**Hannaella pagnoccae** sp. nov., a tremellaceous yeast species isolated from plants and soil

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**Abbreviations:** ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domain and ITS region of the LSU rRNA gene of strain BI118T are KC169793 and FJ828959, respectively.

One supplementary figure is available with the online version of this paper.
The genus *Hannaella* was proposed to accommodate species that belong to the *Bullera sinensis* clade of the *Luteolus* lineage of the *Tremellales* based on sequences of the 18S rRNA gene and the mitochondrial cytochrome *b* gene. Analysis based on these sequences provided support to the monophyletic nature of the *B. sinensis* clade (Wang & Bai, 2008). This genus comprises, at the time of writing, seven recognized species, namely *Hannaella coprosmaensis*, *Hannaella kunmingensis*, *Hannaella luteola*, *Hannaella oryzae*, *Hannaella sinensis*, *Hannaella surugaensis* and *Hannaella zeae*. These species occur widely on plant leaf surfaces and are considered important phyllosphere-inhabiting yeasts (Wang & Bai, 2008; Boekhout et al., 2011). Despite the monophyletic nature of the genus, it has been demonstrated that *Hannaella kunmingensis* shows phenotypic and genotypic variability (Dayo-Owoyemi et al., 2013).

Scorzetti et al. (2002) reported the phylogenetic placement of a strain (CBS 8369) isolated from a flower of *Pimenta dioica* (Myrtaceae) in the *Bullera sinensis* clade, presently classified as a member of the genus *Hannaella*. Subsequently, independent yeast surveys in Rio Grande do Sul (Brazil), Minas Gerais (Brazil), Rio de Janeiro (Brazil), Tocantins (Brazil), Oklahoma (USA), Hsinchu (Taiwan) and Bangkok (Thailand) identified 31 additional yeast isolates (Table 1) with similar physiological profiles, and sequences of the D1/D2 domains and internal transcribed spacer (ITS) region of the large subunit (LSU) of the rRNA gene. Analysis of the sequences showed that these isolates belong to a novel species of the genus *Hannaella*, for which we propose the name *Hannaella pagnoccae* sp. nov.

Details of the localities and substrates of isolation are summarized in Table 1.

Strains BI118T, BI264 and BI334 were isolated from leaves of the bromeliads *Vriesea gigantea*, *Tillandsia geminiiflora* and *Vriesea friburgensis*, respectively. Samples were collected aseptically in May 2004, January 2005 and May 2007 in Itapuá Park, southern Brazil (approx. coordinates: 30° 22’ S 51° 04’ W). Isolation of these strains was achieved according to the method described by Landell et al. (2009, 2010). The samples were spread on YM agar (1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% agar, acidified to pH 4.0 with hydrochloric acid, and supplemented with 0.04% chloramphenicol) plates and incubated at 20–25 °C for up to 7 days.

Strains UFMG-F7C2, UFMG-F12C3, UFMG-F23C2 and UFMG-F23C3 were isolated from flower bracts of *Heliconia psittacorum* collected in the riparian forest of Formiga Falls, a Cerrado ecosystem in the Jalapão Region, Brazil (approx. coordinates: 10° 22’ S 46° 40’ W), in April 2009. Extraloral nectaries were gently scraped with a sterile inoculation loop and streaked on plates of YM agar supplemented with 0.02% chloramphenicol. Plates were incubated at room temperature (25 ± 3 °C) for 3–8 days (Barbosa et al., 2012).

Strains UFMG-ABT 30, UFMG-ABT 680 and UFMG-ABT 714 were isolated from the phytotelmata of *Encholirium* sp. (Bromeliaceae), and strain UFMG-ABT 625 from the phytotelmata of *Bromelia karatas* (Bromeliaceae). Strains UFMG-ABT 720, UFMG-ABT 729 and UFMG-ABT 750 were isolated from water inside rock holes. All these strains were collected in Aurora, south-eastern Tocantins State, Brazil (approx. coordinates: 12° 42’ S 46° 24’ W), in December 2011 and February 2012.

Strains UFMG-BRO 162 and UFMG-BRO 159 were isolated from the phytotelmata of *Vriesea minarum*, collected at the Serra da Piedade region, Caeté city, Minas Gerais State, Brazil (approx. coordinates: 19° 49’ S 43° 40’ W), in September 2008. Water samples were collected aseptically with a sterile pipette, transferred to sterile flasks and transported to the laboratory in ice for processing within 24 h. Aliquots of 0.1 ml of appropriate decimal dilutions were spread on YM agar supplemented with 0.02% chloramphenicol and 0.0033% rose Bengal. The plates were incubated at 25 °C for 3–8 days (Safar et al., 2013).

Strains IMUFRJ 51959, IMUFRJ 51960, IMUFRJ 51961, IMUFRJ 51962 and IMUFRJ 51981 were isolated from sugar cane (*Saccharum* spp.) in Brazil, leaves of *Arundinaria pusilla*, *Bromelia karatas* and *Vitis vinifera* in Thailand, soil samples in Taiwan, and prairie soil in the USA. Sequence analysis of the D1/D2 domains of the large subunit of the rRNA gene showed that the novel species differs from *Hannaella coprosmaensis* and *Hannaella oryzae* by 36 and 46 nt substitutions, respectively. A novel species is suggested to accommodate these isolates, for which the name *Hannaella pagnoccae* sp. nov. is proposed. The type strain is BI118T (=CBS 11142T=ATCC MYA-4530T).

Several independent surveys of yeasts associated with different plant materials and soil led to the proposal of a novel yeast species belonging to the *Tremellales* clade (*Agaricomycotina*, *Basidiomycota*). Analysis of the sequences of the D1/D2 domains and internal transcribed spacer region of the large subunit of the rRNA gene suggested affinity to a phylogenetic lineage that includes *Hannaella coprosmaensis*, *Hannaella oryzae* and *Hannaella sinensis*. Thirty-two isolates were obtained from different sources, including bromeliads, nectar of *Heliconia psittacorum* (Heliconiaceae), flowers of *Pimenta dioica* (Myrtaceae), roots and leaves of sugar cane (*Saccharum* spp.) in Brazil, leaves of *Cratoxylum maingayi*, *Arundinaria pusilla* and *Vitis vinifera* in Thailand, soil samples in Taiwan, and prairie soil in the USA. Sequence analysis of the D1/D2 domains of the large subunit of the rRNA gene showed that the novel species differs from *Hannaella coprosmaensis* and *Hannaella oryzae* by 36 and 46 nt substitutions, respectively. A novel species is suggested to accommodate these isolates, for which the name *Hannaella pagnoccae* sp. nov. is proposed. The type strain is BI118T (=CBS 11142T=ATCC MYA-4530T).
22° 45′ S 43° 40′ W). Samples were taken after 9 and 12 months of plant growth, respectively, in September 2007, during the dry season, and in January 2008, during the wet season, in the final stage of plant growth before flowering. Roots (5 g) or leaves (20 g) were added to 200 ml of sterile 0.9% NaCl, 0.02% Tween 80 and 20 g of 2–3 mm (irregular...
The yeasts were grown on YM agar at 24°C. Soil samples collected in Taiwan. Isolation of these strains was achieved according to Fungsin et al. (2006).

Strains TY-143, TY-148 and TY-176 were isolated from leaves of *Cratosylum maingai*, *Arundinaria pusilla* and *Vitis vinifera*, respectively, in Thailand. Isolation of these strains was achieved according to Fungsin et al. (2006).

Strains SJ14S03, SF3S09 and GE7S10 were isolated from soil samples collected in Taiwan. Isolation of these strains was according to the method described by Lee et al. (2008). The yeasts were grown on YM agar at 24 °C for 3 days.

Strains NFMay08DNXL-Y7, TGP907D-Y13, TGPJul08DX1-Y6 and TGP907AX-Y11 were isolated from soil samples collected in Oklahoma, USA. Isolation of these strains was according to the method of Vishniac (1985).

Strain CBS 8369 was isolated from a flower of *Pimenta dioica* (Myrtaceae) in Pau da Fome at Pedra Branca State Park, Rio de Janeiro, Brazil by G. Capriotti (Dipartimento di Biologia Vegetale dell'Università di Perugia Industrial Yeasts Collection, Italy). Details concerning the isolation of this strain are not available. DNA extraction, amplification and sequencing were performed according to Fell et al. (2000).

All yeast isolates were purified by repeated streaking on YM agar plates and preserved at −80 °C or in liquid nitrogen for later identification. The yeasts were characterized by standard methods (Kurtzman et al., 2011). Carbon and nitrogen assimilation assays were carried out in solid plates.

DNA from strains BI118T, BI264 and BI334 was extracted and purified according to Ramos et al. (2001). The divergent D1/D2 domain of the LSU rRNA gene was amplified with primers NL1 and NL4 (O'Donnell, 1993) and as described by Kurtzman & Robnett (1998). The ITS region (ITS1, 5.8S rRNA gene and ITS2) was amplified with primers ITS1 and ITS4 and sequenced as described by Pèter et al. (2009). The sequences of strains BI118T, BI264 and BI334 were obtained using Amersham MegaBACE 1000 automated sequencers using standard protocols at the facilities of the Brazilian Genome Network at the Center of Biotechnology, Chiotic UFRGS-RS and Instituto Nacional do Cancer, Rio de Janeiro.

DNA of the UFMG strains was amplified as described by Lachance et al. (1999). The sequences were sequenced using an ABI3130 capillary electrophoresis apparatus, using BigDye v3.1 and POP7 polymer.

DNA extraction, amplification and sequencing of strains IMUFRJ 51959, IMUFRJ 51960, IMUFRJ 51961, IMUFRJ 51962 and IMUFRJ 51981 employed procedures described by Ribeiro et al. (2011), while DNA manipulations of strains TY-143, TY-148 and TY-176 followed Fungsin et al. (2006), and those of strains SJ14S03, SF3S09 and GE7S10 followed Lee et al. (2008). For the Oklahoma strains NFMay08DNXL-Y7, TGP907D-Y13, TGPJul08DX1-Y6 and TGP907AX-Y11, DNA extraction, amplification and sequencing were performed according to Vishniac (1985).

The sequences obtained were compared with sequences deposited at the GenBank database with the BLAST tool (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990). The ITS and D1/D2 sequences were concatenated with the SequenceMatrix software. To estimate phylogenetic relationships, a neighbour-joining tree was generated that used the Kimura two-parameter model to correct for genetic distances (Kimura, 1980) with the MEGA software, version 5.1 (Tamura et al., 2011). Gaps were excluded from the analysis. The robustness of trees was calculated with 1000 bootstrap pseudoreplicates (Felsenstein, 1985). To test the reproducibility of the results, the Bayesian Markov chain Monte Carlo method of phylogenetic inference was applied, as implemented in the computer program MrBayes (Ronquist & Huelsenbeck, 2003). This method allows estimation of the a posteriori probability that groups of taxa are monophyletic given the DNA alignment (i.e. the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 100 000 generations using the general time-reversible model (six rate classes) of DNA substitution, additionally assuming a portion of invariable sites with gamma-distributed substitution rates of the remaining sites (GTR + I + G), random starting trees and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations, which resulted in an overall sampling of 10 000 trees. From those trees that were sampled after the process had reached a stationary stage (burnin + 2000), a consensus tree was computed to obtain estimates for the a posteriori probabilities. All sequences generated in the present study were deposited at GenBank and accession numbers are shown in Table 1.

Sequence comparisons of the ITS region and the D1/D2 domains of the LSU rRNA gene indicated that all strains belong to a novel yeast species within the genus *Hannaella*. Pairwise comparisons between the sequences demonstrated that all the strains shared more than 99% similarity in the D1/D2 region (0–3 substitutions and 0–2 indels) and between 98 and 100% in the ITS region (0–7 substitutions and 0–3 indels). The only exception was the comparison between the ITS sequence of strains UFMG-ABT 680 and UFMG-F12C3, which presented nine substitutions and four indels. Nevertheless, all of them shared more than 99% similarity with strain CBS 11142T (= BI118T) in both D1/D2 (0–3 substitutions and 0–2 indels) and ITS (0–5 substitutions and 0–1 indels) regions. Intraspecific variation within members of *Hannaella kunmingensis* has been recently reported (Dayo-owoymeni et al., 2013), and strains of *Rhodospiridium toruloides* were considered conspecific despite showing 94.8–97.8% ITS sequence similarity (Hamamoto et al., 2002). The ITS region has been proposed as the barcode for fungi due to its superior resolving power for species discrimination, and the more clearly defined
barcode gap between interspecific and intraspecific variation (Schoch et al., 2012). Interspecific ITS similarity between Hannaella pagnoccae sp. nov. and other species of the genus was 87–94%, and therefore we considered all the strains analysed in the present study to be conspecific despite some variations in the ITS sequences.

The phylogenetic placement of Hannaella pagnoccae sp. nov. is in agreement with the placement of strain CBS 8369 within the Hannaella (Bullera) sinensis clade as previously reported by Scorzetti et al. (2002) (Fig. 1). The Bayesian consensus tree obtained from the ITS and D1/D2 concatenated dataset (Fig. S1, available in the online Supplementary Material) is congruent with the neighbour-joining tree (Fig. 1). Tree probabilities from the Bayesian analysis were calculated from credible sets of trees (887 trees sampled), where: the 50% credible set contained 68 trees, the 90% credible set contained 647 trees, the 95% credible set contained 68 trees, and the 100% credible set contained 65 trees.

**Fig. 1.** Phylogenetic placement of Hannaella pagnoccae sp. nov. obtained by neighbour-joining (Kimura two-parameter distance method) analysis of the combined ITS and D1/D2 nucleotide datasets. Bootstrap values higher than 50% are shown (1000 replicates). Bar, 0.02 substitutions per nucleotide position.
credibly set contained 767 trees and the 99% credible set contained 863 trees. Three principal groups could be identified in the neighbour-joining and the Bayesian consensus trees: the Dioszegia and Derxomyces groups and a third group composed of species in the genus Hannaella, including Hannaella pagnoccae sp. nov.

Hannaella pagnoccae sp. nov. was isolated from distant locations with similar tropical to subtropical climates, associated with different plants and soil (Table 1). Most strains were associated with plant substrates, except the isolates from soil in Oklahoma and in Taiwan. Hannaella strains were associated with plant substrates, except the isolates from soil in Oklahoma and in Taiwan. Hannaella pagnoccae sp. nov. was isolated from distant locations with similar tropical to subtropical climates, associated with different plants and soil (Table 1). Most strains were associated with plant substrates, except the isolates from soil in Oklahoma and in Taiwan.

The novel species can be distinguished from all known members of the genus Hannaella, except for Hannaella coprosmaensis, based on its ability to grow in vitamin-free medium and from Hannaella coprosmaensis and Hannaella luteola by growth at 30 °C. The other species (Hannaella coprosmaensis, Hannaella kunmingensis, Hannaella luteola, Hannaella oryzae, Hannaella sinensis, Hannaella surugaensis and Hannaella zeae) related to Hannaella pagnoccae sp. nov. can be distinguished from the novel species based on their ability to grow on vitamin-free medium and from starch formation.

Table 2. Differential physiological and biochemical characteristics between Hannaella pagnoccae sp. nov. and its phylogenetically closest relatives

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*Strain numbers tested for physiological profiles were: BI1187; UFMG-BRO 159; UFMG-ABT 680; UFMG-ABT 729; UFMG-ABT 750; UFMG-F7C2; UFMG-F12C3; UFMG-F23C2; UFMG-F23C3; all IMUFRJ strains; TY-143; TY-148; TY-176; GE7S10; NFMay08DNX1 Y7; TGP907D-Y13; TGPJul08DX-1-Y6; TGP907AX-Y11.

Description of Hannaella pagnoccae sp. nov.

Hannaella pagnoccae (pag.noc’cae. N.L. gen. n. pagnoccae named in honour of Fernando Carlos Pagnocca, Universidade Estadual Paulista Júlio de Mesquita Filho, UNESP, who dedicated his life to the study of yeast ecology and taxonomy). In PDA broth after 5 days at 25 °C, cells are globose to ovoid and occur singly or in pairs (3.5–7.1 × 2.0–5.3 μm) (Fig. 2) with a surrounding capsule. On YM agar after 3 days at 25 °C, colonies are smooth, mucous to butyrous, glistening...
and cream-coloured with an entire margin. After 3 weeks in Dalmau plate culture on cornmeal agar, pseudohyphae and true hyphae are not formed. Sexual reproduction is not observed. Ballistoconidia production is variable. Fermentation ability is negative. Assimilates the following carbon compounds: D-glucose, inulin (variably), sucrose, raffinose, melibiose, galactose, lactose (variably), trehalose, maltose, melezitose, methyl α-D-glucopyranoside, soluble starch (variably), cellobiose, L-rhamnose, xylose, L-arabinose, D-arabinose, ribose, glycerol (variably), meso-erythritol, ribitol, D-galactitol, mannitol, glucitol, inositol, L-arabinonitol, xyitol, lactate (variably), succinic acid, citrate (variably), D-gluconic acid, D-glucono-1,5-lactone, N-acetylglucosamine, Tween 20 and Tween 80; no growth on D-gluconic acid, D-glucosamine. Assimilates the following nitrogen compounds: sodium nitrite (variably), cadaverine, ethylamine and lysine; no growth on creatine, creatinine, D-glucosamine. Growth at 30 °C is positive but not at 37 °C. Growth on vitamin-free medium is positive. No growth is observed on glucose-yeast extract-peptone with 10 or 16% NaCl. Growth on 0.01% cycloheximide is positive but not on 0.1%. Production of starch-like compounds is variable. Urease activity is positive. Diazonium blue B reaction is positive.

The type strain, BI118<sup>T</sup> (=CBS 11142<sup>T</sup> = ATCC MYA-4530<sup>T</sup>), was isolated from leaves of the bromeliad Vriesea gigantea. The Mycobank number is MB805548.

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References


