Nakazawaea Odontotermitis f.a., sp. nov., a Novel Yeast Isolated from the Gut of *Odontotermes Horni* in India.

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Abstract

Three strains SMT1.3, SMT1.10, and SMT2.2, representing a novel asexual ascomycetous yeast species, were isolated from the gut of a termite *Odontotermes horni* in Maharashtra, India. Phylogenetic analyses of the LSU, ITS and SSU sequences revealed that they belonged to the genus *Nakazawaea*, with *N. siamensis* as the closest relative. The new species differed from the type strain of *N. siamensis* (DMKU-RK467^T) by 1.93% nucleotide substitutions in the D1/D2 region of the large subunit (LSU) rRNA gene, 0.53% nucleotide substitutions in the small subunit (SSU) rRNA gene and 12.6% nucleotide substitutions in the internal transcribed spacer (ITS) region. Notable physiological differences were also observed between *N. siamensis* and the new species. Hence, the species *Nakazawaea odontotermitis* f.a., sp. nov. is proposed. The type strain is SMT1.3^T (MTCC 13105 = NFCCI 5011). The GenBank accession numbers of the LSU and ITS and SSU sequences of *Nakazawaea odontotermitis* f.a., sp. nov. are MZ234240, MZ234239 and OK384663. The MycoBank number is MB 841926.

Introduction

The genus *Nakazawaea* of the order Saccharomycetales was first proposed in 1994 by Yamada et al (Yamada et al. 1994), with *Nakazawaea holstii* as the type species. This ascomycetous genus was introduced to reassign *Pichia holstii* to a new genus, *Nakazawaea*, based on certain notable characteristics that distinguished it from the other hat-shaped, ascospore-forming and nitrate-assimilating species of the genus *Pichia*. However, phylogenetic analyses based solely on the partial sequences of the D1/D2 region of the LSU and SSU rRNA genes of all known species of *Pichia* did not validate the transfer of *P. holstii* to the new genus, since very few species were considered for rRNA analysis (Kurtzman and Robnett 1998). Later, a reclassification based on multigene phylogeny of the sequences of several protein-coding genes including actin (*ACT1*), largest subunit and second-largest subunit of the RNA polymerase II gene (*RPB1 and RPB2*), the second subunit of mitochondrial cytochrome oxidase (*COX2*), along with the D1/D2 region of the LSU rRNA gene provided sound support for the proposal the genus *Nakazawaea* (Tsui et al. 2008). Combined analysis of the LSU (D1/D2) rRNA gene, translation elongation factor-1α (EF-1α) gene, SSU rRNA gene, and *RPB1* and *RPB2* gene sequences separated the genus *Pichia* from *Nakazawaea* and in 2014, several asexual species of the genus *Candida* were transferred to the genus *Nakazawaea*, based on multigene phylogeny (Kurtzman and Robnett 2014). The species that were accommodated in the genus *Nakazawaea* were *N. anatomiae*, *N. ernobii*, *N. ishiwadae*, *N. laoshanensis*, *N. molendini-olei*, *N. peltata*, *N. pomicola*, *N. populi*, *N. wickerhamii*, and *N. wyomingensis*. Since this reorganization, only three new species viz. *N. siamensis*, *N. todaengensis* and *N. ambrosiae* have been described so far (Kaewwichian and Limtong 2014; Polburee et al. 2017; Crous et al. 2019). The type species *N. holstii* is the sole ascospore-producing species in this genus. Initially, *C. ernobii* was considered a synonym of *N. holstii*, based on the similarities between the D1/D2 domain and SSU rRNA gene sequences (Kurtzman 2011). But due to significant variations in the *EF-1a*, *RPB1*, and *RPB2* gene regions, they have been designated as two distinct species, *N. ernobii* and *N. holstii* (mycobank.org). Currently, there are 14 legitimate species of *Nakazawaea* listed in Mycobank.

The gut of insects, especially the xylophagous kind, is a niche for several ascomycetous and a few basidiomycetous yeasts, which may share a mutualistic relationship with the insect host (Blackwell 2017). The exact role of yeast-insect associations is not entirely understood, but the most plausible explanation is that the yeast symbiont assists the insect with nutrition, while the insect protects the yeast from unfavourable environments and helps in dispersal to new habitats (Stefanini 2018). It is well-known that the gut of beetles and wood roaches harbour many novel yeasts (Suh et al. 2005a, b). In recent years the termite gut has also been established as a potential habitat for diverse yeast species, including various novel yeast taxa (Handel et al. 2016; Ali et al. 2017; Tiwari et al. 2021). Termites represent one of the most significant wood-degrading species, which can break down complex polymers into simpler monomers with the help of enzyme complexes secreted by the gut symbionts in combination with the host's endogenous enzymes. In a recent survey of the termite gut-associated yeasts in India, yeasts belonging to the genera *Vishniacozyma*, *Kodamea*, *Pseudozyma*, *Hannaella*, and *Cystobasidium* were reported for the first time from the gut of termites *Coptotermes heimi* and *Odontotermes javanicus* (Tiwari et al. 2020). Termites of the genus *Odontotermes* are the most dominant in the tropical and subtropical regions of Africa and Asia, especially the Indian subcontinent (Shanbhag and Sundararaj 2013).

In the present study, while investigating the yeast communities residing in the gut of wood-feeding termites, we isolated thirty ascomycetous yeast strains belonging to *Yamadazyma* sp., *Cyberlindnera bimundalis*, *Cy. fabianii*, *Candida silvanorum*, and *C. insectorum* from the gut of *Odontotermes horni*. We also obtained three strains representing a novel species of the genus *Nakazawaea*, for which the name *Nakazawaea odontotermitis* f. a., sp. nov. is proposed.

Materials And Methods

Termite collection and yeast isolation

Worker termites (30 adults per sample) feeding on fallen pieces of wood were collected from Kapare, Maharashtra (17.551372° N, 73.434554° E), India, while investigating the yeast diversity of the termite gut in parts of the Western Ghats of India, in December 2020. Two separate termite-infested wooden twigs were sampled from the same area, 50 m apart from each other. The two samples were temporarily designated as SMT1 and SMT2. Molecular identification of the host termites was achieved by sequencing the mitochondrial 16s rRNA gene (Clark and Kambhampati 2003) and the sequences were deposited in NCBI GenBank. Previously described protocols were followed for termite dissection and preparation of homogenous gut suspensions (Tiwari et al. 2020). The gut suspension (100 µl) was plated on yeast-extract peptone dextrose (YPD) agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) containing antibiotics (100 µg ml^{-1} streptomycin and 100 µg ml^{-1} ampicillin) in replicates. Plates were incubated at 25 °C until yeast colonies emerged. YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, and 2% agar) was used for routine subculturing and maintenance at 25 °C. For long-term preservation, yeast cultures were stored at −80 °C in broth culture supplemented with 20% (w/v) glycerol.

Morphological, physiological, and biochemical characterization

The yeast strains were characterized morphologically, biochemically and physiologically by standard methods (Barnett et al. 2000; Kurtzman et al. 2011). Pseudohyphae or true hyphae formation was investigated on potato dextrose agar (PDA; 20% potato infusion, 2% glucose and 2% agar) and cornmeal agar (HiMedia Laboratories, LLC, India) in slide culture at 25 °C for up to 14 days. Ascospore formation was investigated on YPD agar, 5% malt extract agar (5% malt extract and 1.5% agar), McClary's acetate agar (0.1% glucose, 0.18% potassium chloride, 0.82% sodium acetate trihydrate, 0.25% yeast extract and 1.5% agar), Gorodkowa agar (0.1% glucose, 0.5% sodium chloride, 1% peptone and 2% agar) and Fowell's acetate agar (0.5% sodium acetate trihydrate and 2% agar) at 15 and 25 °C for up to 4 weeks. Photomicrographs were created using a DIC microscope (Olympus BX53) equipped with an Olympus DP73 camera and cellSens 1.13 imaging software. Carbon and nitrogen assimilation tests were performed in duplicate, and results were read up to 14 days of incubation.

Phylogenetic analyses

Genomic DNA was isolated using a simple yet effective protocol (Aamir et al. 2015). The yeast isolates were screened by MSP-PCR (microsatellite-primed PCR) fingerprinting technique using the $(GTG)_5$ primer. The MSP-PCR was performed following standard reagents and cycling conditions (Ramírez-Castrillón et al. 2014). A few representative strains of each cluster (fingerprint-based grouping) were selected for molecular identification by sequencing the barcode DNA regions. The ITS region, SSU and the D1/D2 region of the LSU rRNA gene were amplified using the primers ITS1 and ITS4; SSU1f, SSU4r, SSU3f and SSU2r; NL1 and NL4 (White et al. 1990; Kurtzman and Robnett 2003; Polburee et al. 2017). The amplicons were purified with the FavorPrep^{TM} GEL/PCR Purification Kit (FAVORGEN Biotech Corporation, Taiwan) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) by Sanger sequencing. All the sequences generated were deposited in NCBI GenBank. The D1/D2 and SSU rRNA gene sequences of closely related species were retrieved from GenBank, and alignments were made using the MUSCLE program (Edgar 2004). The phylogenetic tree was constructed based on concatenated sequences of SSU rRNA and the D1/D2 region of the LSU rRNA genes, using the Maximum Likelihood (ML) algorithm and the GTR+F+R2 model in IQ-TREE software v. 1.6.7 (Nguyen et al. 2015).

Results And Discussion

Termite identification and yeast isolation

The termite heads were used for molecular identification. The mitochondrial 16s rRNA gene sequencing identified both the host termites as *Odontotermes horni* (SMT1 OL629227, SMT2 OL629228). Several yeast colonies appeared on YPD agar plates after two days of incubation at 25 °C. Thirteen yeast strains were obtained from one termite sample (SMT1), while 17 yeast strains were obtained from the other sample (SMT2) (Table 1). The three strains SMT1.3 and SMT1.10 were isolated from the SMT1 sample, and the strain SMT2.2 was isolated from the SMT2 sample.

Phylogenetic analyses and yeast identification

All thirty yeast strains were subjected to MSP-PCR based screening, and eleven representative yeast strains were proceeded for identification based on D1/D2 LSU rDNA sequencing (Supplementary Fig. 1). The strains SMT1.3, SMT1.10, and SMT2.2 were isolated from the termite *O. horni*, amongst other ascomycetous yeast species (Table 1). These three strains were identified up to species level by analysing the D1/D2 LSU rDNA sequences. Phylogenetic analyses based on the combined sequences of the SSU and D1/D2 domain of rDNA supported the placement of a novel species in the genus *Nakazawaea* of Saccharomycetales, as it formed a separate clade from its close relatives *N. siamensis* and *N. ambrosiae* (Fig. 1). When comparing the sequences of the D1/D2 domains, the new species differed from the closely related species *N. siamensis* DMKU-RK467^{T} (NG_060235) by 1.93% sequence variation (11 substitutions 0 gaps) and *N. holstii* CBS 6225 by 2.9% variation (16 substitutions and 1 gap) in a 569 bp aligned region. When comparing the SSU rDNA sequences, the new species showed 0.61% variation when compared to *N. siamensis* DMKU-RK467T (8 substitutions and 1 gap), and 2.2% (29 substitutions and 1 gap) sequence variation with *N. holstii* NRRL Y-2155 in a 1312 bp SSU rDNA region. The strains SMT1.3, SMT1.10, and SMT2.2 showed 100% sequence similarity with similar phenotypic characteristics, indicating that they might be conspecific. In the phylogenetic tree also, the three novel strains were placed into one single clade. These nucleotide substitutions and phylogenetic analysis warrant considering these strains as a novel species for which the name *Nakazawaea odontotermitis* f.a. sp. nov. is proposed.

Morphological and physiological characteristics

The colony of the new species appears circular, white with an opaque sector, shiny, and smooth textured (Fig 2a). The cells of *N. odontotermitis* are globose to subglobose measuring 2.5 – 5 × 3 – 5 µm (Fig. 2b), while those of *N. siamensis* measured 3 – 5 × 3 – 5 µm. Both species show multilateral budding, but in *N. odontotermitis* sp. nov., the buds are predominantly polar (Fig 2b & c). Ascospores, hyphae or pseudohyphae formation were not observed in the new species. Furthermore, the new species can be discriminated from *N. siamensis* based on its ability to assimilate nitrate and D-glucosamine, while *N. siamensis* failed to assimilate these as sole nitrogen and carbon sources. Moreover, *N. siamensis* could assimilate inulin, methyl-α-D-glucoside, erythritol, galactitol, while the new species could not utilize these compounds (Table 1). The new species could grow up to 37 °C, but *N. siamensis* could grow up to 40 °C.

Members of the genus *Nakazawaea* have been isolated from various habitats but predominantly associated with plant material and insects. However, a few species like *N.anatomiae*, *N. ishiwadae* and *N. peltata* have been isolated from some unusual habitats like corpses embalmed in formalin, deep core of stratigraphic drillings, and mastitis milk, respectively (Kurtzman 2011). Several strains of *N. populi*, *N. wyomingensis* and *N. pomicola* were recovered from sap fluxes of trembling aspen (*Populus tremuloides*), black cottonwood (*Populus trichocarpa*), birch (*Betula* sp.), red oak (*Quercus rubra*) and old fustic (*Maclura tinctoria*) (Lachance et al. 2011). Strains of *N. wickerhamii* were isolated from silage made of olive husks and olive oil wastewaters (alpechín), while *N. molendini-olei* was obtained from olive

oil and its by-products (Lachance et al. 2011; Čadež et al. 2012). The species *N. laoshanensis* was recovered from decayed wood in China (Wang et al. 2010). Two species were discovered in Thailand, *N. siamensis* was isolated from the surface of sugar cane leaves, and *N. todaengensis* from peat in a swamp forest (Kaewwichian and Limtong 2014; Polburee et al. 2017). Three species of *Nakazawaea* namely *N. holstii*, *N. ernobii*, and most recently isolated *N. ambrosiae* have been isolated from bark beetles infesting pine, spruce and fir trees (Lachance et al. 2011; Crous et al. 2019). Similarly, in the present study, three strains of the novel species *N. odontotermitis* were isolated from the gut of termites (*O. horni*) feeding on wood. The occurrence of multiple isolates of the new species in the termite gut suggests that it may be a frequent inhabitant of the gut along with other symbiotic microbes and may contribute to the host's nutrition. The majority of the species of this genus have been isolated from wood materials or insects, suggesting that these might be potential habitats for the recovery of *Nakazawaea* species.

Taxonomy

Description of Nakazawaea odontotermitis **f.a. sp. nov. (S. Tiwari, B.C. Behera and A. Baghela)**

Nakazawaea odontotermitis (odon.to.ter'mi.tis. N.L. gen. n. *odontotermitis* of the host termite genus *Odontotermes*).

After 10 days at 25°C in YM agar, the colony is circular, white with an opaque sector, shiny, smooth, butyrous with an entire margin (Fig. 2a). After three days at 25°C in YM broth, vegetative cells are globose to subglobose (2.5–5 × 3–5 µm) and occur singly or in groups (Fig. 2b). Budding is multilateral, though predominantly polar (Fig. 2b & c). Hyphae or pseudohyphae are not observed on Dalmau (Corn Meal Agar) plates in slide culture, even after 21 days at 25°C. Ascospores are not produced on YPD agar, 5% malt extract agar, McClary's acetate agar, Gorodkowa agar or cornmeal agar after 4 weeks at 15 and 25°C. Fermentation of glucose is positive, but negative for D-galactose, sucrose, maltose, raffinose, lactose, α α-trehalose, xylose and cellobiose. The following carbon compounds are assimilated: D-glucose, D-galactose (weak), L-sorbose, sucrose, maltose, cellobiose (slow and weak), salicin, α α-trehalose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose (weak), D-ribose, ethanol, glycerol, ribitol (weak), D-mannitol, D-glucitol, arabinitol, D-gluconic acid, (slow), succinic acid, citric acid, arbutin, *N*-acetyl-D-glucosamine, D-glucosamine, D-glucono-*δ*-lactone, xylitol, 2-keto-D-gluconic acid, ethylamine (weak), L-lysine (slow and weak), nitrate and cadaverine. But lactose, melibiose, raffinose, inulin, methyl-α-D-glucoside, galactitol, erythritol, DL-lactate, methanol, D-glucuronic acid, *myo*-inositol, 5-keto-D-gluconic acid, hexadecane, and nitrite are not assimilated (Table 2 and Table S2). Growth in amino acid-free medium is positive, but no growth in vitamin-free medium. Growth on medium containing 50% glucose, 60% glucose and 10% sodium chloride/5% glucose is positive. Growth with 0.01 and 0.1% cycloheximide is positive. Grows at 25, 30, 35 and 37°C, but not at 40°C (Table 2 and Supplementary Table 2). Starch-like compounds and acid production are absent. Diazonium blue B colour and urease reactions are negative.

The type of *N. odontotermitis* f.a. sp. nov. is MTCC 13105^{T} (=SMT1.3), was isolated from the gut of the termite *O. horni* in Kapare, Pune, Maharashtra (India). It is preserved in a metabolically inactive state at Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH-Chandigarh, India), and also at the National Fungal Culture Collection of India (NFCCI), Pune, India (NFCCI 5012). The GenBank accession numbers of the ITS, LSU and SSU rDNA sequences are MZ234239, MZ234240 and OK384663. The Mycobank number is MB 841926.

Declarations

Acknowledgement

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]. The strains used in the present study are available at the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH- Chandigarh, India), and at the National Fungal Culture Collection of India (NFCCI), India.

Compliance with ethical standards

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1. Details of yeast strains isolated during the study

Termite	No. of isolates obtained	Representative strains		Yeast species	GenBank accession number
		Yeast strain code	% similarity (D1/D2 LSU rDNA) with type species		
SMT1 *Odontotermes horni*	13	SMT1.1	98.36	*Yamadazyma* sp.	MZ234241
		SMT1.2	99.45	*Cyberlindnera bimundalis*	MZ983382
		SMT1.3	98.07	*Nakazawaea* sp.	MZ234240
		SMT1.4	99.63	*Cyberlindnera fabianii*	MZ983391
		SMT1.7	100	*Candida silvanorum*	MZ983393
		SMT1.8	99.80	*Candida insectorum*	MZ983392
		SMT1.10	98.07	*Nakazawaea* sp.	MZ674393
SMT2 *Odontotermes horni*	17	SMT2.1	98.35	*Yamadazyma* sp.	MZ983395
		SMT2.2	98.02	*Nakazawaea* sp.	MZ674394
		SMT2.3	100	*Candida silvanorum*	MZ983394
		SMT2.17	99.43	*Cyberlindnera bimundalis*	MZ983383

Table 2. Physiological characteristics differentiating *Nakazawaea odontotermitis* f.a. sp. nov. from *N. siamensis* DMKU-RK467

Characteristics	*N. odontotermitis*	*N. siamensis* *
Growth on carbon compounds		
Inulin	−	+
D-Galactose	W	S
Cellobiose	S[a, b]/W[c]	+
L-Sorbose	+	S
L-Rhamnose	W	+
L-Arabinose	+	L
D-Arabinose	+	S
Methyl-α-D-glucoside	−	+
Erythritol	−	+
Ribitol	W	+
Galactitol	−	L
D-Glucosamine	+	−
Growth on nitrogen compounds		
Nitrate	+	−
Ethylamine	W	+
L-Lysine	S[a, c]/W [b]	W
Growth on other compounds		
2-keto-D-gluconate	+	W
Cycloheximide (0.1%)	+	W
Amino acid-free	+	ND
Growth at 40°C	−	+

+ positive; − negative; ND not determined; W weak; V variable; S: slow; L: latent

[a] SMT1.3= MTCC 13105[T]

[b] SMT1.10

[c] SMT2.2

* Data obtained from R. Kaewwichian and S. Limtong et al. 2014.

Figures

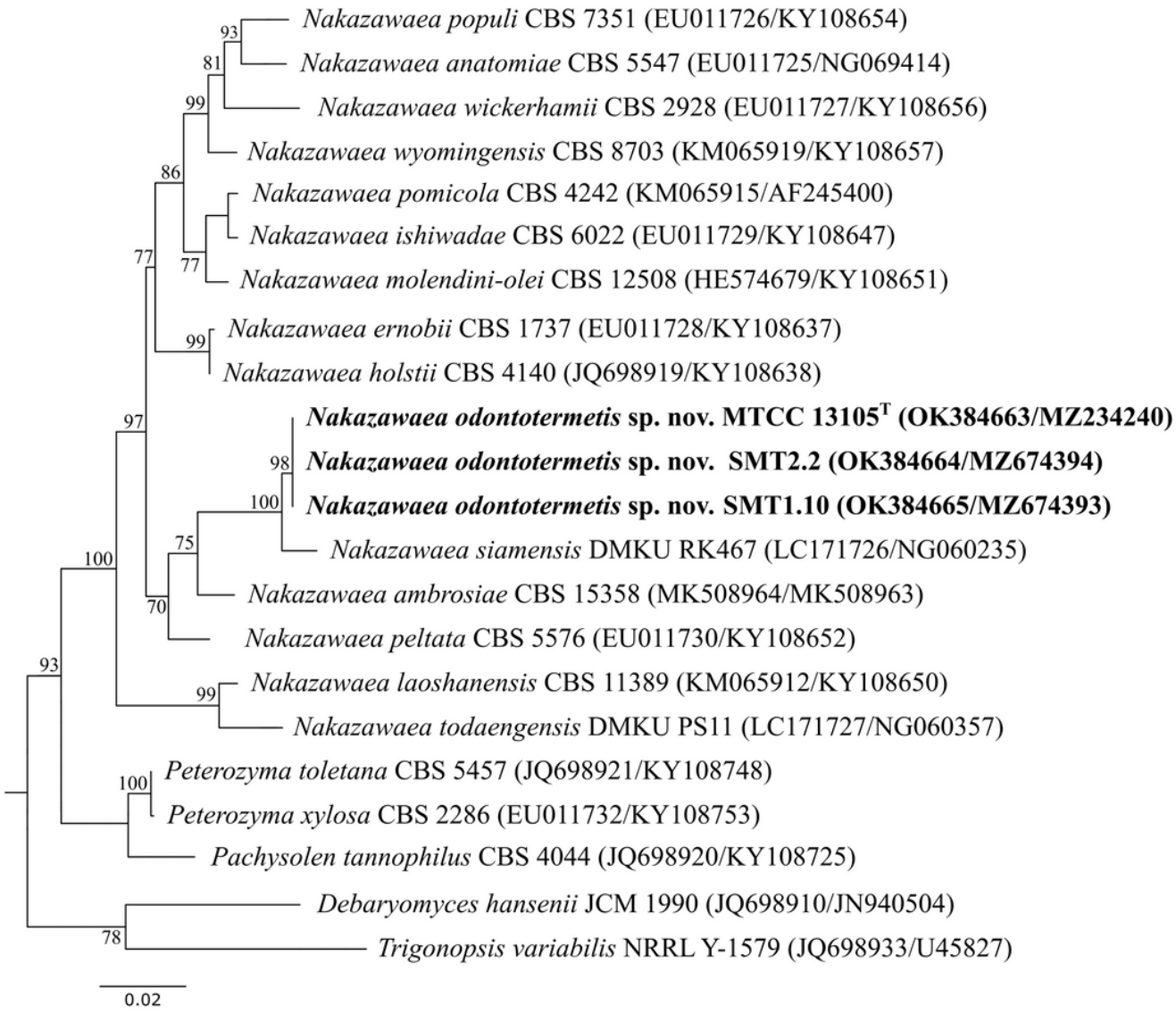

Figure 1

Maximum-likelihood tree based on the SSU rRNA and the D1/D2 region of the LSU rRNA genes of Nakazawaea odontotermitis f.a., sp. nov. and other related species. The tree was constructed using IQ-TREE v.1.6.7 (Nguyen et al. 2015), employing the GTR+F+R2 model. The scale bar indicates the number of expected substitutions per site. The numbers provided on branches are frequencies with which a given branch appeared in 1000 bootstrap replications. The tree was rooted with Trigonopsis variabilis NRRL Y-1579T.

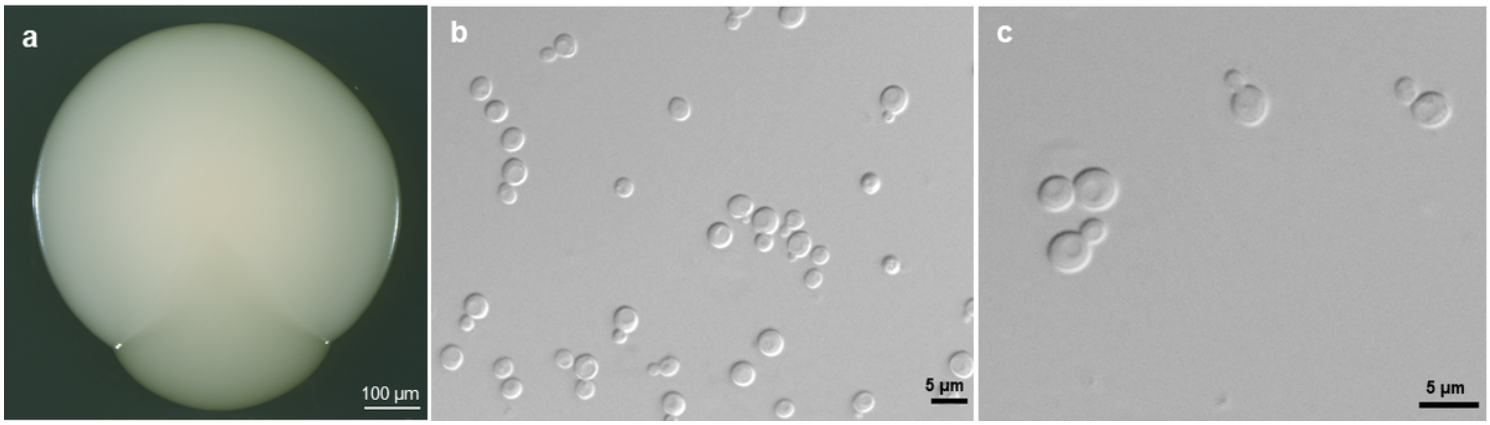

Figure 2

A 10-day old circular, white with an opaque sector, shiny, and smooth textured colony (12.5 mm) of Nakazawaea odontotermitis f.a., sp. nov. grown on YM agar at 25 °C (a), Micrograph showing vegetative cell morphology of 3 days old culture on YM agar (b), Micrograph of budding cells seen on YM agar (c).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation25.11.2021.docx