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Hannaella oleicumulans sp. nov. and *Hannaella higashiohmiensis* sp. nov., two novel oleaginous basidiomycetous yeast species

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Abstract

Three strains of novel oleaginous yeast species were isolated from soil samples collected in Shiga Prefecture, Japan. The sequences of the internal transcribed spacer (ITS) region and the D1/D2 region of the large subunit (LSU) of the rRNA genes indicated that these novel yeast species are members of the genus *Hannaella*. The results of molecular phylogenetic analysis indicated that strains 38–3 and 8s1 were closely related to *Hannaella oryzae*. They differed by 10 nucleotide substitutions and one gap (1.77%) in the D1/D2 region of the LSU of the rRNA genes and by 17–18 nucleotide substitutions and 10–11 gaps (5.45–5.85%) in the ITS region. Strain 51–4 differed from the type strain of the most closely related species, *Hannaella pagnoccae*, by 26 nucleotide substitutions (4.46%) in the D1/D2 region of the LSU of the rRNA genes and by 20 nucleotide substitutions and six gaps (5.42%) in the ITS region. The names proposed for these previously undescribed species are *Hannaella oleicumulans* sp. nov. and *Hannaella higashiohmiensis* sp. nov.

The genus *Hannaella* was established by F.Y. Bai and Q.M. Wang in 2008 for members of the Tremellales, with *Hannaella sinensis* as the type species [1]. Closely related genera include *Bulleribasidium*, *Derxomyces*, *Dioszegia*, *Nielozyma* and *Vishniacozyma*, forming the *Bulleribasidiaceae* clade [2, 3]. A clade of species formerly classified as members of the genus *Cryptococcus* has been previously placed in the genus *Hannaella*, including *Hannaella luteola*, *Hannaella surugaensis*, *Hannaella taiwanensis* and so on [1, 4, 5]. At the time of writing, the genus contains 15 species [1, 5–10].

Oleaginous yeasts can accumulate lipids to more than 20% of their dry cell mass [11]. The lipids are similar in composition to the vegetable oils used in biodiesel production and are becoming an attractive alternative to conventional petroleum diesel fuel [12]. Oleaginous yeast strains represent broad taxonomic diversity; however, many strains are members of the genera *Rhodotorula*, *Cutaneotrichosporon* and *Lipomyces* [12, 13]. Thus far, about 160 species have been reported to be oleaginous [12].

During a study of soil yeast diversity in Shiga Prefecture, Japan, three strains of unidentified basidiomycetous yeasts were isolated. On the basis of the results of morphological and physiological tests and phylogenetic analyses reconstructed with the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, it was determined that these isolates represent two kinds of novel yeast species. Here we describe these species as *Hannaella oleicumulans* sp. nov. and *Hannaella higashiohmiensis* sp. nov.

METHODS

Yeast isolation

The three yeast strains studied were collected in May to June 2021 from three sites in Shiga prefecture: at the roadside station Kutsuki Shin-Honjin (35.35111° N, 135.91555° E), at Ryukoku University (34.96390° N, 135.94065° E) and at Yukinoyama

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Keywords: Basidiomycota; *Hannaella*; oleaginous yeast; phylogeny; taxonomy.
Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.
The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU of the rRNA genes of strains 38-3, 51-4 and 8s1 are LC672604, LC672605 and LC672606, respectively. Those for the internal transcribed spacer region are LC672607, LC672608 and LC672609, respectively.
Two supplementary figures are available with the online version of this article.
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Rekishi Park (35.08250° N, 136.14662° E). Each sample (approximately 1 g) was suspended in 10 ml sterilized distilled water and diluted to 10^{-1} in sterilized distilled water. Then, 100 µl aliquots were spread on dichloran rose bengal chloramphenicol agar [14]. The plates were incubated aerobically at 15 °C for 1 week. The yeast colonies were selected and purified on YM agar (Difco) supplemented with 0.001% chloramphenicol.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the LSU rRNA gene and the ITS region were amplified by PCR using KOD FX Neo (Toyobo). Amplification of the D1/D2 region of the LSU rRNA gene was performed by PCR with the forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG) [15]. The amplification of the ITS region was performed with forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC) [16]. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The purified products were submitted to Takara Bio for Sanger dideoxy sequencing with a 3730xl DNA Analyzer (Applied Biosystems). The sequences were assembled and aligned with the MEGA 11 programme [17], which was also used to reconstruct phylogenetic trees. Phylogenetic analyses using the D1/D2 region of the LSU rRNA gene and the ITS region were conducted using neighbor-joining analysis [18]. Bootstrap values were obtained from 1000 replications. Gaps were completely deleted. Comparisons with sequences from the GenBank database (www.ncbi.nlm.nih.gov) were performed using the BLASTN search programme [19]. The DNA sequences have been deposited at GenBank/EMBL/DDBJ under accession numbers LC672604–LC672606 (D1/D2 region) and LC672607–LC672609 (ITS region).

Measurement of fatty acids

To evaluate lipid-accumulating ability, strains of the novel species were cultivated in 500 ml Erlenmeyer flasks containing 100 ml SS2 medium [3% glucose, 0.5% ammonium sulphate, 0.05% magnesium sulphate, 0.01% sodium chloride, 0.01% calcium chloride and 0.01% yeast extract (Difco)] at 25 °C on a rotary shaker at 150 r.p.m. Cells from 5 ml of culture broth were harvested after 10 days of cultivation. Lipid extraction, methylation and gas chromatography analyses were carried out as described previously [20].

Phenotypic characterization

Physiological properties and morphological characteristics were examined by standard methods as described by Kurtzman *et al.* [21].

RESULTS AND DISCUSSION

Physiological characterization

Strains 38–3 (NBRC 115584=CBS 18152), 8s1 (NBRC 115586^T = CBS 18154) and 51–4 (NBRC 115585^T = CBS 18153) all formed cream-coloured, butyrous colonies with entire margins on YM agar medium at 25 °C. NBRC 115585^T had glistening and flat colonies, whereas the colonies of NBRC 115584 and NBRC 115586^T were smooth and had slightly depressed centres. NBRC 115585^T did not grow at temperatures above 30 °C, but NBRC 115584 and NBRC 115586^T grew at up to 35 °C. Physiologically, NBRC 115584 and NBRC 115586^T could be differentiated from NBRC 115585^T on the basis of assimilation of lactose, melezitose, methyl α-D-glucoside, L-sorbose, erythritol, DL-lactate, succinate, citrate, D-gluconate, gluconolactone, D-glucosamine, hexadecane and sodium nitrite. Therefore, the three strains could be classified into two groups.

Molecular phylogenetic analyses

The results of neighbor-joining analysis [18] of the D1/D2 region of the LSU rRNA gene and the ITS region indicated that the three strains represented novel species belonging to the *Hannaella* clade (Fig. 1). NBRC 115584 and NBRC 115586^T differed from NBRC 115585^T by 7.14% (45 substitutions out of 630 nucleotides) and 12.73–12.93% (42 substitutions and 21–22 gaps out of 495 nucleotides) in the D1/D2 region of the LSU rRNA gene and the ITS region, respectively, which indicated that they probably represent different species. Yeasts that differ by more than 1% in either the D1/D2 region of the LSU rRNA gene or the ITS region should be regarded as separate species [22–24]. The sequences of the D1/D2 region of the LSU rRNA gene were identical between NBRC 115584 and NBRC 115586^T, and those of the ITS region differed from each other by one substitution and one gap; therefore, the two strains can be considered to represent the same species. Sequence alignments indicated that the two strains were closely related to *Hannaella oryzae* CBS 7194^T, but NBRC 115584 and NBRC 115586^T each differed from *H. oryzae* CBS 7194^T by 1.77% (10 substitutions and one gap out of 621 nucleotides) in the D1/D2 region of the LSU rRNA gene and by 5.45–5.85% (17–18 substitutions and 10–11 gaps out of 495–496 nucleotides) in the ITS region. Similarly, NBRC 115585^T was most closely related to the type strain of *Hannaella pagnoccae* (CBS 11142^T) but was quite distant from the type strain in terms of nucleotide diversities, with the diversity being 4.46% (26 substitutions out of 583 nucleotides) in the D1/D2 region of the LSU rRNA gene and 5.42% (20 substitutions and six gaps out of 480 nucleotides)

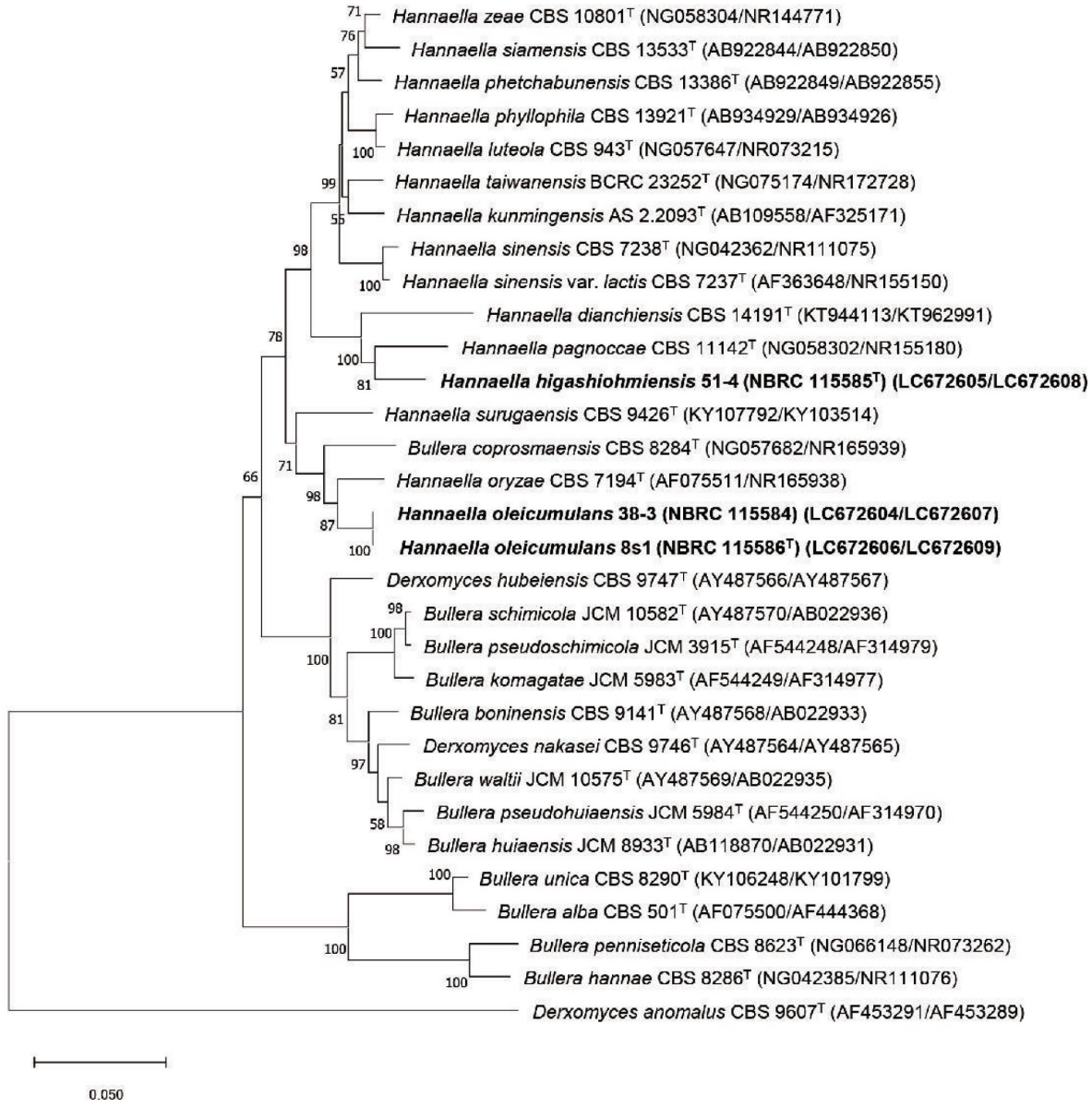

Fig. 1. Phylogenetic tree based on the concatenated sequence of the ITS region and the D1/D2 region of the LSU rRNA gene of *Hannaella oleicumulans*, *Hannaella higashiohmiensis* and closely related species. Bootstrap values higher than 50% are shown. The numbers in parentheses are GenBank accession numbers. *Derxomyces anomalus* CBS 9607^T was used as the outgroup in the analysis. Bar, 0.05 substitutions per nucleotide position.

in the ITS region. These comparisons indicated that strains NBRC 115584, NBRC 115585^T and NBRC 115586^T represent two novel species of the genus *Hannaella*, as they are phylogenetically distinct from other species of the genus *Hannaella*

Lipid-accumulating ability

NBRC 115584, NBRC 115585^T and NBRC 115586^T reached lipid contents (percentages of dry cell weight) of 37.72%, 24.37% and 36.47%, respectively (Fig. S1, available in the online version of this article). The results indicate that these novel species are oleaginous [11]. In terms of fatty acid composition, no great differences were observed between the three strains; the major fatty acids were oleic acid (C18:1), which ranged from 44.1 to 49.5%; palmitic acid (C16:0), from 19.7 to 20.9%; stearic acid (C18:0), from 12.9 to 16.7%; and linoleic acid (C18:2) from 12.1 to 13.5% (Fig. S2). A suitable fatty acid composition for biodiesel production consists of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids [25]. All three strains contained these fatty acids at high ratios, ranging from 96.5 to 98.2%. The fatty acids produced by the

selected strains can thus be considered suitable for biodiesel production. Collectively, the above results indicate that strains NBRC 115584, NBRC 115585^T and NBRC 115586^T can be characterized for the first time, to our knowledge, as oleaginous yeasts in the *Hannaella* clade.

Description of *Hannaella oleicumulans* Tanimura & Adachi sp. nov

Hannaella oleicumulans (o.le.i.cu'mu.lans. L. neut. n. *oleum* oil; L. pres. part. *cumulans* accumulating; N.L. part. adj. *oleicumulans* oil-accumulating).

After 3 days in YM broth at 25 °C, cells are ovoid to ellipsoid (3.1–5.4×3.8–7.0 µm) and occur singly, in pairs, or in groups (Fig. 2a). Budding is polar. Sediment and rings are formed. After 3 days of growth on YM agar at 25 °C, colonies are cream-coloured, butyrous, smooth and centrally slightly depressed, with entire margins. Pseudohyphae and hyphae are not observed on Dalmau plate culture on cornmeal agar at 25 °C. Ballistoconidia are not observed on cornmeal agar at 25 °C. No sexual spores are produced on YM agar and cornmeal agar after 4 weeks of incubation at 25 °C.

Fermentation is absent. The following are assimilated as sole carbon sources: glucose, sucrose, raffinose, galactitol, melibiose, D-xylose, galactose, lactose (positive or weakly), trehalose, maltose, cellobiose, ethanol, glycerol, soluble starch (latent), melezitose (weakly), methyl α-D-glucoside (weakly), ribitol, salicin (weakly), L-sorbose (latent), D-ribose (weakly), erythritol, *myo*-inositol, citrate (weakly), D-gluconate (weakly), D-glucosamine (latent), *N*-acetyl-D-glucosamine, hexadecane (weakly), L-rhamnose, L-arabinose, D-arabinose, D-glucitol and D-mannitol. Methanol, DL-lactate, succinate, propane-1,2-diol and gluconolactone are not assimilated as sole carbon sources. Potassium nitrate (weakly), sodium nitrite, ethylamine, L-lysine, cadaverine, imidazole (weakly), D-glucosamine (weakly), creatine (weakly) and creatinine (weakly) are assimilated as sole nitrogen sources. Extracellular starch-like compounds are not produced. Growth in vitamin-free medium is weakly positive. Growth is observed on culture agar with 10% (w/v) NaCl plus 5% (w/v) glucose, but not on a medium with 50% (w/v) glucose. Urease activity is positive. Growth in medium containing 0.1% cycloheximide is positive. Growth is observed at between 5 and 35 °C but not at 37 °C. Diazonium Blue B reaction is positive.

The holotype, NBRC 115586^T, is preserved in a metabolically inactive state at the NITE Biological Resource Centre (NBRC), Kisarazu, Chiba Prefecture, Japan. It was isolated from soil collected from the Seta Campus of Ryukoku University (34.96390° N, 135.94065° E), Otsu, Shiga Prefecture, Japan. Isotype cultures have been deposited at the CBS Yeast Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, as CBS 18154. The D1/D2 domains of the LSU rRNA gene and the ITS regions of strain NBRC 115586^T have been deposited in the GenBank/EMBL/DDBJ database under the accession numbers LC672606 and LC672609, respectively. The MycoBank number is MB 843207.

H. oleicumulans sp. nov. can be distinguished from the most closely related species, *H. oryzae*, not only by the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also by phenotypic characteristics. *H. oleicumulans* sp. nov. assimilates ethanol, glycerol, ribitol, L-sorbose (latent) and D-glucitol, while the type strain of *H. oryzae* does not. The novel species does not assimilate DL-lactate, succinate or gluconolactone but the type strain of *H. oryzae* does. Production of starch-like compounds is negative for the novel species but positive for the most closely related species. Formation of ballistoconidia is negative for *H. oleicumulans* sp. nov. but positive for the type strain of *H. oryzae*.

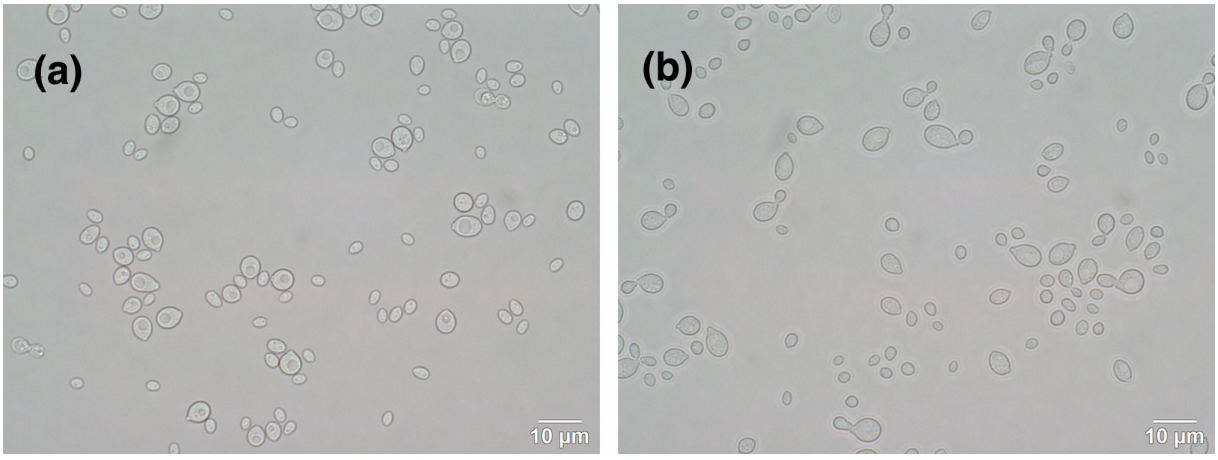

Fig. 2. Cells of *H. oleicumulans* NBRC 115586^T (a) and *H. higashiohmiensis* NBRC 115585^T (b). Optical microscopy: bars, 10 µm.

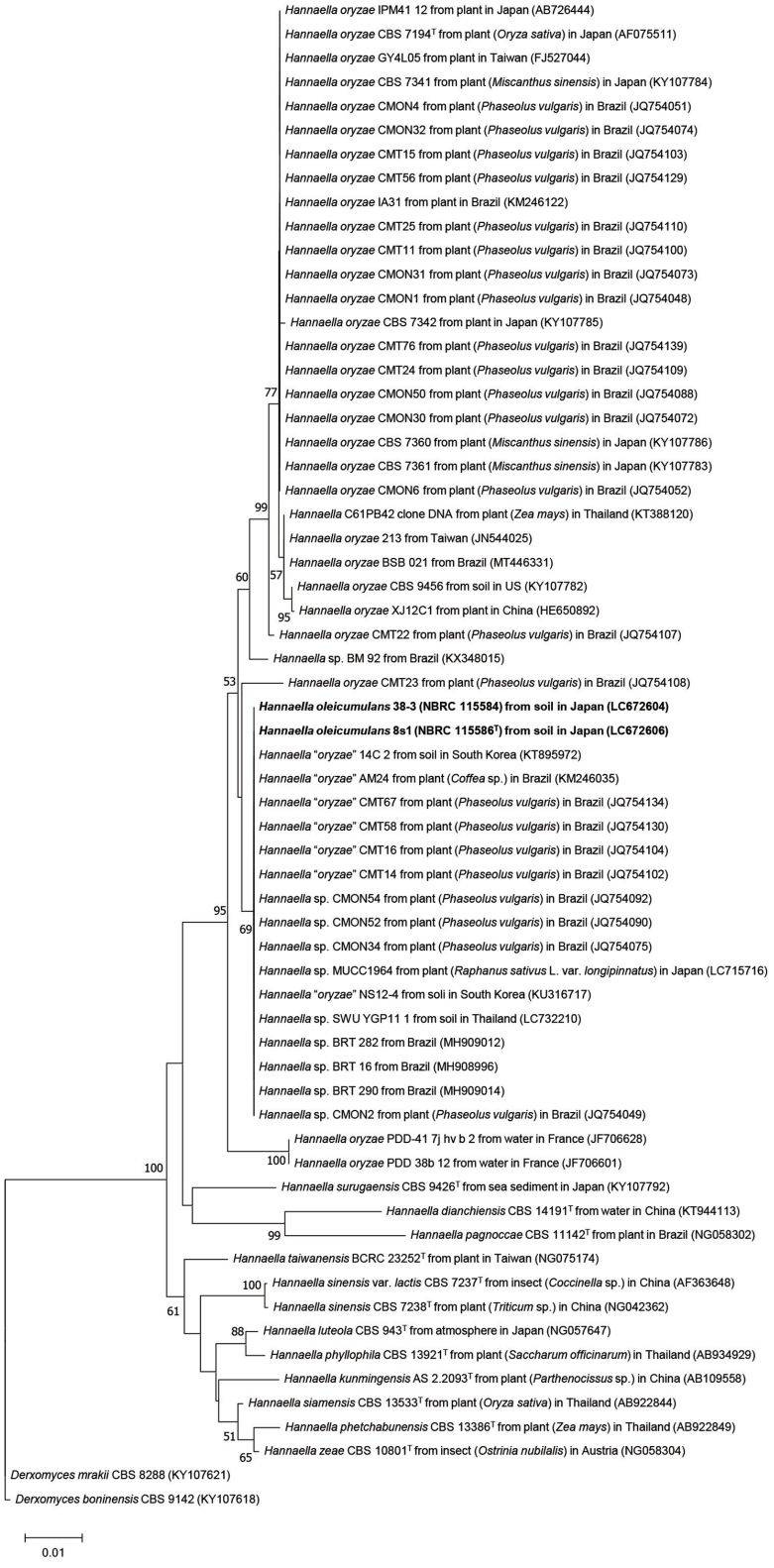

Fig. 3. Neighbor-joining tree based on the D1/D2 region of the LSU rRNA gene of *Hannaella oleicumulans*, its closely related strains (more than 97% nucleotide sequence identity) and the described species of the genus *Hannaella*. Bootstrap values higher than 50% are shown. *Derxomyces mrakii* CBS 8288 and *Derxomyces boninensis* CBS 9142 were used as the outgroup in the analyses. Bar, 0.01 nucleotide substitutions per nucleotide position.

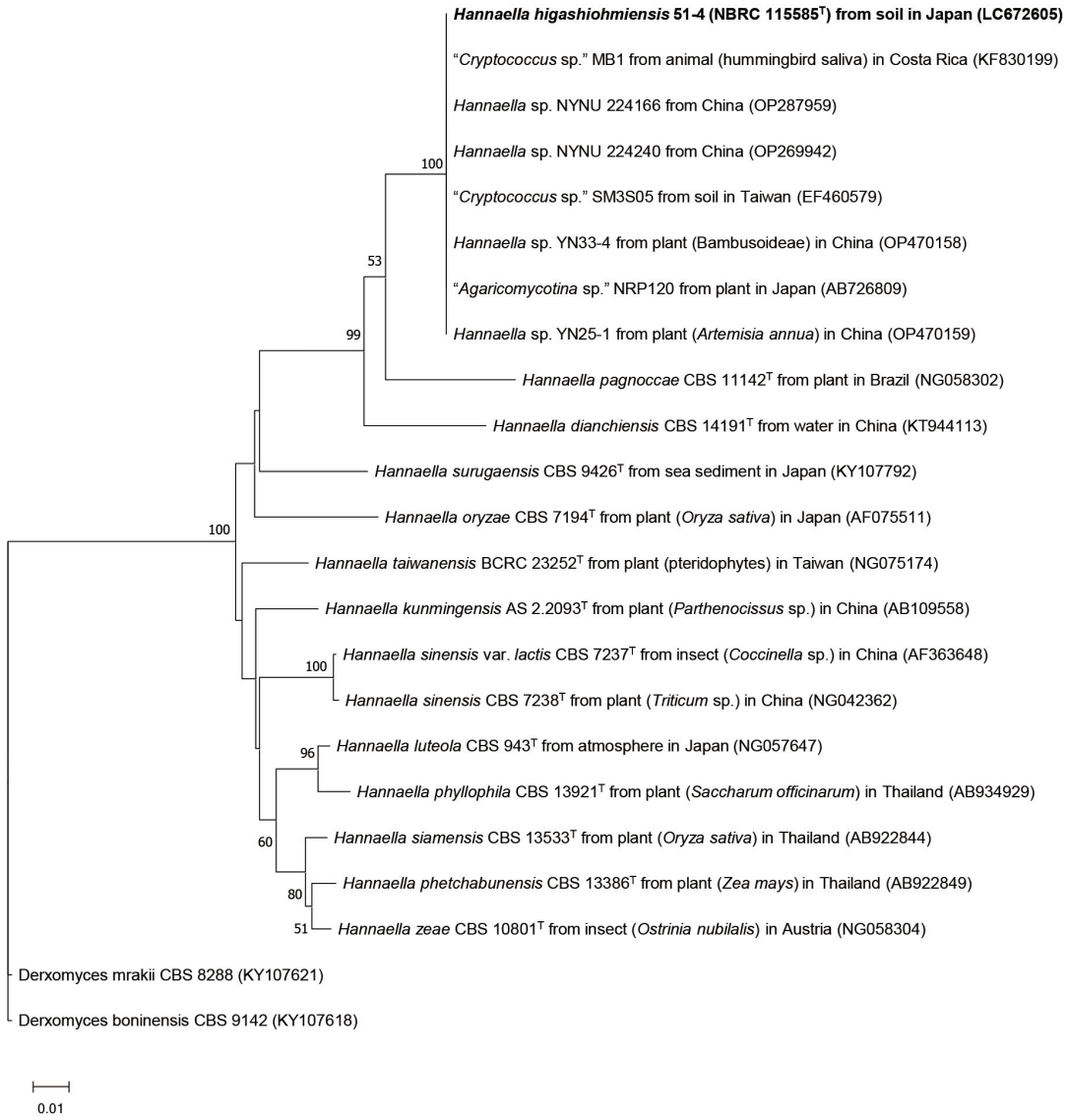

Fig. 4. Neighbor-joining tree based on the D1/D2 region of the LSU rRNA gene of *Hannaella higashiohmiensis*, its closely related strains (more than 97% nucleotide sequence identity) and the described species of the genus *Hannaella*. Bootstrap values higher than 50% are shown. *Derxomyces mrakii* CBS 8288 and *Derxomyces boninensis* CBS 9142 were used as the outgroup in the analyses. Bar, 0.01 nucleotide substitutions per nucleotide position.

Description of *Hannaella Higashiohmiensis* Adachi & Tanimura sp. nov

Hannaella higashiohmiensis (hi.ga.shi.ohmi.en'sis. N.L. fem. adj. *higashiohmiensis* referring to the city Higashiohmi in Shiga prefecture, Japan, where the type strain was isolated).

After 3 days in YM broth at 25 °C, cells are ovoid to ellipsoid (1.5–6.2×3.1–7.1 µm) and occur singly, in pairs, or in groups (Fig. 2b). Budding is polar. Sediment and rings are formed. After 3 days of growth on YM agar at 25 °C, colonies are cream-coloured, butyrous, glistening and flat, with entire margins. Pseudohyphae and hyphae are not observed on Dalmau plate culture on cornmeal agar at 25 °C. Ballistoconidia are not observed on cornmeal agar at 25 °C. No sexual spores are produced on YM agar and cornmeal agar after 4 weeks of incubation at 25 °C.

Fermentation is absent. The following are assimilated as sole carbon sources: glucose, sucrose, raffinose, galactitol, melibiose, D-xylose, galactose, trehalose, maltose, cellobiose, glycerol, DL-lactate (weakly or negative), soluble starch (latent), melezitose, methyl α-D-glucoside, D-mannitol, ribitol, salicin (weakly), L-sorbose, D-ribose, erythritol (weakly), *myo*-inositol, succinate, D-gluconate, gluconolactone, D-glucosamine, *N*-acetyl-D-glucosamine, L-rhamnose, L-arabinose, D-arabinose and D-glucitol. Methanol, ethanol, citrate, propane-1,2-diol and hexadecane are not assimilated as sole carbon sources. Potassium nitrate

(weakly), ethylamine, L-lysine, cadaverine, imidazole (weakly), D-glucosamine, creatine (weakly) and creatinine (weakly) are assimilated as sole nitrogen sources, but sodium nitrite is not. Extracellular starch-like compounds are not produced. Growth in vitamin-free medium is negative. Growth is observed on culture agar with 10% (w/v) NaCl plus 5% (w/v) glucose but not on a medium with 50% (w/v) glucose. Urease activity is positive. Growth in medium containing 0.1% cycloheximide is positive. Growth is observed at between 5 and 30 °C but not at 35 °C. Diazonium Blue B reaction is positive.

The holotype, NBRC 115585^T, is preserved in a metabolically inactive state at the NITE Biological Resource Centre (NBRC), Kisarazu, Chiba Prefecture, Japan. It was isolated from soil collected from Yukinoyama Rekishi Park (35.08250° N, 136.14662° E), Higashiohmi, Shiga Prefecture, Japan. Isotype cultures have been deposited at the CBS Yeast Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, as CBS 18153. The D1/D2 domains of the LSU rRNA gene and the ITS regions of strain NBRC 115585^T have been deposited in the GenBank/EMBL/DDBJ database under the accession numbers LC672605 and LC672608, respectively. The MycoBank number is MB 843208.

H. higashiohmiensis sp. nov. can be distinguished from its phylogenetically closely related species, *H. pagnoccae*, on the basis of the assimilation of L-sorbose, D-glucosamine, creatine and creatinine and growth in 0.1% cycloheximide, which is positive for *H. higashiohmiensis* sp. nov. and negative for *H. pagnoccae*. In addition, the novel species does not assimilate citrate or sodium nitrite, while *H. pagnoccae* does. Growth in vitamin-free medium is negative for the novel species but positive for the most closely related species.

Ecology

Described species of the genus *Hannaella* have been mainly found in phylloplanes (Figs 3 and 4). For example, *Hannaella siamensis* was isolated from rice leaves, composite flower and dead fallen leaves in Thailand and Belize, *Hannaella phetchabunensis* from corn leaves in Thailand [8] and *Hannaella kunmingensis*, *H. pagnoccae* and *Hannaella phyllophila* from plant leaves in PR China, Brazil and Thailand, respectively [6, 7, 9]. In other cases, *Hannaella dianchiensis* was isolated from lake surface water in PR China [10]. There are also many undescribed strains having sequences identical to strains NBRC 115584, NBRC 115585^T and NBRC 115586^T. These have been isolated mostly from samples associated with plant materials: '*Hannaella*' sp. CMON52 (JQ754090), '*Hannaella*' sp. MUCC1964 (LC715716), '*Hannaella*' sp. SWU-YGP11-1 (LC732210), '*Hannaella*' sp. BRT 290 (MH909014), '*Hannaella*' sp. NYNU 224166 (OP287959) and '*Cryptococcus*' sp. SM3S05 (EF460579). Strains NBRC 115584, NBRC 115585^T and NBRC 115586^T were isolated from soil samples collected in Japan. Species of the genus *Hannaella* were thus shown to have diverse habitats and oleaginicity in this study.

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Author contributions
Conceptualization, A.T.; Methodology, A.T.; Validation, K.T., J.O. and J.S.; Investigation, A.T.; Resources, A.T. and H.A.; Data Curation, A.T.; Writing – Original Draft Preparation, A.T.; Writing – Review & Editing, K.T., J.O., H.A. and J.S.; Visualization, A.T.; Supervision, J.S.; Project Administration, K.T., J.O. and J.S.; Funding Acquisition, K.T., J.O. and J.S.

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

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