in agar extracta malti et levidinis continente post 4 dies ad 25 °C, albida cremae et butyrosa. In agar farinae Zea mays post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, L-sorbosum, sucrosum, maltosum, trehalosum, melezitosum, D-xylosum, ethanolum, ribitolum, mannitolum, glucitolum, methyl α-D-glucosidum, acidum gluconicum (variabile), acidum lacticum (lente), acidum succinicum (exiguum), acidum citricum (exiguum), n-hexadecanum (lente), glucono-δ-lactonum, 2-keto-D-gluconatum et xylitolum assimilantur. Non assimilantur galactosum, cellobiosum, lactosum, melibiosum, raffinolum, inulinum, amyllum solubile, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, glycerolum, erythritolum, galactitolum, salicinum, myo-inositolum et 5-keto-D-gluconatum. L-Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35 °C, at non 37 °C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Crescit agar extrato fermenti confecto 50 partes glucosi per centum et lente. Non crescit in medio 100 µg ml⁻¹ cycloheximido addito. Habitat aqua et Heliconia velloziana L. Emigd. (Heliconiaceae). *Typus stirps* UNESP 00-89^T. *In collectione zymotica* Centraalbureau voor Schimmelcultures,

Fig. 2. Photomicrographs of cells of *C. heliconiae* strain UNESP 00-91C1^T (a), *C. pinguabensis* strain UNESP 00-89^T (b) and *C. saopaulonensis* UNESP 00-99^T (c) in yeast extract, glucose broth after 3 days at 25 °C. Bars, 10 µm.

Trajectum ad Rhenum, sub no. CBS 9999^T, *typus stirps deposita est*.

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

Candida picinguabensis (pi.ci.n.gua'ben.sis. N.L. fem. adj., *picinguabensis* pertaining to Picinguaba area, referring to the locality where the species was isolated).

In yeast extract (0.5 %) glucose (2 %) broth after 3 days at 25 °C, cells occur singly or in budding pairs. Cells are spheroidal (3–7 × 4–8 µm). Buds are produced multilaterally (Fig. 2b). On YM agar after 4 days at 25 °C, colonies are cream-coloured or white, low-convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on cornmeal agar, pseudomycelium or true mycelium is not formed. Glucose fermentation is complete after 2–5 days. The carbon compounds glucose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, mannitol, glucitol, methyl α-D-glucoside, D-gluconic acid (variable), DL-lactic acid (slow), succinic acid (weak), citric acid (weak), n-hexadecane (slow), glucono-δ-lactone, 2-keto-D-gluconate and xylitol are assimilated. No growth occurs on galactose, cellobiose, lactose, melibiose, raffinose, inulin, starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, *myo*-inositol or 5-keto-D-gluconate. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35 °C is positive and negative at 37 °C. Acid formation on chalk agar is positive. Urease activity and Diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50 % glucose/yeast extract agar is slow. Growth on YM agar with 10 % NaCl is negative. Growth in the presence of 10 µg cycloheximide ml⁻¹ is positive and negative in 100 µg cycloheximide ml⁻¹. Growth in the presence of 1 % acetic acid is negative.

The type strain, UNESP 00-89^T, was isolated from water accumulated in flower bracts of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 9999^T (= NRRL Y-27814^T).

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

In medio liquido post dies tres ad 25 °C, cellulae globosae, singulae aut binae (3–6.5 × 4–7 µm). *Cultura in agaro extracta multi et levidinis continente post 4 dies ad 25 °C, albida crematae et butyrosa. In agaro farinae Zea mays post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, galactosum, L-sorbosum, sucrosum, maltosum, trehalosum, melezitosum, D-xylosum, ethanolum, ribitolum, mannitolum, glucitolum, methyl α-D-glucosidum, acidum gluconicum, acidum lacticum,*

acidum succinicum (exigue), acidum citricum (exigue), n-hexadecanum, glucono-δ-lactonum, 2-keto-D-gluconatum et xylitolum assimulantur. Non assimilantur cellobiosum, lactosum, melibiosum, raffinolum, inulinum, amyllum solubile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, glycerolum, erythritolum, galactitolum, salicinum, myo-inositolum et 5-keto-D-gluconatum. L-Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35 °C, at non 37 °C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Crescit agaro extrato fermenti confecto 50 partes glucosi per centum raro exigue. Non crescit in medio 100 µg ml⁻¹ cycloheximido addito. Habitat aqua et Heliconia velloziana L. Emigd. (Heliconiaceae). Typus stirps UNESP 00-99^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 10001^T, typus stirps deposita est.

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

Candida saopaulonensis (sao.pau.lo.nen'sis. N.L. fem. adj., *saopaulonensis* pertaining to São Paulo State, referring to the Brazilian state where the species was isolated).

In yeast extract (0.5 %) glucose (2 %) broth after 3 days at 25 °C, cells occur singly or in budding pairs. Cells are spheroidal (3–6.5 × 4–7 µm). Buds are produced multilaterally (Fig. 2c). On YM agar after 4 days at 25 °C, colonies are cream-coloured or white, low-convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on cornmeal agar pseudomycelium or true mycelium is not formed. Glucose fermentation is complete after 2–5 days. The carbon compounds glucose, galactose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, mannitol, glucitol, methyl α-D-glucoside, D-gluconic acid, DL-lactic acid, succinic acid (weak), citric acid (weak), n-hexadecane, glucono-δ-lactone, 2-keto-D-gluconate and xylitol are assimilated. No growth occurs on cellobiose, lactose, melibiose, raffinose, inulin, starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, *myo*-inositol or 5-keto-D-gluconate. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35 °C is positive and negative at 37 °C. Acid formation on chalk agar is positive. Urease activity and Diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50 % glucose/yeast extract agar is weak or absent. Growth on YM agar with 10 % NaCl is negative. Growth in the presence of 100 µg cycloheximide ml⁻¹ is negative. Growth in the presence of 1 % acetic acid is negative.

The type strain, UNESP 00-99^T, was isolated from water accumulated in flower bracts of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures,

Utrecht, the Netherlands, as strain CBS 10001^T (= NRRL Y-27815^T).

Acknowledgements

The authors thank the UNESP-CEIS for supporting this research project and the Secretaria de Meio Ambiente (São Paulo State, Brazil) for permission to collect in the Picinguaba Nucleus at the 'Serra do Mar' State Park (Process. SMA: 42.364/99). This work was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à 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.).

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