**Hannaella dianchiensis** sp. nov., a basidiomycetous yeast species isolated from lake water

Long Han,† Zhi-Ying Li,† Xiao-Fang Guo, Jin-Lian Tan, Shu-Zhuang He, Xiao-Long Cui, and Shao-Lan Li

**Abstract**

Three strains (YIM-HL1107<sup>T</sup>, YIM-HL1045, YIM-HL1112) representing a novel yeast species were isolated from surface water samples collected from the Caohai region of Dianchi Lake in Yunnan, south-western China. On the basis of morphological, physiological and biochemical characteristics and sequence analysis of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region, they were assigned to a novel species of the genus *Hannaella*. The closest relative to the novel species was *Hannaella pagonoccaea*, but it showed 6.3% nucleotide differences (34 nt substitutions out of 541 nt) in the D1/D2 region of the LSU rRNA gene and 9.3–9.6% nucleotide differences (40–41 substitutions and 7–8 gaps out of 430 nt) in the ITS region. The name *Hannaella dianchiensis* sp. nov. is proposed. The type strain is YIM-HL1107<sup>T</sup> (=CCTCC AY 2015009<sup>T</sup>), and the MycoBank number is MB 816297.

**METHODS**

Strains YIM-HL1107<sup>T</sup>, YIM-HL1045 and YIM-HL1112 were isolated from surface water samples collected from three sites (site codes: DCA01, DCA02 and DCA03) in the northern area (Caohai) of Dianchi Lake (25° 1.167′ N 102° 39.934′ E, 25° 0.304′ N 102° 38.807′ E, 24° 58.871′ N 102° 38.529′ E, respectively) on 28 November 2014, on the Yunnan plateau, south-western China. Dianchi Lake is the sixth largest freshwater lake (approximately 309 km<sup>2</sup>) in China, and its average water depth and altitude are 4.4 and 1886 m, respectively. As the lake is contiguous to Kunming city, its water is seriously affected by human activities [10]. Therefore, it is also regarded as one of the three most eutrophic lakes in China [11]. Samples were collected from the subsurface at a depth of approximately 30 cm, stored in sterile buckets and processed as soon as possible within 24 h in the laboratory. Each 20 ml water sample was filtered through one sterile Millipore nitrocellulose membrane (0.45 µm pore size, 47 mm in diameter) with a sterilized filtration system. Ten filtered membranes from each site were placed on the surfaces of the acidified YM agar plates respectively (yeast extract, 3.0 g; malt extract, 3.0 g; peptone, 5.0 g; glucose, 10.0 g; 1 M HCl, 7 ml; distilled water, 1000 ml; pH 3.8), and incubated at 25 °C for up to 1 week.

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**Keywords:** Dianchi Lake; yeast; *Hannaella*; phylogenetic analysis; polyphasic taxonomy.

**Abbreviation:** ITS, internal transcribed spacer.

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The GenBank/EMBL/DDBJ accession numbers for the LSU D1/D2 and ITS sequences of strains YIM-HL1107<sup>T</sup>, YIM-HL1045 and YIM-HL1112 are KT944113/KT962991, KX758403/KX758406 and KX758404/KX758405, respectively.

One supplementary table is available with the online Supplementary Material.
The yeasts were chosen for isolation based on colony morphology, and purified by repeated streaking on YM agar plates. Purified yeast strains were maintained in CRYO-BANK tubes (Mast Group) at −20 °C.

The genomic DNA was extracted using Lysis Buffer for Microorganism to Direct PCR [TaKaRa Biotechnology (Dalian)] according to the manufacturer’s instructions. The D1/D2 region of the LSU rRNA gene was amplified by PCR with the primer set NL-1 and NL-4 [12]; the ITS region including the 5.8S rRNA gene was amplified by PCR with the primer set ITS-1 and ITS-4 [13]. The PCR products were checked by agarose gel electrophoresis and purified with the Mag-MK PCR Products Purification Kit (Sangon Biotech). The purified PCR products were sequenced by using an automated DNA sequencer (ABI 3730XL; Thermo Fisher Scientific) with the same primer sets as sequencing primers. The sequences were compared pairwise using a BLAST N search [14] and aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL X, version 2.0 [15]. Phylogenetic trees were reconstructed using the neighbour-joining method with the same primer sets as sequencing primers. The sequences were compared pairwise using a BLAST N search [14] and aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL X, version 2.0 [15]. Phylogenetic trees were reconstructed using the neighbour-joining method with the same primer sets as sequencing primers. Bootstrap analysis with 1000 resamplings [19] was used to evaluate tree topology.

The three strains were characterized morphologically, physiologically and biochemically according to the standard methods described by Kurtzman et al. [20]. Assimilation of sole carbon sources was tested using liquid yeast nitrogen base (BD Biosciences) medium with one carbon compound

Fig. 1. Phylogenetic tree, based on the combined sequences of the D1/D2 region of the LSU rRNA gene and the ITS region, showing the positions of strains of *H. dianchiensis* sp. nov. with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura’s two-parameter correction [18], using the neighbour-joining method and MEGA version 6.0. Numbers at nodes indicate percentages of bootstrap support derived from 1000 replicates; bootstrap percentages higher than 50% are shown. *Bulleromyces albus* CBS 501T was the outgroup. Bar, 0.01 substitutions per nucleotide position (K_{nu}).
added separately. The method used for nitrogen assimilation tests was similar to that described for carbon assimilation tests, using liquid media, with yeast carbon base (Sigma-Aldrich) instead of yeast nitrogen base. Formation of pseudohyphae and true hyphae was examined on Dalmau plates consisting of cornmeal agar at 25°C for up to 21 days. Growth at various temperatures (5, 10, 20, 25, 30 and 35°C) was determined by cultivation in YM broth [20]. Ballistococnidia formation was investigated on cornmeal agar at 25°C for up to 30 days [20, 21]. Microscopic observations of the yeast cells grown in YM broth were performed using a light microscope (Leica DM2000).

RESULTS AND DISCUSSION

The sequences of the D1/D2 region of the LSU rRNA gene of the three strains (YIM-HL1107^T, YIM-HL1045, YIM-HL1112) were identical. The nucleotide sequences of the ITS region of the three strains differed slightly from each other by up to 1 nt and one gap. Pairwise sequence alignments showed that the three strains were closely related to *H. pagnoccae* CBS 11142^T (D1/D2 LSU rRNA=FJ828959, ITS=KC169793) with a sequence similarity of 94.1%, but showed 6.3% nucleotide differences (34 nt substitutions out of 541 nt) in the D1/D2 region of the LSU rRNA gene and 9.3–9.6% nucleotide differences (40–41 substitutions and 7–8 gaps out of 430 nt) in the ITS region.

Phylogenetic analysis of the combined nucleotide sequences of the D1/D2 and ITS regions further revealed that the three strains were placed in the *Hannaella* clade, and represented a novel species of the genus *Hannaella*. It grouped together with its closest relative, *H. pagnoccae*, with a high bootstrap support (99%; Fig. 1). The BLAST search results of the D1/D2 region sequence of strain YIM-HL1107^T showed an undescribed *Cryptococcus* sp. NBRC 105034 (GenBank: AB462340) and a *Cryptococcus* sp. LCF-20 (GenBank: HQ623516) to be even more closely related to strain YIM-HL1107^T (based on 98% sequence similarity in the D1/D2 region) than *H. pagnoccae*. Similarly, the undescribed *Cryptococcus* sp. LCF-20 was also the closest relative of strain YIM-HL1107^T in the BLAST search results of the ITS region (based on 98% sequence similarity) (Fig. 1). *Cryptococcus* sp. LCF-20 may represent another undescribed novel species belonging to the genus *Hannaella*.

Members of the genus *Hannaella* were reported to exhibit high intraspecific sequence variation, but high interspecific

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**Table 1.** Physiological characteristics that differentiate *Hannaella dianchiensis* sp. nov. from known species in the genus *Hannaella*

Species: 1, *H. dianchiensis* sp. nov.; 2, *H. pagnoccae*; 3, *H. sinensis*; 4, *H. kunmingensis*; 5, *H. phyllophila*; 6, *H. luteolus*; 7, *H. phethubunensis*; 8, *H. zeae*; 9, *H. siamensis*; 10, *H. surugaensis*; 11, *H. coprosmaeensis*; 12, *H. oryzae*. Data for species 2–12 were taken from Surussawadee et al. [8], Kaewwichian et al. [7], Landell et al. [6], Bai et al. [24], Fonseca et al. [9], Molnár and Prillinger [25] and Nagahama et al. [22]. +, Positive; −, negative; w, weak; v, variable; d, delayed positive; ND, no data.

<table>
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<td>ND</td>
<td>ND</td>
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<td>Growth with 50 % (w/v) glucose</td>
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<td>−</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>Production of starch-like compounds</td>
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**Fig. 2.** A micrograph of budding cells of *Hannaella dianchiensis* sp. nov. YIM-HL1107^T grown in YM broth for 2 days at 25°C. Bar, 10 µm.
sequence variation was found only in the ITS region, not in the D1/D2 region of the LSU rRNA gene [6, 8, 22, 23]. A comparison of the phylogenetic tree based only on the D1/D2 region sequences with that based on the ITS region sequences revealed that the phylogenetic positions of strain YIM-HL1107\(^T\) in the **Hannaella** clade were different (data not shown). However, the differences between the three strains in this study and the type strain of *H. pagnoccae*, CBS 11142\(^T\), were considered sufficient to recognize them as representing two species (sequence dissimilarity 5.9\%) (Fig. 1).

Cells of the three strains in YM broth were ellipsoidal to long-ovoid, occurred singly or in parent–bud pairs and proliferated by polar budding (Fig. 2). Colonies on YM agar were convex, smooth, glistening, mucoid, white and with entire margins. Pseudohyphae and hyphae were not formed on Dalmau plates consisting of cornmeal agar at 25°C for up to 3 weeks. Ballistoconidia were not formed on cornmeal agar at 25°C after 1 month. The physiological and biochemical characteristics of strain YIM-HL1107\(^T\) were compared with the relevant properties of known species in the genus **Hannaella** (Table 1). The ability to utilize a wide variety of carbon sources was a distinctive trait of this genus. **Hannaella dianchiensis** YIM-HL1107\(^T\) differed from the closely related *H. pagnoccae* by the ability to assimilate L-sorbose and salicin, and the inability to assimilate nitrite and L-lysine. Moreover, although the conventional growth temperature range of most mesophilic yeasts is between 20 and 30°C, the three novel strains grew well at 5°C and between 20 and 30°C, and no growth was observed at 35°C. The traits, such as the spectrum of nitrogen and carbon sources, resistance to high osmotic pressures and necessity of vitamins, varied among species of the genus (Table 1). Additionally, a distinguishing cultivation characteristic of the genus, namely a white (or almost white) mucoid fluidic yeast culture in a potato-dextrose agar slant [3], which differs from the closely related genera *Dexromyces* and *Dioszegia*, was also observed during cultivation of the novel strains under the same conditions. A comparison between strain YIM-HL1107\(^T\) and the two other novel strains showed that most of their features are similar except the ability to utilize carbon sources (Table S1, available with the online Supplementary Material).

On the basis of the analysis of the combined sequences of the ITS region and the D1/D2 domains of the LSU rRNA gene and various morphological, physiological and biochemical characteristics, we therefore concluded that the three strains represent a novel species within the genus **Hannaella**, for which we propose the name **Hannaella dianchiensis** sp. nov. In practice, *H. dianchiensis* can be distinguished from its closest relative *H. pagnoccae* not only on the basis of sequences of the D1/D2 region of the LSU rRNA gene and the ITS region, but also by several phenotypic characteristics (Table 1).

**DESCRIPTION OF HANNAELLA DIANCHIENSIS**

**HAN, LI, GUO, TAN, HE, CUI AND LI SP. NOV.**

**Hannaella dianchiensis** (dian.chi.en’sis. N.L. fem. adj. *dianchiensis* pertaining to Dianchi Lake, where the type strain was isolated).

In YM broth after 3 days at 25°C, cells are ellipsoidal to long-ovoid (5–7 × 3–5µm) and occur singly or in parent–bud pairs (Fig. 2). Budding is polar. On YM agar after 3 days at 25°C, colonies are convex (1.0–1.5 mm in diameter), smooth, glistening, mucoid, white and with entire margins. After 1 month in YM broth, sediment is formed, and no pellicle is observed. On Dalmau plates consisting of cornmeal agar, pseudohyphae and true hyphae are not formed. Ballistoconidia are not formed on cornmeal agar. Fermentation is not observed. The following carbon sources are assimilated: glucose, inulin (weakly), sucrose, raffinose, melibiose, galactose, trehalose, maltose, melezitose, methyl α-D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, L-ribose, D-glucose, D-fructose, inositol, sucrose, L-lysine, gluconolactone and 2 keto-D-gluconate, but not methanol, ethanol, lactose, galactitol, DL-lactate, D-glucosamine and N-acetyl-D-glucosamine are not assimilated. The nitrogen compounds ethylamine and cadaverine are assimilated, but nitrate, nitrite and L-lysine are not. Vitamins are not necessary for growth. The diazonium blue B reaction and urease activity are positive. Extracellular starch-like compounds are not produced. Growth is observed on culture agar with 50% glucose, but not on a medium with 10% (w/v) NaCl plus 5% (w/v) glucose or with 60% glucose. Growth is not inhibited in the presence of 0.01% (w/v) cycloheximide, but no growth occurs in the presence of 0.1% (w/v) cycloheximide. Growth is observed between 5 and 30°C, but not at 35°C.

Strain YIM-HL1107\(^T\) is the holotype of **Hannaella dianchiensis**. The strain was isolated from surface water samples collected from the northern area (Caohai region) of Dianchi Lake in Kunming, Yunnan, south-western China. The holotype strain permanently preserved in a metabolically inactive state by lyophilization was deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, as CBS 14191\(^T\) and in the China Centre for Type Culture Collection (CCTCC), Wuhan, China, as CCTCC AY 2015009\(^T\). The MycoBank number is MB 816297.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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