Biodiversity studies focusing on yeasts inhabiting the Brazilian Atlantic rainforest have revealed the rich yeast diversity of this ecosystem (da Cunha et al., 1957; Morais et al., 1992; Prada & Pagnocca, 1997; Araujo et al., 1998; Ruivo et al., 2005; Landell et al., 2006; Pimenta et al., 2009; Morais et al., 2013). Although this biome has not been intensively studied, the discovery of many novel species has been reported. Examples include *Saturnispora hagleri* (Morais et al., 2005), *Candida (Kodamaea) leandreae* (Ruivo et al., 2004), *Candida (Metschnikowia) ubaturbensis* (Ruivo et al., 2005), *Candida (Metschnikowia) saapaulonensis*, *Candida (Nakazawaeia) helconiae* and *Candida (Metschnikowia) picinguabensis* (Ruivo et al., 2006), *Candida (Scheffersomyces) queiroziae* (Santos et al., 2011) and *Spathaspora arborariae* (Cadete et al., 2009). Some of the species discovered in the Atlantic rainforest may be considered rare or to be habitat specific because they have not reportedly been isolated elsewhere since their first discovery. During a survey of yeasts associated with diverse plant substrates in the São Sebastião do Ribeirão Grande agricultural land, located in the municipality of Pinda-monhangaba, south-eastern Brazil, various species, the majority with ascomycetous affinity, including three strains of two putative novel yeast species were identified. Based on analysis of sequences of the D1/D2 domains of the LSU rRNA gene, these strains belong to the *Wickerhamiella* clade.

Two converging characteristics among species in the *Wickerhamiella* clade are their physiological restrictions (in terms of carbon assimilation) and strong associations with ephemeral flowers and floricolous insects, particularly *Drosophila* and nitidulid beetles (Lachance et al., 1998; Lachance & Kurtzman, 2011). Because of the strong associations of *Wickerhamiella lipophila* (anamorph: *Candida lipophila*) and *Wickerhamiella occidentalis* with drosophilids found in morning glories, it was suggested that the yeasts may be involved in enhancing the nutritional values of the

The GenBank/EMBL/DDBJ accession numbers for the LSU and ITS sequences of *Wickerhamiella kiyanii* FB1-1DASPT = CBS 12905T = CBMAI 1613T and *Wickerhamiella fructicola* f.a., sp. nov. (type strain H10YT = CBS 12902T = CBMAI 1614T) are proposed in the *Wickerhamiella* clade (Saccharomycetaceae, Saccharomycetales) to accommodate three strains isolated from flowers and fruits typical of the Brazilian Atlantic rainforest. The novel status of these yeast species was established by sequence divergence observed in the D1/D2 domains of the LSU rRNA gene from the most closely related, described species as well as by phylogenetic analysis. *Wickerhamiella kiyanii* sp. nov. differs from its nearest phylogenetic neighbours *W. pagnoccae* CBS 12178T, *Candida jalapaonensis* CBS 10935T and *Candida drosophilae* CBS 8459T by 2.2–4.2 % in the D1/D2 sequences. By contrast, a sequence divergence of 13.2–13.8 % was observed between *W. fructicola* sp. nov. and its closest, described phylogenetic relative *Candida kazoui* JCM 12558T and *Candida hasegawae* JCM 12559T. Taxonomic descriptions of the two novel species are given.

Wickerhamiella kiyanii f.a., sp. nov. and Wickerhamiella fructicola f.a., sp. nov., two yeasts isolated from native plants of Atlantic rainforest in Brazil

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Two novel species, *Wickerhamiella kiyanii* f.a., sp. nov. (type strain FB1-1DASPT = CBS 12905T = CBMAI 1613T) and *Wickerhamiella fructicola* f.a., sp. nov. (type strain H10YT = CBS 12902T = CBMAI 1614T) are proposed in the *Wickerhamiella* clade (Saccharomycetaceae, Saccharomycetales) to accommodate three strains isolated from flowers and fruits typical of the Brazilian Atlantic rainforest. The novel status of these yeast species was established by sequence divergence observed in the D1/D2 domains of the LSU rRNA gene from the most closely related, described species as well as by phylogenetic analysis. *Wickerhamiella kiyanii* sp. nov. differs from its nearest phylogenetic neighbours *W. pagnoccae* CBS 12178T, *Candida jalapaonensis* CBS 10935T and *Candida drosophilae* CBS 8459T by 2.2–4.2 % in the D1/D2 sequences. By contrast, a sequence divergence of 13.2–13.8 % was observed between *W. fructicola* sp. nov. and its closest, described phylogenetic relative *Candida kazoui* JCM 12558T and *Candida hasegawae* JCM 12559T. Taxonomic descriptions of the two novel species are given.
flies’ diet by converting flower lipids into a nutritionally richer biomass (Lachance & Kurtzman, 2011). Recent samplings of yeasts revealed that the sugar cane plant may be a new habitat for some yeasts in this clade. *Wickerhamiella slavikovae* and *Wickerhamiella goessii* were isolated from sugar cane plants (Hagler et al., 2013) while *Wickerhamiella dulcicola* and *Wickerhamiella cachassae* were isolated from sugar cane juice and must, respectively (Badotti et al., 2013). A common link between these yeasts and sugar cane is probably their association with insects, bees and drosophilid flies. Based on the low degree of sequence relatedness observed among some described species in the genus *Wickerhamiella*, Lachance & Kurtzman (2011) predicted that many unknown species might yet be discovered in this clade. In the present study, we provide the description of two additional species, *Wickerhamiella kiyanii* f.a., sp. nov. and *Wickerhamiella fructicola* f.a., sp. nov. to be accommodated in the *Wickerhamiella* clade.

The proposed novel species were isolated in 2006 from flowers and fruits collected at the São Sebastião do Ribeirão Grande agricultural land in the Atlantic rainforest of Pindamonhangaba, São Paulo State, Brazil (22° 44’ 28” S 45° 28’ 19” W). Strain FB1-1DASP T (=CBS 12905T) was isolated from flower bracts of *Siphocampylus* sp. (Campanulaceae) while strains H10YT (=CBS 12902T) and H10-10AY were recovered from fruits of the white garland-lily (*Hedychium coronarium* Koenig). Nectar was collected aseptically using a sterile inoculating loop and streaked on the surface of yeast-malt extract (YM) agar (yeast extract 0.3 %, malt extract 0.3 %, mycological peptone 0.5 %, glucose 1 %, agar 2 % and chromamphilon 0.015 %). The plates were incubated at 22 °C for 5 days. Yeast colonies were purified on YM agar and maintained at 4 °C and by cryopreservation in 15 % glycerol (v/v) at −80 °C. For yeast isolation from the fruits of *H. coronarium*, mature fruits were inoculated in YM broth; after incubation for 5 days, aliquots of 100 µl were plated on YM agar. Isolation was also carried out by scraping the surface of mature fruits with a swab followed by streaking on YM agar.

The yeast isolates were characterized by standard procedures described by Kurtzman et al. (2011). Ascosporation was investigated by inoculating the yeast strains on cornmeal, YM, 5 % malt extract and acetate agar, respectively, and incubating at 15 and 25 °C. The cultures were examined weekly for up to three months. Physiological and biochemical tests were performed by replica plating on solid and in liquid media according to the protocols of Kurtzman et al. (2011). Test samples were incubated at 25 °C and results were read weekly for up to 28 days. In addition, the strains were subjected to screening of their potential for amylase (Buzzini & Martini, 2002), cellulase (Strauss et al., 2001), xylanase (Pointing, 1999), pectinase (Oliveira et al., 2006) and a possible lipase activity according to the methods of Kouker & Jaeger (1987) and Hou & Johnston (1992).

Genomic DNA was extracted following the protocol described in Sampaio et al. (2001). The primer pair NL1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL4 (5’-GGTCGGTGTTCAGACGG-3’) was used to amplify the approximately 600 bp fragment of the LSU rRNA gene. A 25 µl PCR was performed using Ready-to-Go beads (GE Healthcare) and 5.0 µl of diluted DNA template (1 : 750). The PCR thermal cycler conditions followed those described by Pagnocca et al. (2008). Amplification and sequencing of the ITS region, which includes ITS1–5.8S–ITS2, were carried out using the primer pair ITS1 and ITS4 (White et al., 1990). Each PCR product was subsequently purified using Nucleospin Gel and PCR Clean-up kits (Macherey-Nagel) and sequenced on an ABI 3130 Genetic Analyzer (Life Technologies) using the BigDye Terminator v. 3.1 sequencing chemistry. Contig comparisons with other sequences were carried out in the databases of MycoBank (www.mycobank.org) and the NCBI – GenBank database (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed in Mafft v. 7 (Katoh & Standley, 2013) and visualized in BioEdit v.7.0.5.3 (Hall, 1999). The alignment contained sequences of 38 yeast species (including the 2 proposed novel species) and consisted of 416 positions (all gaps and missing data were excluded). Evolutionary relationships based on sequences of the D1/D2 regions of the LSU rRNA gene were reconstructed using the neighbour-joining algorithm under the maximum composite likelihood as the substitution model. The tree was inferred in MEGA5 (Tamura et al., 2011). Branch support was calculated based on 1000 bootstrap iterations.

*W. kiyanii* sp. nov. differed from its closest relative *W. pagnoccae* by 12 nt substitutions in the D1/D2 region and by 27 substitutions and three gaps in the ITS region. Phylogenetic analysis of D1/D2 sequences placed *W. kiyanii* sp. nov. in a position different from previously described species in the *Wickerhamiella* clade (Fig. 1).

Due to its limited range of growth responses, which is also a common characteristic among species in the *Wickerhamiella* clade, *W. kiyanii* sp. nov. is difficult to separate from related species on the basis of phenotypic characteristics; however, some phenotypic differences exist between *W. kiyanii* sp. nov. and *W. pagnoccae*. The former grew in sucrose, glycerol, D-glucanate, ethylamine and cadaverine as well as in the presence of 0.1 % cycloheximide, while the latter did not. Table 1 summarizes growth tests where differences were observed from related species. Differences were also observed between the cellular morphologies of *W. kiyanii* sp. nov. and related species. Most species in the *Wickerhamiella* clade do not form pseudomycelium. Cells of *W. pagnoccae* are ovoid but do not form pseudomycelium, whereas *W. kiyanii* sp. nov. forms elongated to ovoid cells and produces extensive pseudohyphae even without induction on a nutritionally poor medium. The cells of *W. kiyanii* sp.
nov. are also unusually larger compared with those of related species. In addition, whereas W. kiyanii sp. nov. did not grow at 30 °C, W. pagnoccae grew at 37 °C.

A BLAST search of the GenBank database with D1/D2 sequences identified Candida kazuoi JCM 12558T, isolated from insect frass in Thailand (Nakase et al., 2007), as the

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Fig. 1. Phylogenetic placement of W. kiyanii sp. nov. and W. fructicola sp. nov. in the Wickerhamiella clade (Saccharomycetes, Saccharomycetales) determined by neighbour-joining analysis of sequences from the LSU rRNA gene. Bootstrap support values on branches are from 1000 pseudoreplicates (only values above 50% are shown). Names of yeast species are followed by culture collection voucher numbers and sequence accessions are indicated in parentheses. Bar, 0.02 substitutions per site.
from drosophilids collected from flowers of morning glory on the island of Maui, which led to the description of the teleomorph *W. lipophila*. *W. pagnoccae* (Barbosa et al., 2012) and its closest relative, *C. jalapaonensis* (Rosa et al., 2009), were also isolated from flowers of *Heliconia psittacorum* and *Centropogon cornutus* (Campanulaceae) in the Cerrado ecosystem, in the state of Tocantins, Brazil. It is probable that *W. kiyani* sp. nov. and *W. fructicola* sp. nov. will also be associated with floricolous insects that visit the plants from which they were isolated. Discovery of additional strains in the future may reveal ascosporic states of these species as well as provide information on intraspecific physiological and genetic variations. The mention *forma asexualis* (f.a.) is added as a reminder that a sexual state is not known for these two novel yeast species, although the formal species description is intended to include eventual sexual strains (Lachance & Kurtzman, 2013).

**Description of Wickerhamiella kiyani** Pagnocca, Rosa, Dayo-Owoyemi & Rodrigues sp. nov.

Etymology: *kiyan*i. N.L. gen. n. *kiyanii*, in honour of Professor Emeritus Choiti Kiyan of UNESP – Univ Estadual Paulista, Campus de Rio Claro, São Paulo State, Brazil.

**Growth on YM agar.** After 3 days at 25 °C, colony growth is white, convex and butyrous with entire margin. After 21 days, colonies are light brown, raised, butyrous and folded or smooth with entire margins.

**Growth in YM broth.** Cells are oval and elongated. Cells divide by multilateral budding, occurring in budded pairs or chains of pseudohyphae and measuring 4–8 μm (Fig. 2a). After 7 days, a white, creeping pellicle is formed.

**Table 1.** Physiological characteristics differentiating *Wickerhamiella kiyani* sp. nov. from closely related species

Species: 1, *W. kiyani* sp. nov.; 2, *W. pagnoccae* (data from Barbosa et al., 2012); 3, *C. jalapaonensis* (Rosa et al., 2009); 4, *C. drosophila* (Lachance et al., 1998). *W. kiyani* nov. also differed from members of the *Wickerhamiella* clade, which are frequently found in association with flowers and floricolous insects (Lachance & Kurtzman, 2011). Lachance et al. (1998) described five species in the *Wickerhamiella* clade including two asexual taxa, namely *C. drosophilae* and *C. (W.) lipophila* isolated from flowers of *Ipomoea acuminata* and its associated insect *Drosophila florica* (2000) later isolated conjugating strains of *C. lipophila* from drosophilids collected from flowers of morning glory on the island of Maui, which led to the description of the teleomorph *W. lipophila*. *W. pagnoccae* (Barbosa et al., 2012) and its closest relative, *C. jalapaonensis* (Rosa et al., 2009), were also isolated from flowers of *Heliconia psittacorum* and *Centropogon cornutus* (Campanulaceae) in the Cerrado ecosystem, in the state of Tocantins, Brazil. It is probable that *W. kiyani* sp. nov. and *W. fructicola* sp. nov. will also be associated with floricolous insects that visit the plants from which they were isolated. Discovery of additional strains in the future may reveal ascosporic states of these species as well as provide information on intraspecific physiological and genetic variations. The mention *forma asexualis* (f.a.) is added as a reminder that a sexual state is not known for these two novel yeast species, although the formal species description is intended to include eventual sexual strains (Lachance & Kurtzman, 2013).

**Table 2.** Physiological characteristics differentiating *Wickerhamiella fructicola* sp. nov. from closely related species

Species: 1, *W. fructicola* sp. nov.; 2, *C. kazuoi* (data from Nakase et al., 2007); 3, *C. hasegawae* (Nakase et al., 2007). +, Positive; −, negative; l, latent (rapid development of a positive reaction after a lag period); w, weakly positive; s, positive but slow; w/−, weakly positive or negative; s/l−, slow or negative.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Yeast species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on:</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
</tr>
<tr>
<td>Ethanol</td>
<td>−</td>
</tr>
<tr>
<td>Glycerol</td>
<td>w</td>
</tr>
<tr>
<td>β-Glucan</td>
<td>w</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>−</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>+</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>w</td>
</tr>
<tr>
<td>Cycloheximide 0.01 %</td>
<td>−</td>
</tr>
<tr>
<td>Cycloheximide 0.1 %</td>
<td>−</td>
</tr>
<tr>
<td>Growth at 30 °C</td>
<td>+</td>
</tr>
</tbody>
</table>
Dalmau plate culture on cornmeal agar. Growth under a cover glass shows abundant pseudohyphae that consist of long, branched chains of elongated cells but no mycelium. No sexual structures were observed in pure cultures plated on cornmeal agar, 5 % malt extract and acetate agar.

Fermentation and growth reactions. Glucose is not fermented. Glucose, L-sorbitose, sucrose, glycerol (weakly), D-mannitol (weakly), D-glucitol and D-gluconate (weakly) are assimilated. Carbon compounds not assimilated include galactose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylene, D-arabinose, L-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, ethanol, erythritol, ribitol, galactitol, methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, myo-inositol, 2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, D-glucuronate, glucono-δ-lactone, xylitol and L-arabinitol. Nitrogen compounds assimilated are cadaverine, lysine and ethylamine (weakly). Nitrate, nitrite, creatine and creatinine are not assimilated. Extracellular amyloid compound is absent. Growth occurred in the presence of 0.01 and 0.1 % cycloheximide. Growth is not observed in medium containing 10 % NaCl/5 % glucose or in vitamin-free medium. Diazonium blue B and urease reactions are negative. Positive for lipase production. Growth occurs at 25 and 28 °C but not at 30 °C.

The type strain, FB1-1DASPT T, was isolated from a flower of Siphocampylus sp. (Campanulaceae) collected in the Pindamonhangaba municipal area of São Paulo State, Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 12905 T and in the Brazilian Collection of Environmental and Industrial Micro-organisms (Coleção Brasileira de Micro-organismos de Ambiente e Indústria, CBMAI), Campinas, São Paulo, Brazil, as strain CBMAI 1613 T. The Mycobank number is MB805400.

Description of Wickerhamiella fructicola
Pagnocca, Rosa, Dayo-Owoyemi & Rodrigues sp. nov.

Etymology: fruc’tus. L. n. fructus fruit; L. suff.-cola (from L. n. incola) a dweller, inhabitant; N.L. masc. n. fructicola an inhabitant of fruit.

Growth on YM agar. After 3 days at 25 °C, colony growth is white, raised and butyrous with entire margin. After 21 days, colonies are cream, raised, butyrous and smooth with entire margins.

Growth in YM broth. Cells are oval, occurring in single budded pairs and measuring 1.6–3.6 × 2–7 μm. Cells divide by multilateral budding (Fig. 2b). After 7 days, a white, creeping pellicle is formed.

Dalmau plate culture on cornmeal agar. Growth under a cover glass shows rosettes of joined cells that form short pseudomycelium but no mycelium. No sexual structures are observed in pure cultures plated on cornmeal agar, 5 % malt extract and acetate agar.

Fermentation and growth reactions. Glucose is not fermented. Glucose, sucrose (weakly), ethanol (weakly), D-mannitol, D-glucitol, D-glucuronate (weakly) and glucono-δ-lactone (weakly) are assimilated. Carbon compounds not assimilated include galactose, L-sorbitose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylene, L-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, ribitol galactitol, methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, myo-inositol, 2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, D-glucuronate, xylitol and L-arabinitol. Nitrogen compounds assimilated are cadaverine, lysine and ethylamine (weakly). Nitrate, nitrite, creatine and creatinine are not assimilated. Extracellular amyloid compound is absent.

Fig. 2. Phase-contrast micrographs showing budding cells and pseudomycelium of W. kiyani sp. nov. (a) and budding cells of W. fructicola sp. nov. (b), both after 3 days on YM agar. Bars, 10 μm (a) and 5 μm (b).
Growth does not take place in the presence of 0.01 or 0.1 % cycloheximide. Growth in medium containing 10 % NaCl/5 % glucose or 50 % glucose is weak. Growth in vitamin-free medium is negative. Diazonium blue B and urease reactions are negative. Lipase production is negative. Growth occurs at 25 °C and weakly at 28 or 30 °C.

The type strain, H10YT, was isolated from fruits of Hedychium coronarium collected in the Pindamonhangaba municipal area of São Paulo State, Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 12902T and in the Brazilian Collection of Environmental and Industrial Micro-organisms (Coleção Brasileira de Micro-organismos de Ambiente e Indústria, CBMAI), Campinas, São Paulo, Brazil, as strain CBMAI 1614T. The Mycobank number is MB805401. The additional strain H10-10AY was examined in the present study.

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