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TAXONOMIC DESCRIPTION

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Zygotorulaspora chibaensis sp. nov. and *Zygotorulaspora danielsina* sp. nov., novel ascomycetous yeast species from tree bark and soil

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Abstract

Multiple isolates belonging to the ascomycetous genus *Zygotorulaspora* were obtained from forest soils and tree bark in Shiba Prefecture in Japan, and Lake Daniels, Lewis Pass, in New Zealand. Phylogenetic analyses employing combined sequences of the D1/D2 domain and ITS region support the recognition of two new species: *Zygotorulaspora chibaensis* sp. nov. (type strain PYCC 6970^T=CBS 15364^T) and *Zygotorulaspora danielsina* sp. nov. (type strain PYCC 6984^T=CBS 15365^T). Both species are able to grow on D-xylose and L-arabinose and at 35 °C, unlike *Zygotorulaspora florentina* and *Zygotorulaspora mrakii*, the other two species in the genus.

The genus *Zygotorulaspora* (Saccharomycetales, Saccharomycetaceae) was created in 2003 to accommodate two species, *Zygotorulaspora florentina* and *Zygotorulaspora mrakii* segregated from the genus *Zygosaccharomyces* based on a multigene phylogenetic analysis of the '*Saccharomyces* complex' [1]. As its name implies, *Zygotorulaspora* is phylogenetically related to *Zygosaccharomyces* and to *Torulaspora* [1–3]. In the latest revision of the genus, *Z. florentina* was represented by four strains that were found in Italy (fruits), Japan (*Rhododendron* species) and the USA (*Drosophila pseudoobscura*) [4]. For *Z. mrakii*, only two strains were known and both were isolated from silage in Italy [4]. It appears that strains (and species) of the genus *Zygotorulaspora* are not frequent or, alternatively, that the natural habitats of *Zygotorulaspora* have not yet been sampled, although a recent search in the database of the collection of the Westerdijk Fungal Biodiversity Institute (CBS) revealed that five additional strains of *Z. florentina,* isolated mainly from soft drinks, are known.

The isolation of yeasts of the genus *Saccharomyces* from natural samples normally involves an enrichment step that employs liquid medium with ethanol to inhibit other yeasts [5]. In some cases, especially if incubations are carried out at low temperatures (10 °C), some non-*Saccharomyces* yeast species can tolerate the selective conditions imposed by ethanol and multiply. Here we report on two new *Zygotorulaspora* species, isolated using the *Saccharomyces* enrichment protocol and low temperatures from tree bark and soil. *Zygotorulaspora chibaensis* sp. nov. was found in association with trees of the Fagaceae (*Castanopsis* and *Quercus*) in Chiba, Japan and *Zygotorulaspora danielsina* sp. nov., was found in association with *Nothofagus* (southern beech) near Lake Daniels, New Zealand.

Samples of tree bark and soil underneath trees were collected in oak forests in Chiba Prefecture, Japan, in June 2008 and in a *Nothofagus* forest near Lake Daniels, Lewis Pass, New Zealand in November 2009. Bark samples (3–6 cm^2) were collected aseptically and subsequently cut into small pieces. Similar amounts of bark or soil (~3 g) were introduced into 20 ml sterile flasks containing selective enrichment medium. The selective medium consisted of yeast nitrogen base (YNB; Difco) supplemented with 1 % (w/v) raffinose and 8 % (v/v) ethanol. The flasks were tightly capped and incubated at 10 °C without shaking. The flasks were surveyed periodically for turbidity and gas formation (indicative of fermentation), for 2 months. Samples exhibiting yeast growth after observation under the microscope were plated onto low pH (3.5) YMA (1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract, 2 % agar) and purified on YMA. Molecular identification relied on

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The GenBank/EMBL/DDBJ accession numbers of the LSU and ITS sequences of *Zygotorulaspora chibaensis* sp. nov. PYCC 6970^T and *Zygotorulaspora danielsina* sp. nov. PYCC 6984^T are MH055367, MH055358, MH055369 and MH055360, respectively. The MycoBank accession numbers are MB824884 and MB824885, respectively.
Two supplementary figures are available with the online version of this article.

rRNA gene sequencing as described before [6]. The phylogenetic analysis was based on a combined alignment of the ITS region, including the 5.8S rRNA gene, and the D1/D2 domains of the 26S rRNA gene (LSU), and was made with MEGA 7 [7] using the maximum-likelihood algorithm and Kimura's two-parameter model, as suggested by the implemented model test. Assimilation tests were performed in liquid media following standard procedures [8]. For microscopy, cultures were grown at 25 °C on YM agar or on V8 agar and studied with phase contrast optics.

The new species were found during ecological surveys primarily designed to isolate members of the genus *Saccharomyces*. Both new species were isolated in incubations carried out at 10 °C of soil and bark samples as shown in Table 1. The phylogenetic placement of the new isolates was based on a combined alignment of the D1/D2 domains of the 26S rRNA gene (LSU) and complete ITS region and is shown in Fig. 1. *Zygotorulaspora chibaensis* sp. nov. emerged as the closest relative of *Z. florentina* with eight nucleotide substitutions in the D1/D2 region and two–four substitutions in the ITS region. *Zygotorulaspora danielsina* sp. nov. was positioned as the closest relative of *Z. mrakii*, but with low bootstrap support, and with 24 nucleotide substitutions in the D1/D2 region and more than 50 substitutions in the ITS region. The separate phylogenetic trees of D1/D2 and ITS regions are shown in Figs S1 and S2 (available in the online version of this article), respectively. The phylogenetic tree of Fig. 1 revealed that besides the two novel species proposed here, another new *Zygotorulaspora* species is represented by D1/D2 and ITS sequences available in GenBank and that refer to strain MM1, a strain isolated from grapes in the Republic of Korea. BLAST searches revealed also additional *Zygotorulaspora* sequences but in those cases only D1/D2 (Fig. S1) or ITS (Fig. S2) sequences were available. Whereas the additional strains for which D1/D2 sequences are available appear to be conspecific with strain MM1, the additional strains for which ITS sequences are available appear to represent additional representatives of *Z. florentina*.

In our study, the same enrichment strategy allowed also the isolation of *Z. florentina* and of members of the sister genus *Torulaspora*, namely *T.* cf. *delbrueckii* and *T.* cf. *microellipsoides* recovered from tree bark of Fagaceae or soil underneath those trees in Japan (Fig. 1, Table 1). Whereas the molecular identification of the *Torulaspora* strains based on D1/D2 sequences offered no doubts (Fig. S1), the same did not happen with the ITS region (Fig. S2), since the new isolates had sequences that differed considerably (32–35 substitutions) from the sequences of the type strains. Moreover GenBank searches did not hold similar sequences. In accordance to these findings our identifications of the *Torulaspora* isolates should be viewed as tentative.

DESCRIPTION OF *ZYGOTORULASPORA CHIBAENSIS* SP. NOV. C. CARVALHO, A. TOMÁS AND J.P. SAMPAIO

Zygotorulaspora chibaensis (chi.ba.en′sis. N.L. fem. adj. *chibaensis* refers to Shiba Prefecture, Japan, the locality where the strains were isolated).

After 1 week on YM agar at 25 °C cultures are cream-coloured and butyrous. After 3 days of growth on YM agar at 25 °C, cells are globose (5×3.5 µm) to sub-globose (6–4×4–3 µm) (Fig. 2a) and proliferation is by multilateral budding on a narrow base. On Dalmau plates after 2 weeks at 25 °C, rudimentary pseudohyphae are present but true hyphae are not formed. Sexual reproduction is observed on V8 agar and the studied strains appear to be homothallic. Asci are persistent and form after cell-to-cell conjugation involving either a cell and its bud or two independent cells.

Table 1. Yeast strains isolated during this study and relevant information pertaining to them (*T., Torulaspora; Z., Zygotorulaspora*)

Species	Strain	Origin	D1/D2 accession no.	ITS accession no.
T. cf. *delbrueckii*	ZP 686=PYCC 8099	Bark of *Lithocarpus edulis*, Shiba Prefecture, Japan	MH055372	MH055363
T. cf. *delbrueckii*	ZP 689=PYCC 8100	Soil underneath *Quercus acuta*, Shiba Prefecture, Japan	MH055373	MH055364
T. cf. *delbrueckii*	ZP 700=PYCC 8101	Soil underneath *Quercus salicina*, Shiba Prefecture, Japan	MH055374	MH055365
T. cf. *microellipsoides*	ZP 701=PYCC 8102	Soil underneath *Castanopsis sieboldii* Shiba Prefecture, Japan	MH055375	MH055366
Z. chibaensis sp. nov.	ZP 699=PYCC 6970^T	Soil underneath *Castanopsis sieboldii*, Shiba Prefecture, Japan	MH055367	MH055358
Z. chibaensis sp. nov.	ZP 733=PYCC 7009	Bark of *Quercus salicina*, Shiba Prefecture, Japan	MH055368	MH055359
Z. danielsina sp. nov.	ZP 982=PYCC 6984^T	Bark of *Nothofagus menziesii*, Lake Daniels, Lewis Pass, New Zealand	MH055369	MH055360
Z. danielsina sp. nov.	ZP 999=PYCC 6989	Soil underneath *Nothofagus menziesii*, Lake Daniels, Lewis Pass, New Zealand	MH055370	MH055361
Z. florentina	ZP 696=PYCC 6843	Soil underneath *Quercus serrata*, Shiba Prefecture, Japan	MH055371	MH055362

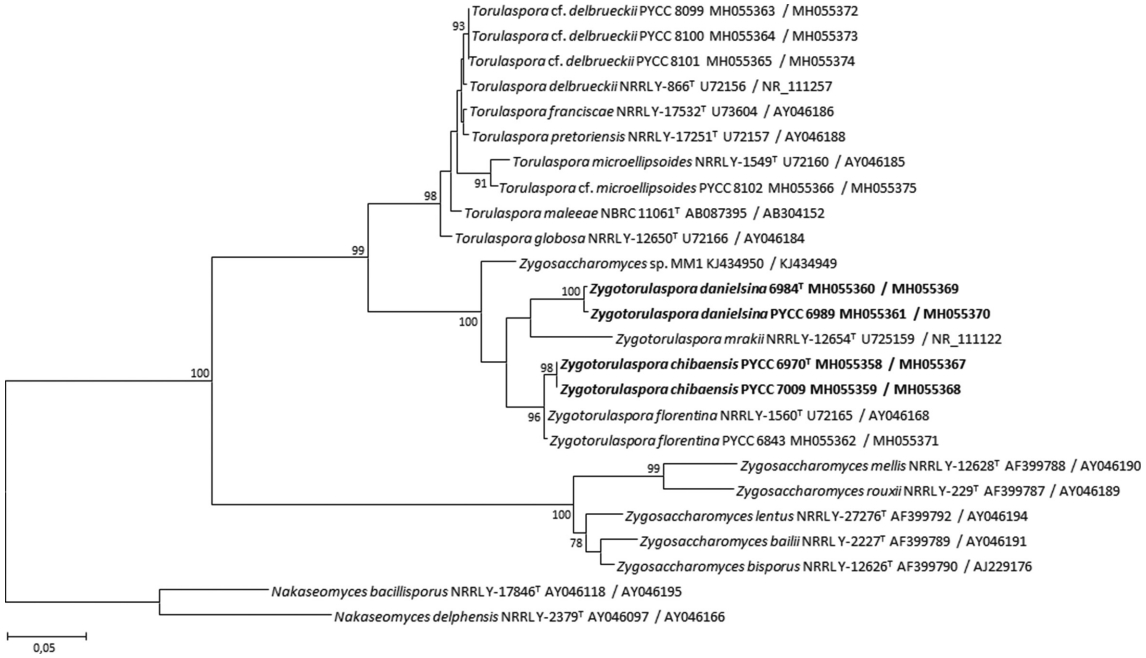

Fig. 1. Phylogenetic placement of *Zygotorulaspora chibaensis* sp. nov. and *Zygotorulaspora danielsina* sp. nov. among the species of the genera *Zygotorulaspora* and *Torulaspora*, including also additional strains of these two genera isolated during this study. The phylogenetic tree was based on a concatenated alignment of the D1/D2 domain of 26S rRNA gene and complete ITS region and used the maximum-likelihood method and Kimura's two-parameter model of sequence evolution. The tree was rooted with *Nakaseomyces bacillisporus* and *Nakaseomyces delphensis*. The numbers provided on branches are frequencies with which a given branch appeared in 1000 bootstrap replications (values below 70 % not shown). Bar, number of expected of substitutions per site.

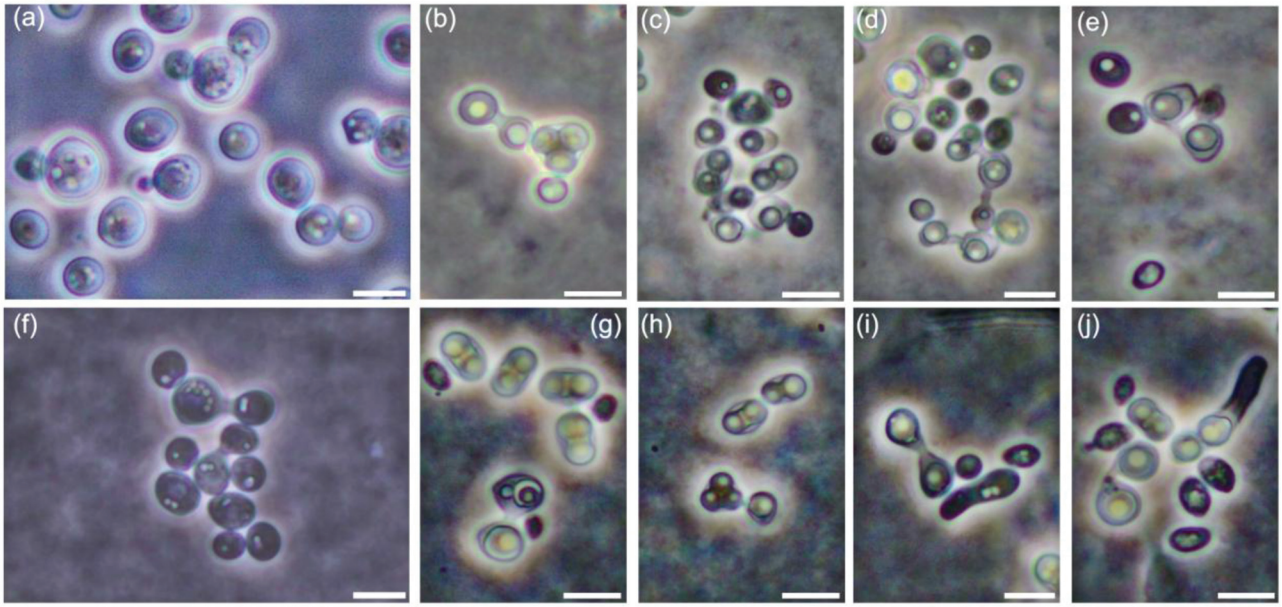

Fig. 2. Micrographs of *Zygotorulaspora chibaensis* sp. nov. and *Zygotorulaspora danielsina* sp. nov. (a) Yeast cells of *Z. chibaensis* PYCC 6970^T on YM agar. (b–e) Asci and ascospores of *Z. chibaensis* on V8 agar. (f) Yeast cells of *Z. danielsina* PYCC 6984^T on YM agar. (g–j) Asci and ascospores of *Z. danielsina* on V8 agar. Bars, 10 µm.

Asci produce one to four smooth, spherical ascospores, measuring 3.5–2.5 µm in diameter (Fig. 2b–e).

Carbon compounds fermented: D-glucose, D-galactose, maltose, sucrose, melibiose, melezitose and raffinose. No fermentation of α,α-threalose, lactose or cellobiose. Carbon compounds assimilated: D-glucose, D-galactose, L-sorbose, D-xylose, L-arabinose, sucrose, maltose, α,α-threalose, methyl α-D-glucoside, melibiose, raffinose, melezitose, inulin (delayed), xylitol, D-glucitol, D-mannitol, D-glucono-δ-lactone (delayed), succinate and ethanol. No growth on D-glucosamine, D-ribose, D-arabinose, L-rhamnose, cellobiose, salicin, lactose, soluble starch, glycerol, erythritol, ribitol, galactitol, myo-Inositol, D-gluconate, D-glucuronate, DL-lactate, succinate, citrate, methanol, L-malic acid or L-tartaric acid. Nitrogen compounds assimilated: L-lysine and cadaverine. No growth on nitrate, nitrite, ethylamine, creatine or creatinine. Grows in the presence of 0.01 and 0.1% cycloheximide. Grows at 37 °C (weak) but negative at 42 °C. Grows in the absence of vitamins. Hydrolysis of urea and DBB reaction are negative.

The holotype is maintained in a metabolically inactive state in the Portuguese Yeast Culture Collection, Caparica, Portugal, and the type strain was deposited in the same collection (PYCC 6970^T) and in the collection of the Westerdijk Fungal Biodiversity Institute (CBS 15364^T), Utrecht, the Netherlands. The strains ZP 699 (PYCC 6970^T) and ZP 733 (PYCC 7009) were isolated from soil underneath *Castanopsis sieboldii* and bark from *Quercus salicina*, respectively, in Shiba Prefecture, Japan, in June 2008.

DESCRIPTION OF *ZYGOTORULASPORA DANIELSINA* SP. NOV. C. CARVALHO, A. TOMÁS AND J.P. SAMPAIO

Zygotorulaspora danielsina (da.ni.els.i'na. N.L. fem. adj. *danielsina* refers to Lake Daniels, New Zealand, the locality where the strains were isolated).

After 1 week on YM agar at 25 °C cultures are beige and butyrous. After 3 days of growth on YM agar at 25 °C, cells are globose (5×4 µm) to sub-globose (6–5×4–3 µm) (Fig. 2f) and proliferation is by multilateral budding on a narrow base. On Dalmau plates after 2 weeks at 25 °C, rudimentary pseudohyphae are present but true hyphae are not formed. Sexual reproduction is observed on V8 agar and the studied strains appear to be homothallic. Asci are persistent and form after cell-to-cell conjugation, either between a cell and its bud or between independent cells. Asci produce one to four smooth, spherical ascospores, measuring 3–2 µm in diameter (Fig. 2g–j).

Carbon compounds fermented: glucose, galactose, maltose, sucrose, melibiose (delayed), melezitose and raffinose. No fermentation of trehalose, lactose, cellobiose or melezitose. Carbon compounds assimilated: D-glucose, D-galactose, D-xylose, L-arabinose sucrose, maltose, α,α-threalose (variable), methyl α-D-glucoside, melibiose (variable), raffinose, melezitose (variable), xylitol (delayed) and ethanol. No growth on L-sorbose, D-glucosamine, D-ribose, D-arabinose, L-rhamnose, cellobiose, salicin, lactose, inulin, soluble starch, glycerol, erythritol, ribitol, D-mannitol, galactitol, myo-inositol, D-glucono-δ-lactone, D- gluconate, D-galacturonate, DL-lactate, succinate, citrate, methanol, L-malic acid or L-tartaric acid. Nitrogen compounds assimilated: L-lysine and cadaverine. No growth on nitrate, nitrite, ethylamine, creatine and creatinine. Grows in the presence of 0.01 and 0.1% cycloheximide. Growth at 37 °C (weak) but negative at 42 °C. Grows in the absence of vitamins. Hydrolysis of urea and DBB reaction are negative.

The holotype is maintained in a metabolically inactive state in the Portuguese Yeast Culture Collection, Caparica, Portugal, and the type strain was deposited in the same collection (PYCC 6984^T) and in the collection of the Westerdijk Fungal Biodiversity Institute (CBS 15365^T), Utrecht, the Netherlands. Strains ZP 982 (PYCC 6984^T) and ZP 999 (PYCC 6989) were isolated from bark from *Nothofagus menziesii* and from soil underneath *Nothofagus menziesii,* respectively, from Lake Daniels, Lewis Pass, New Zealand, in November 2009.

Several physiological tests allow the differentiation of the two new species between themselves and from the other two species in the genus, as shown in Table 2. Overall, *Z. chibaensis* sp. nov. appears to be the most nutritionally versatile species in the genus as it assimilates the largest number of carbon compounds (23 out of 43), whereas *Z. mrakii* appears to be the species with the most limited nutritional spectrum. Moreover, the two novel species are able to grow at 35 °C whereas *Z. mrakii* is unable to grow at 30 °C and *Z. florentina* does not grow at 35 °C.

The two new species appear to share some ecological adaptations like the association with trees (tree bark and soil underneath the trees). Interestingly, some isolates of *Z. florentina* were isolated also from this type of environment (by Herman Phaff from tree exudates and by us), thus suggesting an adaptation of the entire genus to the arboreal niche. Tolerance to ethanol in the enrichment medium is also a

Table 2. Physiological characteristics differentiating *Zygotorulaspora chibaensis* sp. nov. and *Zygotorulaspora danielsina* sp. nov. from the other species of the genus *Zygotorulaspora*

Species: 1, *Z. chibaensis* sp. nov.; 2, *Z. danielsina* sp. nov.; 3, *Z. florentina*; 4, *Z. mrakii*. +, Positive; −, Negative.

Characteristic	Yeast species			
	1	2	3	4
Growth on:				
D-Xylose	+	+	−	−
L-Arabinose	+	+	−	−
Maltose	+	+	+	−
L-Sorbose	+	−	+	−
D-mannitol	+	−	+	+
Growth at 35 °C	+	+	−	−

feature worth noting since most ascomycetous yeasts do not tolerate such stringent conditions.

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

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