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RESEARCH NOTE

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Zygotorulaspora cornina sp. nov. and *Zygotorulaspora smilacis* sp. nov., Two Novel Ascomycetous Yeast Species Isolated from Plant Flowers and Fruits

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ABSTRACT

Three isolates belonging to the ascomycetous genus *Zygotorulaspora* were obtained from the fruits of *Cornus officinalis* and *Smilax china*, and flowers of *Dendranthema zawadskii* var. *latilobum* in Gongju-si, Korea. Phylogenetic Analyses of the LSU D1/D2 domain and ITS region sequences supported the recognition of two new species: *Zygotorulaspora cornina* sp. nov. (type strain NIBRFGC000500475 = KACC93346P^{PP}) and *Zygotorulaspora smilacis* sp. nov. (type strain NIBRFGC000500476 = KACC93347P^{PP}). The two novel species revealed no growth on D-Galactose, unlike the other six species in the genus *Zygotorulaspora*. They are distinguished from each other by their phylogenetic differences and phenotypic characteristics such as assimilation of xylitol, 5-keto-D-gluconate, and ethanol. All species in the genus *Zygotorulaspora* including the two novel species have phenotypic traits of genus *Zygotorulaspora*: asci are persistent, sucrose and raffinose are assimilated, and m-inositol is not required for growth, and they are mainly associated with plants.

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The genus *Zygotorulaspora* (Saccharomycetales, Saccharomycetaceae) was described by Kurtzman [1] to accommodate two species, *Z. florentina* and *Z. mrakii*, which had previously been assigned to the genus *Zygosaccharomyces*. As the clade containing the two species shows weak association with both *Zygosaccharomyces* and *Torulaspora*, Kurtzman suggested the *Zygotorulaspora* as a sister genus [2]. The genus *Zygotorulaspora* is characterized by vegetative reproduction with multilateral budding, persistent asci, assimilation of ribitol, mannitol, glucitol and succinate, and no use of m-inositol for growth [1]. At the time of writing, the genus *Zygotorulaspora* consists of six sexual species. Strains of *Z. florentina* have mainly been found in soft drinks, juices, and plants. The two strains of *Z. mrakii*, the type species of the genus, were isolated from silage. Carvalho et al. [3] reported two new species, *Z. chibaensis* and *Z. danielsina*, which were isolated using a *Saccharomyces* enrichment protocol and low temperatures from tree bark and soil under trees. Moreira et al. [4] described a new species, *Z. cariocana*, which was isolated from tree bark in Brazil. In a recent study [5], *Z. dagestanica* was proposed as new species of the genus *Zygotorulaspora*. The type strain of *Z. dagestanica* was isolated from soil underneath *Georgian honeysuckle* (Lonicera, Caprifoliaceae).

Through a survey of the indigenous yeast species of Korea, we discovered undescribed ascomycetous yeast species. As a result of phylogenetic analyses and physiological testing, we suggest these strains as two novel ascomycetous yeast species in the genus *Zygotorulaspora*. *Zygotorulaspora cornina* sp. nov. was found in the fruits of *Cornus officinalis* and *Z. smilacis* sp. nov. were found in fruits of *Smilax china* and flowers of *Dendranthema zawadskii* var. *latilobum* in Gongju-si, Korea.

Plant materials were collected from a mixed pine-oak forest (*Pinus densiflora* and *Quercus* spp.) in Gongju-si in the Midwest of South Korea (36°26'N, 127°07'E) in October 2017. To isolate yeast strains, wild fruits and flowers were collected aseptically, and approximately 3 g of each sample was added to conical tubes containing 10% malt extract media. The tubes were tightly capped and incubated at 30 °C without shaking. Turbidity and gas formation in the tubes were periodically surveyed for two weeks. Samples exhibiting yeast growth were spread onto YPD media plates were incubated at 30 °C for two weeks. Yeast colonies with different colors and morphologies were isolated. All yeast isolates were

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Supplemental data for this article can be accessed here.

purified by repeatedly streaking the YPD agar plates three times, and the isolates were preserved as 10% glycerol stocks at −80 °C in the culture collection repository at the NIBR (National Institute of Biological Resources), Korea. The isotypes of two novel species were deposited KACC (patent-pending).

DNA was extracted from the yeast colonies using a Nucleospin plant kit (Macherey-Nagel, Düren, Germany). The extracted DNA was amplified using the primers ITS1f [6] and ITS4 [7] for the ITS region, and NL1 and NL4 [8] for the LSU D1/D2 domain with the following thermal cycling parameters: initial denaturation for 5 min at 94 °C, 30 cycles each 1 min at 94 °C, 30 sec at 55 °C, and 1 min at 73 °C, and final elongation for 10 min at 72 °C. The amplicons were sequenced by Macrogen (Seoul, Korea).

Combined alignment of LSU D1/D2 domain and ITS region was used for phylogenetic analysis. The phylogenetic tree was constructed using the program provided with the MEGA7 [9] software package. Phylogenetic analysis of the novel species was based on Tamura-Nei, using a discrete gamma distribution model and the maximum-likelihood method, as suggested by the implemented model test. Bootstrap analysis was carried out using 1000 replicates to estimate the confidence of the tree nodes, and the other parameters retained their default settings.

Carbon assimilation was assessed in glass vials containing yeast nitrogen base liquid media. Nitrogen assimilation was carried out on yeast carbon base agar. Other physiological and chemotaxonomical tests were performed following standard protocols [10]. For microscopy, isolates were grown at 25 °C on YM agar and evaluated using phase contrast optics. Strains were examined for the sexual state after growth and incubation at 25 °C on 5% malt extract agar, McClary acetate agar, corn meal agar (CMA), yeast morphology agar, potato dextrose agar, V8 juice agar, water agar, and YM agar. All experiments were independently carried out in three vials or on three plates. For the analysis of the composition of ubiquinone, type strain of *Z. chibaensis* (CBS 15364) and *Z. danielsina* (CBS 15365) was purchased from CBS.

Thirteen ascomycetous yeast strains were isolated using the enrichment method with 10% malt extract. All of the strains were identified by analyzing the sequences of LSU D1/D2 domain and ITS region. These isolates belonged to *Hanseniaspora opuntiae* (one strain), *Hanseniaspora uvarum* (three strains), *Kazachstania* sp. (one strain), *Lachancea thermotolerans* (three strains), *Pichia kluyveri* (one strain), *Schizosaccharomyces japonicus* (one strain), and two noble *Zygotorulaspora* species (three strains). The strains isolated are listed in Table S1.

Phylogenetic analysis of the novel species strains confirmed the placement of the three strains in the genus *Zygotorulaspora* (order Saccharomycetales, subphylum Saccharomycotina; one *Z. cornina* [NIBRFGC000500475^{T}] and two *Z. smilacis* [NIBRFGC000500476^{T}, NIBRFGC000500477] strains) (Figure 1). Two novel species are closely related with *Z. chibaensis*, *Z. florentina* and *Z. danielsina* group, but exhibit 130–172 substitutions in ITS and 24–28 substitutions in D1/D2 regions. The sequences of the two strains NIBRFGC000500476 and NIBRFGC000500477 of *Z. smilacis* were identical in the ITS and D1/D2 regions. In terms of pairwise sequence similarity, *Z. cornina* (NIBRFGC000500475^{T}) and *Z. smilacis* (NIBRFGC000500476^{T}) were the most closely related species, but had nine nucleotide substitutions in the LSU D1/D2 region and two-nucleotide substitution and one gap in the ITS region (98.5% and 99.5%, respectively). Kurtzman and Robnett [8] reported that strains having greater than 1% substitutions in the nucleotide D1/D2 domain (ca. 600) are likely to be different species, supporting the contention that *Z. cornina* and *Z. smilacis* are different species. The genetic distance of the two novel species is similar to the distance between *Z. chibaensis* and *Z. florentina*, with eight nucleotide substitutions in the LSU D1/D2 region, and between two and four nucleotide substitutions in the ITS region [3]. BLAST search revealed that the D1/D2 region sequence of VdF2-P092, IFO 11070 was identical to that of *Z. cornina*, and CE41 and IFO 11069 were identical to *Z. smilacis*, but only in D1/D2 in those cases (Figure S1). It implies additional strains of *Z. cornina* and *Z. smilacis* Carvalho et al. [3] identified, based on D1/D2 and ITS sequences, a possible new *Zygotorulaspora* species (strain MM1) which we reveal here to constitute an additional representative of *Z. cornina* because of its identical sequences in the LSU D1/D2 domain and ITS region.

Since the two novel species share similar cell morphology with existing *Zygotorulaspora* species (globose to ellipsoid; white to tannish white, beige and cream-colored; multilateral budding), it is difficult to distinguish them by only morphological characteristics, so the phenotypic characteristics must be considered. Carvalho et al. [3] suggested physiological differences among species in the genus such as the assimilation of five sugars (D-xylose, L-arabinose, maltose, L-sorbose, D-mannitol) and growth at 35 °C for species recognition. We suggest the use of additional physiological traits to distinguish the species in this genus (Table 1). The two novel species differ in their fermentation of D-galactose and assimilation of D-galactose, DL-lactate, and ethylamine, compared to other *Zygotorulaspora* species. *Z. cornina* and *Z. smilacis* are distinguished

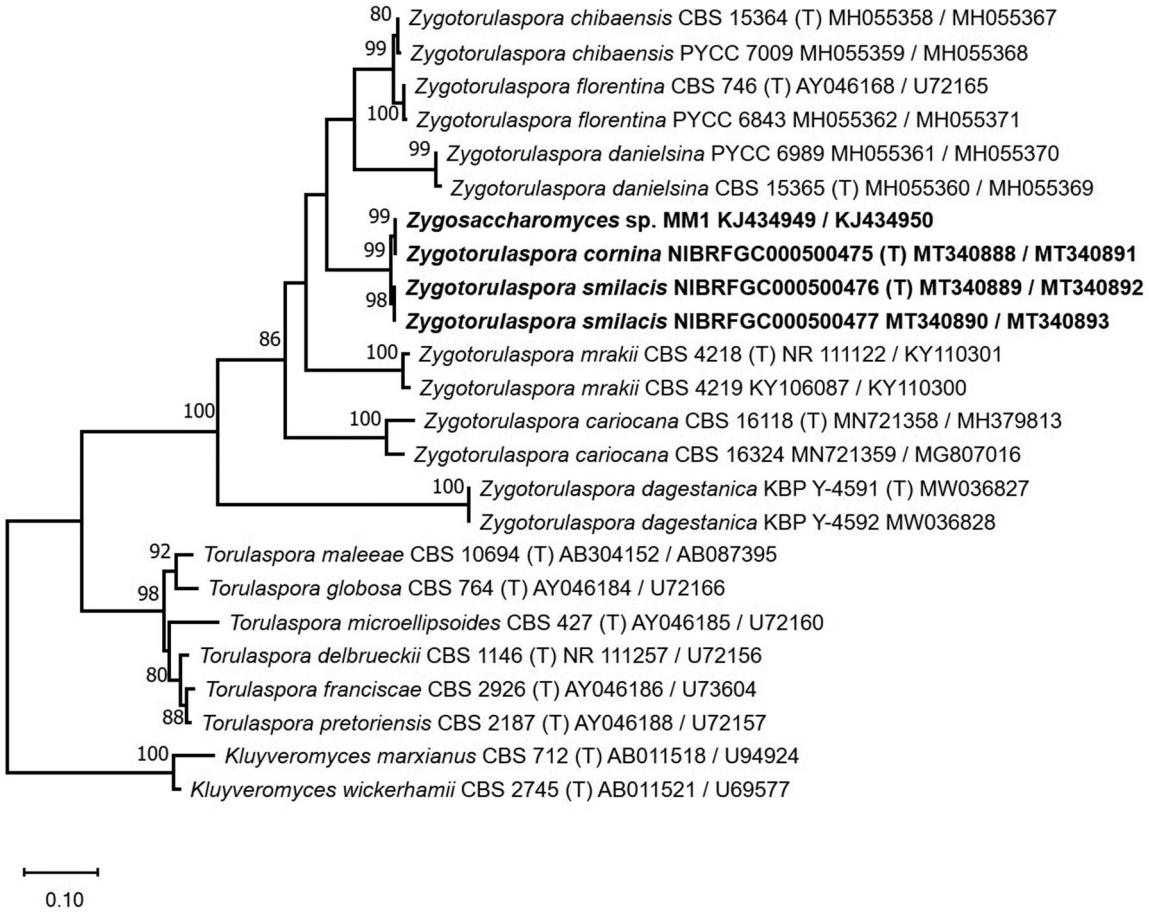

Figure 1. Phylogenetic tree drawn from maximum-likelihood analysis of combined sequences of the LSU D1/D2 domain and ITS regions, showing positions of the two novel species. The tree was rooted with *Kluyveromyces marxianus* and *K. wickerhamii*. Numbers at the nodes indicate bootstrap support percentages, derived from 1000 samples (values below 70% not shown). GenBank accession numbers are indicated after strain designations.

Table 1. Phenotypic characteristics that differentiate the two new species from each other and from the other six species in *Zygotorulaspora*.

	Yeast species							
Characteristics	1	2	3	4	5	6	7	8
Fermentation of:								
D-Galactose	−	−	+	+	+	+	n	+
Maltose	+	+	+	−	+	+	n	−
α,α-Trehalose	+	+	+	−	−	−	n	−
Assimilation of carbon compounds:								
D-Galactose	−	−	+	+	+	+	+	+
L-Sorbose	+	+	+	−	+	−	+	−
Melibiose	−	−	+	+	+	v	+	−
Xylitol	w	−	n	n	+	d	+	n
5-Keto-D-gluconate	−	+	−	−	n	n	n	−
DL-Lactate	+	+	−	−	−	−	+	−
Ethanol	−	+	+	v	+	+	+	−
Assimilation of nitrogen compounds:								
Ethylamine	+	+	n	n	−	−	n	n
Major ubiquinone	7	7	6	6	7	7	n	n

Species: 1, *Zygotorulaspora cornina* sp. nov.; 2, *Z. smilacis* sp. nov.; 3, *Z. florentina*; 4, *Z. mrakii*; 5, *Z. chibaensis*; 6, *Z. danielsina*; 7, *Z. cariocana*; 8, *Z. dagestanica*. Data for species 1 and 2 are from the present study, for species 3 and 4 are from Kurtzman [11], for species 5 and 6 are from Carvalho et al. [3], for 7 is from Moreira et al. [4], and for species 8 is from Kachalkin et al. [5]. Growth reactions: +, strong growth; d, delayed growth; w, weak growth; −, no growth; n, no data; v, variable results.

from each other not only based on the sequences of LSU D1/D2 domain and ITS region but also based on phenotypic characteristics such as the assimilation of xylitol, 5-keto-D-gluconate, and ethanol. Kurtzman described the genus *Zygotorulaspora* to accommodate two species, *Z. florentina* and Z. *mrakii*, having Q-6 as major ubiquinone [11]. Since then, four additional species have been described in *Zygotorulaspora*. Their composition of ubiquinone has not been studied. As a result of analysis the ubiquinone of four type strains (*Z. chibaensis*, CBS 15364; *Z. danielsina* CBS 15365; *Z. corninna* NIBRFGC000500475; *Z. smilacis* NIBRFGC000500476), it was found that Q-7 is the major ubiquinone of four species. Genus *Zygotorulaspora* may have six or seven isoprene units in the side chain.

All species in the genus *Zygotorulaspora*, including the two novel species, are associated with plants. The species were isolated from plants (bark, exudate, flower, and fruit), plant products (silage, soft drinks, and juice), and soil under trees. Known cultures of this genus, except for *Z. cariocana*, are from the temperate region of the northern hemisphere in Europe (Italy, Austria, France, Netherlands and Russia), Asia (Korea and Japan), and the southern hemisphere (New Zealand). *Z. cariocana* strains were isolated from a rainforest in Brazil.

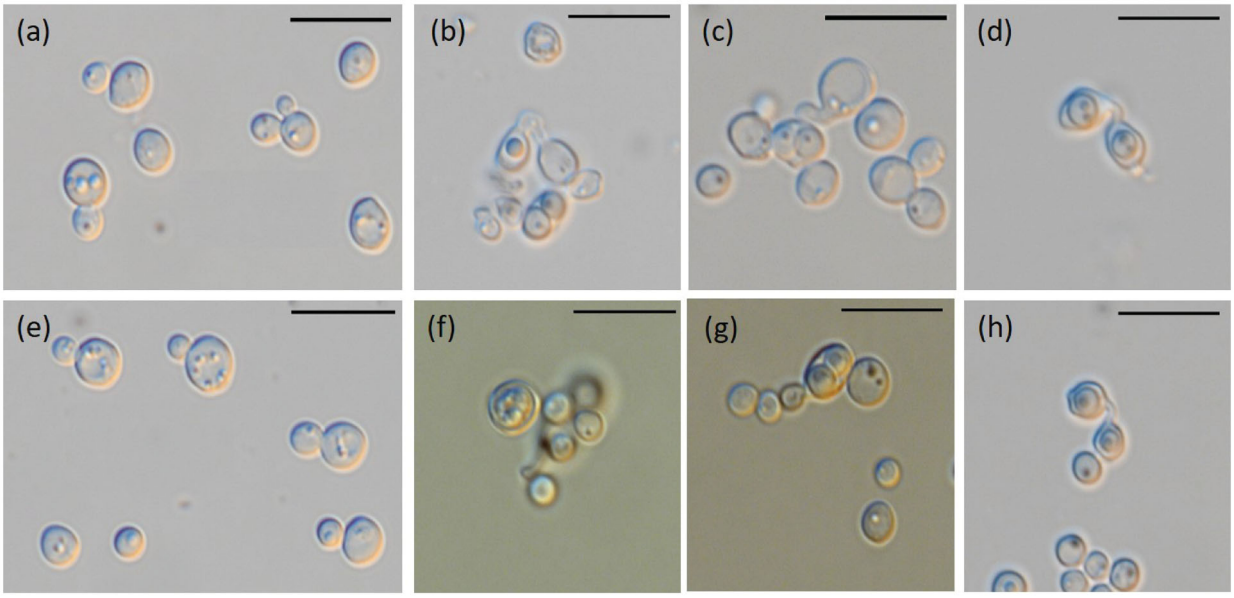

Figure 2. Micrographs of *Zygotorulaspora cornina* sp. nov. NIBRFGC000500475^{T} (a–d) and *Zygotorulaspora smilacis* sp. nov. NIBRFGC000500475^{T} (e–h). (a) Budding cells on YM agar 3 days at 25 °C. (b–d) Asci and ascospores on corn meal agar. (e) Budding cells on YM agar after 3 days at 25 °C. (f–h) Asci and ascospores on corn meal agar. Bar, 10 µm.

Taxonomy

Zygotorulaspora cornina sp. nov. C. Ahn and C. Kim

Zygotorulaspora cornina (cor.ni'na. N.L. fem. n. *cornina* of *cornus* refers to the host plant *Cornus officinalis* from which this yeast was isolated).

After 1 week on YM agar at 25 °C, colonies are shiny, smooth, with an entire margin and white to tannish-white in color. After 3 days on YM agar at 25 °C, cells are globose to sub-globose (2.9–6.0 × 2.8–5.4 µm) and proliferation is by multilateral budding on a narrow base (Figure 2a). On Dalmau plates after 2 weeks at 25 °C, rudimentary pseudohyphae are present but true hyphae are not formed. Sexual reproduction is observed on corn meal agar plate after 7 days at 25 °C. Asci are persistent and form after conjugation between either a cell and its bud or two independent cells. Asci produce one to two smooth, globose ascospores, measuring 2–3 µm in diameter (Figure 2b–d). The studied strains appear to be homothallic.

Carbon compounds fermented: D-glucose, maltose, α-methyl-D-glucoside, sucrose, α,α-trehalose, melezitose, raffinose, and inulin. No fermentation of D-galactose, melibiose, lactose, cellobiose, soluble starch, or D-xylose.

Carbon compounds assimilated: D-glucose, L-sorbose, sucrose, maltose, α,α-trehalose, methyl α-D-glucoside, raffinose, melezitose, inulin, xylitol(weak), D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, DL-lactate, succinate, palatinose, and L-malic acid (weak).

No growth on D-galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, cellobiose, salicin, arbutin, melibiose, lactose, soluble starch, glycerol, erythritol, ribitol, L-arabinitol, galactitol, myo-Inositol, 5-Keto-D-gluconate, D-glucuronate, D-galacturonate, citrate, methanol, ethanol, propane 1,2 diol, butane 2,3 diol, quinic acid, D-glucarate, levulinate, L-tartaric acid, D-tartaric acid, meso-tartaric acid, galactaric acid, uric acid, gentobiose, ethylene glycol, Tween 40, Tween 60, or Tween 80.

Nitrogen compounds assimilated: ethylamine, L-lysine, cadaverine, D-tryptophan, D-proline, and putrescine. No growth on nitrate, nitrite, creatine, creatinine, glucosamine, or imidazole. Growth on 0.01 % and 0.1% cycloheximide containing medium was positive. Grows at 35 °C but negative at 37 °C. Hydrolysis of urea and DBB reaction are negative. Grows in the absence of vitamins. The major ubiquinone system is Q-7.

Mycobank: MB837980

Type: Korea, Gongju-si, 18 Oct 2017, isolated from fruit of *Cornus officinalis* (holotype: NIBRFGC000500475 preserved in a metabolically inactive state at the National Institute of Biological Resources [NIBR], Incheon, Korea; isotype culture: KACC93346P^{PP}); ex-type: ITS (MT340888) and LSU D1/D2 (MT340891) sequences.

Zygotorulaspora smilacis sp. nov. C. Ahn and C. Kim

Zygotorulaspora smilacis (smi.la'cis. N.L. fem. n. *smilacis* of *smilax*, refers to the host plant *Smilax china* from which this yeast was isolated).

After 1 week on YM agar at 25 °C, colonies are shiny, smooth, with an entire margin and white to tannish-white in color. After 3 days on YM agar at 25 °C,

cells are globose to sub-globose (3.1–6.4 × 2.7–6.1 µm) and proliferation is by multilateral budding on a narrow base (Figure 2e). On Dalmau plates after 2 weeks at 25 °C, rudimentary pseudohyphae are present but true hyphae are not formed. Sexual reproduction is observed on corn meal agar plate after 7 days at 25 °C. Asci are persistent and form after conjugation between either a cell and its bud or two independent cells. Asci produce one to two smooth, globose ascospores, measuring 2–3 µm in diameter (Figure 2f–h). The studied strains appear to be homothallic.

Carbon compounds fermented: D-glucose, maltose, α-methyl-D-glucoside, sucrose, α,α-trehalose, melezitose, raffinose, and inulin. No fermentation of D-galactose, melibiose, lactose, cellobiose, soluble starch, or D-xylose.

Carbon compounds assimilated: D-glucose, L-sorbose, sucrose, maltose, α,α-trehalose, methyl α-D-glucoside, raffinose, melezitose, inulin, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, DL-lactate, succinate, ethanol, and palatinose.

No growth on D-galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, cellobiose, salicin, arbutin, melibiose, lactose, soluble starch, glycerol, erythritol, ribitol, xylitol, L-arabinitol, galactitol, myo-Inositol, D-glucuronate, D-galacturonate, citrate, methanol, propane 1,2 diol, butane 2,3 diol, quinic acid, D-glucarate, levulinate, L-malic acid, L-tartaric acid, D-tartaric acid, meso-tartaric acid, galactaric acid, uric acid, gentobiose, ethylene glycol, Tween 40, Tween 60, or Tween 80. Nitrogen compounds assimilated: ethylamine, L-lysine, cadaverine, D-tryptophan, D-proline, and putrescine. No growth on nitrate, nitrite, creatine, creatinine, glucosamine, or imidazole.

Growth on 0.01 % and 0.1% cycloheximide containing medium was positive. Grows at 35 °C but negative at 37 °C. Hydrolysis of urea and DBB reaction are negative. Grows in the absence of vitamins. The major ubiquinone system is Q-7.

Mycobank: MB837981

Type: Korea, Gongju-si, 18 Oct 2017, isolated from the fruit of *Smilax china* (holotype: NIBRFGC000500476 preserved in a metabolically inactive state at National Institute of Biological Resources [NIBR], Incheon, Korea; isotype culture: KACC93347P^{PP}; paratype: NIBRFGC 000500477); ex-type: ITS (MT340889) and LSU D1/D2 (MT340892) sequences.

Author contributors

Chorong Ahn: Investigation; Writing – Original Draft Preparation, Minkyeong Kim: Investigation; Review and Editing, Changmu Kim: Supervision; Writing – Review and Editing and Funding.

Repositories

The GenBank accession numbers of the LSU and ITS sequences of *Zygotorulaspora cornina* NIBRFGC000500475^{T} and *Zygotorulaspora smilacis* NIBRFGC000500476^{T} are MT340891, MT340888, MT340892 and MT340889, respectively. The MycoBank accession numbers are MB837980 and MB837981, respectively.

Disclosure statement

The type strains described in this paper are patent-pending (details of the patent application).

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