Hanseniaspora nectarophila sp. nov.,
a yeast species isolated from ephemeral flowers

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Seven apiculate yeast strains that were isolated from the flowers of Syphocampylus corymbiferus Pohl in Brazil are genetically, morphologically and phenotypically distinct from recognized species of the genera Hanseniaspora and Kloeckera. Genetic discontinuities between the novel strains and their closest relatives were found using a networking approach based on the concatenated sequences of the rRNA gene (internal transcribed spacer and D1/D2 of the LSU), and the protein-coding genes for actin and translation elongation factor-1α. Phylogenetic analysis based on the rRNA and the actin gene placed the novel species represented by the strains in close relationship to Hanseniaspora meyeri and Hanseniaspora clermontiae. PCR fingerprinting with microsatellite primers confirmed the genetic heterogeneity of the novel species. The name Hanseniaspora nectarophila sp. nov. is proposed, with UFMG P OG a.1T (=ZIM 2311T =CBS 133835) as the type strain; MycoBank no. MB807210. As the current description of the genus does not allow the presence of multilateral budding, an emended diagnosis of the genus Hanseniaspora Zikes is proposed.

Apiculate yeasts that belong to the genus Hanseniaspora and its anamorph Kloeckera can cause fermentative spoilage once they are introduced onto overripe and senescent fruit (Phaff & Starmer, 1987). The vectors for their dispersal are generally Drosophila species that are attracted to certain fermented substrates, where they feed, oviposit and pick up spoilage microbiota (Brito da Cunha et al., 1957; Miller & Phaff, 1962; Morais et al., 1992; Chandler et al., 2012). Apiculate yeasts belong to the early colonizers of decaying fruit because they can grow rapidly, and this ecological advantage enables them to predominate over the fruit surface microbiota (Brito da Cunha et al., 1957). Later in the process of fruit deterioration, these Hanseniaspora–Kloeckera species are replaced by other yeast species, due to their limited ability to assimilate various carbon sources (Morais et al., 1995).

The genera Hanseniaspora and Kloeckera comprise, at the time of writing, 17 recognized species that have been delineated based on DNA–DNA hybridization of their whole genomes (Meyer et al., 1978; Cadez et al., 2003; Jindamorakot et al., 2009) or on sequencing of the D1/D2 domain of the LSU rRNA gene (Chang et al., 2012). This classification has been confirmed by several phylogenetic studies (Yamada et al., 1992; Boekhout et al., 1994; Kurtzman, 2003), although a single phylogenetic marker that can reliably reconstruct the relationships between species of the genus Hanseniaspora has not yet been described (Cadez et al., 2006). As the rates for nucleotide substitutions in the LSU D1/D2 region between closely related species of Hanseniaspora are lower than between the majority of yeast species (Kurtzman & Robnett, 1998), a polyphasic approach for species delineation must be applied.

In the present study, we report on the isolation of seven apiculate yeast strains from flowers of Syphocampylus corymbiferus (Campanulaceae) in Brazil that are genetically, morphologically and phenotypically distinct from recognized species of the genera Hanseniaspora and Kloeckera.

All seven strains of apiculate yeasts were isolated from flowers collected at the São Sebastião do Ribeirão Grande farm in the Atlantic rainforest of the Pindamonhangaba municipal area in the State of São Paulo, Brazil (22° 44’ 28” W 45° 28’ 19” S), in June 2006. Seven flowers of S. corymbiferus were aseptically sampled using sterile plastic bags. Yeasts were cultured within a few hours of sampling. The nectar region of the flowers was scraped gently with a
sterile loop and streak-inoculated on yeast extract-malt extract agar (YMA; glucose 1 %, peptone 0.5 %, malt extract 0.3 %, yeast extract 0.3 %, agar 2 % and chloramphenicol 10 mg%). The strains were deposited in the Culture Collection of Microorganisms and Cells of the Universidade Federal de Minas Gerais, Brazil (UFMG). Details of the strains used in this study, their origins and GenBank accession numbers are listed in Table S1 available in the online Supplementary Material. The strains were phenotypically characterized by standard methods, according to Kurtzman et al. (2011). Sporulation was investigated on yeast-potato-glucose (Sigma), 3 and 5 % malt extract (Difco; Becton, Dickinson and Company) and YMA at 26 °C over 3 weeks. DNA was extracted from the cultures grown on yeast-potato-glucose agar plates for 2 days, using MasterPure Yeast DNA Purification kits (Epicentre). Three microsatellite primers, (ATG)₃₅, (GTG)₂₃ and (GACA)₄₆, were used in PCR amplification reactions, as described previously (Cadex et al., 2002). The thermal cycler was programmed for 35 cycles of 1 min at 94 °C, 1 min at 48 °C for primer (ATG)₃₅, 1 min at 52 °C for (GTG)₂₃ or 1 min at 43 °C for (GACA)₄₆, followed by 2 min at 72 °C. The PCR products were separated on 2.5 % agarose gels by electrophoresis at 180 V for 40 min. Ethidium-bromide-stained gels were documented by GelDoc 2000 (Bio-Rad) and processed using BioNumerics 7.1 (Applied Maths). Similarities between the combined fingerprints were calculated using the Pearson’s product moment correlation coefficient (r), based on the overall densitometric profiles of the banding patterns. Cluster analysis of the pair-wise values was performed using the UPGMA algorithm. The internal transcribed spacer (ITS) and the LSU D1/D2 domain of the rRNA gene, the protein-coding genes for actin and translation elongation factor 1 alpha (EF-1α) were amplified and sequenced as detailed by Cadex et al. (2006), except that the sequences were determined by a commercial sequencing facility (Macrogen Inc., South Korea). The relationships among the yeast strains were established by the minimum spanning tree (MST) network creation method, using BioNumerics 7.1, and by parsimony network analysis, using the TCS 1.21 program (Clement et al., 2000). Gapped positions were excluded from the analysis. For the phylogenetic analysis, the sequences were aligned using CLUSTAL X (Thompson et al., 1997). The most-parsimonious trees were generated by the PAUP* 4.0b10 software package (Swofford, 2002). Bootstrap support for the trees was determined from 1000 replications. **Species delineation, phylogenetic placement and ecology** The seven strains formed ascospores on all of the media tested, although sporulation was most abundant on 3 % malt agar. The strains differed by two or fewer nucleotide substitutions (≤0.35 % sequence divergence) in their ITS region and D1/D2 LSU rRNA gene, and by up to five substitutions (≤0.7 % divergence) in the partial sequences of the protein-coding genes for actin and EF-1α. Further evidence for the conspecificity of the strains was confirmed by PCR fingerprinting with three microsatellite primers (Fig. S1). The seven strains shared similar PCR fingerprint profiles, but were different from the type strains and other representative strains of species of the genera Hanseniaspora and Kloeckera. Furthermore, a BLAST similarity search with the D1/D2 LSU confirmed that the Brazilian strains belong to the apiculate yeast family Saccharomycodaceae. Their closest relatives were Hanseniaspora meyeri, Hanseniaspora clermontiae, Hanseniaspora opuntiae and Hanseniaspora guilliermondii from which they differed by four nucleotide substitutions (0.7 % sequence divergence). Although four nucleotide substitutions is not indicative of a separate species according to the generalizations of Kurtzman & Robnett (1998), we sought for further evidence of a novel species because lower evolutionary rates in the D1/D2 region in comparison to the overall genetic divergence for the Hanseniaspora uvarum–H. meyeri–H. clermontiae and H. guilliermondii–Hanseniaspora lachancei–H. opuntiae–Hanseniaspora pseudo guilliermondii species complexes have been observed (Cadex et al., 2003, 2006). As the discriminating capacity of the protein-coding genes for resolving relationships between closely related species is better than that of the ribosomal gene (Daniel & Meyer, 2003; Cadex et al., 2006) we used actin and EF-1α genes as additional phylogenetic markers. Speciation can be operationally observed by significant genetic discontinuities between populations as a consequence of the interruption of gene flow between species (Lachance et al., 2010). To observe these genetic discontinuities among the Brazilian strains and 21 strains of diverse origin belonging to closely related species, we applied an MST networking approach that combined all strains in a single, most-parsimonious network (Bandelt et al., 1999; Posada & Crandall, 2001). The distances between the strains reflected the genetic relatedness based on the concatenated sequences of the rRNA gene (ITS and D1/D2 LSU) and the protein-coding genes for actin and EF-1α (Fig. 1). The strains from Brazil segregated in a well-separated subnetwork that was connected by a long branch to its closest relative (H. meyeri; 79 nt, 2.7 %). Divergences between the other three species (H. clermontiae, H. opuntiae and H. guilliermondii) differing by four nucleotide substitutions from the novel strains along the D1/D2 sequences were even higher, and ranged from 85 substitutions (2.9 %) for H. clermontiae to 109 substitutions (3.7 %) for H. guilliermondii (connections not shown). The boundary between the Brazilian strains and their relatives was confirmed by using a statistical parsimony network analysis (Posada & Crandall, 2001) of concatenated protein-coding and ribosomal datasets at the 95 % connection limit. However, when the analysis was conducted with the ITS and D1/D2 LSU sequences only, shown by Lachance et al. (2010, 2011) to delimit species, the Brazilian strains remained in a single network with their closest relatives. Therefore, the genetic divergence of the Brazilian strains was further confirmed by PCR fingerprinting. The novel strains segregated from the type
strains and representative strains of all species of the genera *Hanseniaspora* and *Kloeckera* at a low similarity value (60%) (Figs S1–S3).

For the phylogenetic placement of the novel strains, a concatenated dataset of the ITS and D1/D2 LSU of the rRNA gene, and the actin gene was used to construct the most-parsimonious tree with high statistical support (Fig. 2). The novel strains clustered together with *H. meyeri* and *H. clermontiae* within the *Hanseniaspora valbyensis* clade.

The results presented here support the prediction that the studied strains are genetically distinct from other species of *Hanseniaspora*–*Kloeckera*, and therefore that they represent a novel species, for which we propose the name *Hanseniaspora nectarophila* sp. nov. (MycoBank no. MB807210).

The seven strains of *H. nectarophila* were isolated from flowers of *Siphocampylus corymbiferus*. The novel species was isolated from all of the seven flowers sampled, which suggests that this ephemeral substrate is its ecological niche. Pigmented yeasts were also recovered from these flowers. These yeasts can use the nectar produced in the flowers as a nutrient source. This novel species probably uses insects that visit these flowers as their vectors.

**Identification**

As shown in Fig. 3, *H. nectarophila* is morphologically unique, as the mature buds mostly remained adhered to the mother cell. Also, even 48 h of cultivation either in yeast extract–malt extract liquid medium (Fig. 3a) or on YMA plates (Fig. 3b) gave rise to clusters of cells. Furthermore, budding of the cells was not strictly bipolar, but arose multilaterally as well. Based on this observation we propose to emend the diagnosis of the genus *Hanseniaspora* Zikes from 'Budding is bipolar' to 'Budding is mostly bipolar'.

*H. nectarophila* produced one to two round ascospores per ascus (Fig. 3c), a feature shared with *H. uvarum*, but not with its closely related taxa.

Physiologically, the strains of *H. nectarophila* can be differentiated from their closest relatives *H. meyeri*, *H. clermontiae*, *H. opuntiae*, *H. lachancei*, *H. pseudoguilliermondii* and *H. guilliermondii* by their ability to ferment and assimilate trehalose (both slowly) and from *Hanseniaspora thailandica* by their inability to assimilate D-gluconate. Nevertheless, for the unambiguous discrimination of *H. nectarophila* from other species of *Hanseniaspora*–*Kloeckera*, sequencing of the ITS and D1/ D2 LSU rRNA gene is suggested.
**Description of Hanseniaspora nectarophila sp. nov.**

Hanseniaspora nectarophila [nec.ta.ro’phi.la; Gr. n. nectar the drink of the gods, honey; Gr. adj. philos loving; N.L. fem. adj. nectarophila referring to the isolation source (nectar) of the strains of the species].

In yeast extract-malt extract liquid medium after 48 h at 25 °C, cells are apiculate, ovoid to elongate, 3.5–8.0 μm × 1.8–5.0 μm, and occur singly, in pairs or in short chains. Budding is mostly bipolar. A sediment is present. After 1 month, a very thin ring is formed. After 1 month at 25 °C, streak culture on malt agar is cream coloured, butyrous, smooth, glossy, and flat to slightly raised at the centre, with

*Wickerhamomyces anomalus* CBS 5759T was used as the outgroup. Bar, number of nucleotide substitutions.
an undulate margin. On slide culture with corn meal agar after 7 days at 25 °C, a rudimentary pseudomycelium is formed. Ascii containing one to two warty, spherical ascospores (1.4–3.4 μm) after 2 weeks or more are observed on 3 and 5 % malt extract, and on potato-glucose agar and YMA, at 26 °C. Ascospores are not released from the ascus. Glucose and α,α-trehalose (slow) are fermented; D-galactose, maltose, sucrose and lactose are not fermented. The carbon compounds that are assimilated are glucose, α, α-trehalose (weakly or weakly and slowly), cellobiose, salicin, arbutin, glucono-δ-lactone and 2-ketogluconate; no growth occurs on galactose, L-sorbitose, glucosamine, D-ribose, D-xylene, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, methyl α-D-glucoside, melibiose, lactose, raffinose, melezitose, starch, glycerol, erythritol, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, galactitol, myo-inositol, D-gluconate, D-glucuronate, D-galacturonate, DL-lactate, succinate, citrate, methanol, ethanol, 1,2-propanediol, 2,3-butanediol and hexadecane. Assimilation of nitrogen compounds is positive for ethylamine, lysine and cadaverine; negative for potassium nitrate, sodium nitrite, creatine, creatinine, glucosamine and imidazole. No growth in vitamin-free medium. Growth occurs at 30 °C, but not at 35 °C. Growth with 10 % NaCl and with 0.1 % cycloheximide is positive, but growth is absent with 16 % NaCl, on 50 % (w/w) glucose-yeast extract agar and with 1 % acetic acid. The diazonium blue B reaction is negative.

The type strain, UFMG POG a.1T (=ZIM 2311T=CBS 13383), was isolated from a flower of Siphocampylus corymbiferus collected at the São Sebastião do Ribeirão Grande farm in the Atlantic rainforest of the Pindamonhangaba municipal area in the State of São Paulo, Brazil, in June 2006. The MycoBank number is MB 807210.

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References


