Nakazawaea odontotermitis f.a., sp. nov., a novel yeast isolated from the gut of Odontotermes horni in India

Snigdha Tiwari1,3 · Bhaskar C. Behera2 · Abhishek Baghela1,3

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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-RK467T) by 11 substitutions in the D1/D2 region of the large subunit (LSU) rRNA gene and by 8 substitutions and one gap in the small subunit (SSU) rRNA gene. Notable biochemical and 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 13,105 = NFCCI 5011 = PYCC 9153). GenBank accession numbers of the LSU, ITS and SSU sequences of Nakazawaea odontotermitis f.a., sp. nov. are MZ234240, MZ234239, and OK384663. The MycoBank number is MB 841926. 

Keywords

Ascomycetous yeast · Asexual · Nakazawaea · New yeast species · Insect gut

Introduction

The genus Nakazawaea of the order Saccharomycetales was first proposed in 1994 by 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 support the creation of a 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 of 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, RPB1 and RPB2 gene sequences separated the genus Pichia from Nakazawaea. 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 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 (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). However, due to significant divergence in the EF-1α, RPB1, and RPB2 gene regions, they are regarded as two distinct species, N. ernobii and N. holstii (mycobank.org). Currently, there are 14 legitimate species of Nakazawaea listed in Mycobank. Most strains of these species have been recovered from plant material, decayed wood, and wood-feeding insects like beetles.

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 insect hosts (Blackwell 2017). The exact role of yeast–insect associations is not entirely understood, but the most plausible explanation is that the yeast symbionts assist insects with nutrition, while the host insects protect yeasts from unfavorable environments and help in their dispersal to new habitats (Stefanini 2018). It is well known that the gut of beetles and wood roaches harbor 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 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 prevalent in tropical and subtropical regions of Africa and Asia, especially the Indian subcontinent (Shanbhag and Sundararaj 2013).

In the present study, while investigating yeast communities residing in the gut of wood-feeding termites, we isolated 30 ascomycetous yeast strains identified as Yamadazyma sp., Cyberlindnera bimundalis, Cy. fabianii, Candida silvano-rum, 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 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, but 50 m apart. 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). Thirty termite individuals (per sample) were surface sterilized with 95% ethanol, rinsed with 0.9% saline, and dissected with forceps or needles. Gut contents were transferred to a sterile tube containing 0.9% saline (500 µl) and crushed with a pestle. The gut mixture was briefly centrifuged to separate the debris, and 100 µl aliquots were spread on yeast-extract peptone dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) containing antibiotics (100 µg ml⁻¹ streptomycin and 100 µg ml⁻¹ ampicillin). Three YPD plates per sample were inoculated and incubated at 25 °C until yeast colonies emerged. Single yeast colonies were picked and streaked for purification. 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 analysis

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 ITS4 and ITS5; SSU1f, SSU4r, SSU3f, and SSU2r; NL1 and NL4 (White et al. 1990; Kurtzman and Robnett 2003; Polburee et al. 2017). Amplicons were puriﬁed with the FavorPrep™ 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 sequences generated during the study 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). A phylogenetic tree was constructed based on concatenated sequences of SSU rRNA and D1/D2 region of LSU rRNA genes, using the Maximum-Likelihood (ML) algorithm and GTR + F + R2 model in IQ-TREE software v. 1.6.7 (Nguyen et al. 2015).

Results and discussion

Termite identification and yeast isolation

Termite heads were used for molecular identification. Mitochondrial 16S rRNA gene sequencing identified the host termites as Odontotermes horni (SMT1 OL629227, SMT2 OL629228). Several yeast colonies appeared on YPD agar plates after 2 days of incubation at 25 °C. Thirteen yeast strains were obtained from SMT1, while 17 were obtained from SMT2 (Table 1). Strains SMT1.3 and SMT1.10 were isolated from the SMT1 sample on two separate plates, while SMT2.2 was isolated from the SMT2 sample.

Sequence comparison and species delineation

All 30 yeast strains were subjected to MSP-PCR-based screening, and 11 representative yeast strains were processed for identification based on D1/D2 LSU rDNA sequencing (Table 1 and Fig. S1). The strains SMT1.3, SMT1.10, and SMT2.2 were isolated from the termite O. horni, among other ascomycetous yeast species. These three strains were identified up to species level by analyzing the D1/D2 LSU rDNA sequences (Table 1). When comparing sequences of the D1/D2 domains, the new species differed from the closely related species N. siamensis DMKU-RK467^T (NG_060235) by 1.9% sequence divergence (11 substitutions 0 gaps) and N. holstii CBS 6225 by 2.9% divergence (16 substitutions and 1 gap) in a 569 bp aligned region. The new species showed 0.6% divergence in SSU rDNA sequence compared to N. siamensis DMKU-RK467^T (8 substitutions and 1 gap) and 2.2% (29 substitutions and 1 gap) sequence divergence with N. holstii NRRL Y-2155 in a 1312 bp aligned region. The strains SMT1.3, SMT1.10, and SMT2.2 showed 100% sequence similarity with the same phenotypic characteristics, indicating that they are conspecific. The number of nucleotide substitutions in the D1/D2 region of LSU rDNA and SSU rDNA regions warrants considering these strains as a novel species in the genus Nakazawaeae.

Moreover, the phylogenetic tree reconstructed from the combined sequences of the SSU rRNA and the D1/D2 region of the LSU rRNA genes (Fig. 1) confirmed

| Termite            | No. of isolates obtained | Representative strains | Yeast strain code | % Similarity (D1/D2 LSU rDNA) with type species | Yeast species                  | GenBank accession number |
|--------------------|--------------------------|------------------------|-------------------|-----------------------------------------------|-------------------------------|--------------------------|
| SMT1 Odontotermes horni | 13                       | SMT1.1                 | 98.36             | Yamadazyna sp.                               | MZ234241                      |
|                    |                          | SMT1.2                 | 99.45             | Cyberlindnera bimundalis                      | MZ983382                      |
|                    |                          | SMT1.3                 | 98.07             | Nakazawaeae 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             | Nakazawaeae sp.                              | MZ674393                      |
| SMT2 Odontotermes horni | 17                       | SMT2.1                 | 98.35             | Yamadazyna sp.                               | MZ983395                      |
|                    |                          | SMT2.2                 | 98.02             | Nakazawaeae sp.                              | MZ674394                      |
|                    |                          | SMT2.3                 | 100               | Candida silvanorum                            | MZ983394                      |
|                    |                          | SMT2.17                | 99.43             | Cyberlindnera bimundalis                      | MZ983383                      |
the separation of the new strains SMT1.3, SMT1.10, and SMT2.2 from the other species of the genus *Nakazawaea* and its close relatedness to *N. siamensis*. Following the International Code of Nomenclature rules for algae, fungi, and plants, the designation *forma asexualis* (f.a.) was included, as recommended by Lachance (2012). Therefore, 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 2.5–5.0 × 3.0–5.0 μm (Fig. 2b), while those of *N. siamensis* measured 3.0–5.0 × 3.0–5.0 μm. Both species show multilateral budding, but the buds are predominantly polar in *N. odontotermitis* sp. nov. (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, nitrite, 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, and galactitol, while the new species could not utilize these compounds (Table 2). 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 are predominantly associated with plant material and insects. However, a few species like *N. anatomiae*, *N. ishiwadai*, 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*

| Table 2 Physiological characteristics differentiating *Nakazawaea odontotermitis* sp. nov. from *N. siamensis* DMKU-RK467T |
|---------------------------------------------------------------|
| **Characteristics**                                           | *N. odontotermitis* | *N. siamensis* *a* |
| Growth on carbon compounds                                    |                 |               |
| Inulin                                                        |               | +             |
| d-Galactose                                                   | W              | S             |
| Cellobose                                                     | S d           | W c           |
| D-Sorbitose                                                   | +              |               |
| 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                                                       | +              | −             |
| Nitrite                                                       |                | −             |
| Ethylamine                                                    | W              | +             |
| L-Lysine                                                      | S d           | W c           |
| Growth on other compounds                                     |                 |               |
| 2-keto-d-gluconate                                            | +              | W             |
| Cycloheximide (0.1%)                                          | +              |               |
| 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; b SMT1.10; c SMT2.2

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

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 *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 a novel species, *N. odontotermitis*, were isolated from the gut of termites (*O. horni*) feeding on wood. The occurrence of multiple isolates of this 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 host nutrition. A large proportion of the species in this genus have been isolated from wood materials or insects, indicating that these might be potential habitats for the isolation of *Nakazawaea* species.

**Taxonomy**

**Description of *Nakazawaea odontotermitis* 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 yeast colony is circular, white with an opaque sector, shiny, smooth, butyrous, and an entire margin (Fig. 2a). After 3 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 multi-lateral, 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, d-xylene, and cellobiose. The following carbon compounds are assimilated: d-glucose, d-galactose (weak), l-sorbitose, sucrose, maltose, cellobiose (slow and weak), salicin, α, α-trehalose, melezitose, soluble starch, d-xylene, l-arabinose, d-arabinose, l-rhamnose (weak), d-ribose, ethanol, glycerol, ribitol (weak), d-mannitol, d-sorbitol, 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, nitrite, and cadaverine. However, lactose, melibiose, raffinose, inulin,
methyl-α-D-glucoside, galactitol, erythritol, β-D-lactate, methanol, D-glucuronic acid, myo-inositol, and hexadecane are not assimilated (Table 2 and Table S1). 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, and 37 °C, but not at 40 °C (Table 2 and Table S1). Starch-like compounds and acid production are absent. Diazonium blue B color and urease reactions are negative.

The type strain of *N. odontotermitis* sp. nov. SMT1.3 (=MTCC 13,105) was isolated from the gut of *O. horni* in Kapare, Pune, Maharashtra (India). It is deposited 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). An ex-type was deposited in the Portuguese Yeast Culture Collection, Caparica, Portugal (PYCC 9153). The GenBank accession numbers of the ITS, LSU, and SSU rDNA sequences are MZ234239, MZ234240, and OK384663. The Mycobank number is MB 841926.

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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 this study are available at the Microbial Type Culture Collection and Gene Bank (MTCC)-Institute of Microbial Technology (IMTECH), Chandigarh, India; National Fungal Culture Collection of India (NFCCI), Pune, India; and Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal.

**Declarations**

**Conflict of interest** The authors declare that there are no conflicts of interest.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Ethics approval** Not applicable.

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