# Detection and isolation of a new member of Burkholderiaceae-related endofungal bacteria from a new thermophilic species in Mucorales

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## Abstract

Thermophilic fungi in *Mucorales* (*Mucoromycotina*) have the potential to be opportunistic pathogens, causing mucormycosis. Among them, *Burkholderiaceae*-related endobacteria (BRE) are rarely found and the known range of hosts is limited to *Rhizopus* spp. The phylogenetic divergence of BRE has recently expanded in other fungal groups such as *Mortierella* spp. (*Mortierellomycotina*); however, it remains unexplored in *Mucorales*. Here, we found a thermophilic mucoralean fungus obtained from a litter sample collected from Haha-jima Island in the Ogasawara (Bonin) Islands, Japan. The fungus was morphologically, phylogenetically, and physiologically characterized and proposed as a new species, *Saksenaea boninensis* sp. nov. Besides the fungal taxonomy, we also found the presence of BRE in isolates of this species by diagnostic PCR amplification of the 16S rRNA gene from mycelia, fluorescence microscopic observations, and isolation of the bacterium in pure culture. Phylogenetic analysis of the 16S rRNA gene of BRE revealed that it is distinct from all known BRE. The discovery of a culturable BRE lineage in the genus *Saksenaea* will add new insight into the evolutional origin of mucoralean fungus-BRE associations and emphasize the need to pay more attention to endofungal bacteria potentially associated with isolates of thermophilic mucoralean fungi causing mucormycosis.


## Introduction

Bacterial endosymbionts are commonly found in eukaryotes including broad fungal lineages, and such fungus-bacterium interactions have been widely recognized in mycology and environmental microbiology (Bonfante and Desirò 2017; Robinson et al. 2021). These bacterial endosymbionts are known as endofungal or endohyphal bacteria, in which the family *Burkholderiaceae* associated with *Mucoromycota* is a more extensively studied bacterial lineage (Bonfante and Desirò 2017). In *Mucoromycota*, each bacterial genus of *Burkholderiaceae*-related endobacteria (BRE) such as *Mycetohabitans* spp., *Mycoavidus* spp., and '*Candidatus* Glomeribacter gigasporarum' was found in three subphyla: *Mucoromycotina*, *Mortierellomycotina*, and *Glomeromycotina*, respectively (Bonfante and Desirò 2017). BRE associated with *Mortierella* spp. (*Mortierellomycotina*) and *Gigasporaceae* fungi (*Glomeromycotina*) are phylogenetically diverse, and the host ranges of each BRE lineage cover multiple genera and species (Desirò et al. 2014; Mondo et al. 2012; Takashima et al. 2018). On the other hand, the host range of BRE in *Mucorales* is very limited and BRE such as *Mycetohabitans* spp. are mainly harbored by *Rhizopus microsporus* as its fungal host (Partida-Martinez et al. 2007). *Rhizopus microsporus* is a characteristic host among BRE-harboring fungi because of the nature of thermophilic fungi. Thermophilic fungi are primarily found in *Mucorales* including species obtained in clinical cases of medical mycology; however, the presence of BRE in these species or isolates of thermophilic mucoralean fungi are underinvestigated.

The genus *Saksenaea* is one of the thermophilic genera in *Mucorales*, while *Saksenaea* spp. are occasionally found in soil, water, and clinical specimens of animals and humans (Ajello et al. 1976; Alvarez et al. 2010; Crous et al. 2016; Crous et al. 2017; Saksena 1953; Singh and Kushwaha 2017). Similar to *Rhizopus* spp., some species of *Saksenaea* are capable of growing rapidly at human body temperature and even at over 40°C (Baijal 1967; Alvarez et al. 2010; Crous et al. 2017; Labuda et al. 2019). This characteristic leads to species of *Saksenaea* being opportunistic causal agents of mucormycosis, which is problematic with the pandemic of COVID-19 (Ajello et al. 1976; Chakrabarti and Singh 2014; Chander et al. 2018; Roden et al. 2005).

In the present study, four isolates of *Saksenaea* were obtained from a litter sample collected from Haha-jima Island in the Ogasawara (Bonin) Islands, with uniquely endemic fauna and flora that originated from isolated locations far from the main islands of Japan as oceanic islands. These isolates were morphologically, phylogenetically, and physiologically investigated. As the results, we determined these isolates as undescribed species in this genus; therefore, we proposed *Saksenaea boninensis* sp. nov. Besides the fungal taxonomy, we also detected intracellular bacteria in these isolates by fluorescence microscopy using nucleic staining and fluorescence *in situ* hybridization (FISH), and isolation of the bacteria in pure cultures. Phylogenetic analysis showed that the detected bacteria comprised a single lineage within the family *Burkholderiaceae*, but were distinct from all known BRE.

## Materials And Methods

# Fungal isolation

Litter was collected from the north port of Haha-jima Island in the Ogasawara (Bonin) Islands, Tokyo, Japan (N 26° 41' 40.4", E 142° 08' 49.7") on 9th November, 2018 (Fig. 1). The litter was directly spread onto $_{LC}$A (Miura agar) medium [0.2 g yeast extract (Difco, Sparks, MD, USA), 1 g glucose (Wako Pure Chemical Industries, Osaka, Japan), 2 g $NaNO_3$ (Wako), 1 g $KH_2PO_4$ (Wako), 0.2 g KCl (Wako), 0.2 g $MgSO_4 \cdot 7H_2O$ (Wako), 15 g Bacto agar (Difco) in 1 L distilled water] (Miura and Kudo 1970) and incubated at room temperature (ca. 23°C) under ambient light conditions in the laboratory. After 14-d incubation, sporangia of *Saksenaea* were produced on the medium and four isolates were established by inoculating sporangiospores produced in different sporangia onto fresh $_{LC}$A medium using a frame-sterilized fine needle and incubated at 30°C before use for further analyses. For the preparation of a dried specimen, the representative isolate Sak4 was incubated on Czapek-Dox agar medium (CZA) [30 g sucrose (Wako), 2 g $NaNO_3$ (Wako), 1g $K_2HPO_4$ (Wako), 0.5 g $MgSO_4 \cdot 7H_2O$ (Wako), 0.5 g KCl (Wako), 0.01 g $FeSO_4 \cdot 7H_2O$ (Wako), 15 g Bacto agar (Difco) in 1 L distilled water] for 1 month for sporulation and then inactivated by complete drying using a drying oven at 60°C for 3 d, and deposited in the Kanagawa Prefectural Museum of Natural History (KPM, Kanagawa Pref., Japan). A living culture of the isolate was deposited in the Japan Collection of Microorganisms (JCM, Ibaraki Pref., Japan), the NITE Biological Resource Center (NBRC, Chiba Pref., Japan), and the CBS-KNAW culture collection (CBS, Utrecht, The Netherlands).

# Colony growth measurement

Prior to the measurement, a representative isolate was pre-incubated on $_{LC}$A medium for 4 or 10 d at 30°C. Then, a disc was cut out from the incubated mycelia using an autoclave-sterilized plastic straw (8 mm diam) as a substitute for a cork borer and placed onto CZA (90-mm-diam petri dish) with at least three replicates. The plates were incubated for 4 d at 13, 15, 18, 23, 25, 28, 30, 35, 37, 40, and 42°C and the colony diameter was measured daily.

# Morphological observation

The representative isolate Sak4 was incubated for 1 month on CZA for sporulation at 23°C. Morphological observation was carried out using a stereomicroscope (M205C, Leica, Germany) and a light microscope (BX51, Olympus Corp., Tokyo, Japan) equipped with a digital camera (DP25, Olympus Corp.). The fungal materials were mounted in distilled water or lactic acid.

# DNA extraction, PCR, and sequencing of fungal isolates

Template DNA was extracted from seven-day-old mycelia of each isolate incubated on a sterilized cellophane sheet placed in half-strength cornmeal-malt-yeast agar (1/2 CMMY) agar [8.5 g corn meal agar (Difco), 10 g malt extract (Difco), 1 g yeast extract (Difco), 7.5 g Bacto agar (Difco) in 1 L distilled water] using the Prepman™ Ultra sample reagent (Applied Biosystems, Foster City, CA, USA) in accordance with Sato et al. (2010). The ITS1-5.8S-ITS2 (ITS) and partial large subunit ribosomal RNA (LSU) gene regions and the partial transcription elongation factor 1-α (*tef1*) gene of each isolate were also amplified. Regarding the PCR amplification of ITS and partial LSU regions, 50 μL of a PCR mixture containing 1.0 μL of template DNA, 1.5 μL of each primer solution of the fungal universal primers ITS5 and LR5 (10 pmol $\mu L^{-1}$ each, White et al. 1990; Vilgalys and Hester 1990), 10 μL of 2 mM dNTPs, 1.0 μL of 1.0 U $\mu L^{-1}$ KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan), 25 μL of 2×PCR Buffer for KOD FX Neo DNA polymerase, and 10 μL of sterilized deionized water was prepared. PCR amplification was performed as follows: initially 2 min for 94°C, followed by 30 cycles of 98°C for 10 s, 58°C for 30 s, and 68°C for 1 min using a thermal cycler. Cycle sequence reaction was performed with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. Cycle sequencing of the PCR products of ITS5-LR5 was performed using ITS5, ITS3, ITS4, LR0R, and LR5 primers (White et al. 1990; Vilgalys and Hester 1990). For the partial *tef1* gene, the PCR mixtures as described above with two primer sets, 526F-1567R and 983F-2218R (Rehner and Buckley 2005), were separately prepared. PCR amplification conditions were the same as those shown above except the annealing temperatures were set as 55°C and 58°C, respectively, and the number of cycles was set as 35. Cycle sequencing of the PCR products of 526F-1567R and 983F-2218R primer sets was performed using 526F and 1567R, and 983F, 2218R, and MEF11 primers, respectively (Rehner and Buckley 2005; O'Donnell et al. 2001). Cycle sequencing products were purified by ethanol precipitation, and electrophoresis was performed using the Applied Biosystems 3130xl genetic analyzer (Applied Biosystems) to determine nucleotide sequences. The DNA sequences obtained from each primer of each gene region (DNA sequences using 526F and 983F as sequencing primers were not obtained) were assembled into a single sequence using GeneStudio Professional software version 2.2.0.0 (http://www.genestudio.com/).

# Diagnostic PCR of the endofungal bacterium

DNA extracted from fungal mycelia was also used for detection of the 16S rRNA gene of the endofungal bacteria. For PCR amplification of the 16S rRNA gene, a PCR mixture with the bacterial universal primers 10F and 1541R (Takashima et al. 2018) was prepared and PCR amplification was performed under the same conditions as described in Takashima et al. (2018). Cycle sequencing of PCR products of 10F-1541R was performed using 10F, 341F, 800F, 926R, and 1541R primers (Lane et al. 1991; Muyzer et al. 1993; Takashima et al. 2018). DNA sequencing and assembly of the single sequence were performed in the same way as above.

# Fluorescence microscopy

To observe endofungal bacteria inside fungal cells, we performed fluorescence microscopic observations using the nucleic staining reagent LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) and FISH with the Cy3-labeled bacterial universal probe EUB338 in accordance with Takashima et al. (2018). All fluorescence images were obtained using a fluorescence microscope (BX51, Olympus Corp., Tokyo, Japan) equipped with the digital camera EOS kiss X7i (Canon, Tokyo, Japan).

# Isolation of the endofungal bacterium

Seven-day-old mycelia of each isolate incubated on a sterilized cellophane sheet placed in 1/2 CMMY agar at 30°C were axenically collected from 3 plates (55-mm diam). Fresh mycelia (300–370 mg) of each isolate were homogenized by pestle and filtrated using jointed 8-μm and 3-μm membrane filters following the method of Desirò et al. (submitted). One milliliter of the filtrated bacterial suspension of each isolate was separately added to buffered charcoal yeast extract with 0.1% α-ketoglutalate (BCYEα) medium (Oxoid, Hampshire, UK) and incubated at 23 and 30°C. After 7-d incubation, a drop of the liquid layer of the culture plates incubated at 30°C was streaked onto fresh BCYEα medium and then a single colony was obtained after 5-d incubation at 30°C. Seven-day-old culture on BCYEα medium at 30°C of the endofungal bacterium of each fungal isolate was used for DNA extraction with lysozyme, as in our previous study (Sharmin et al. 2018). Extracted DNA was used for PCR of the 16S rRNA gene, and the sequences were determined in the same way as above.

# Culturability of the endofungal bacterium

The bacterial isolate was pre-incubated on BCYEα medium at 30°C for 7 d. Then, the colony on the medium was inoculated onto media including BCYEα medium, Luria-Bertani (LB) agar [25 g Luria-Bertani broth (Difco), 15 g agar (Difco) in 1 L distilled water], 1/10 nutrient broth agar [0.8 g nutrient broth (Difco), 15 g agar (Difco) in 1 L distilled water], R2A agar [3.2 g R2A Broth "DAIGO" (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 15 g agar (Difco) in 1 L distilled water]. These inoculated plates were incubated at 30°C for 14 d and colony growth was checked after 5, 10, and 14 d.

# Phylogenetic analyses of the fungal host and endofungal bacterium

For the fungal host, ITS, LSU, and *tef1* gene sequences of *Saksenaea* spp. and *Apophysomyces elegans* were obtained from GenBank (Table 1). These sequences and ITS, LSU, and *tef1* gene sequences obtained from the fungal isolates were aligned independently for each region using MAFFT v7.212

(Katoh and Standley 2013). The obtained alignment blocks were subject to Gblocks 0.91b (Castresana 2000) to remove poorly aligned positions with the relaxed selection setting described in Talavera and Castresana (2007) using the following parameters (-t = d -b2 = 9 -b3 = 10 -b4 = 5 -b5 = h). After automatically removing gaps, the alignment blocks were viewed using MEGA 6.06 software (Tamura et al. 2013) and poorly aligned positions at either end of the alignments were removed manually. The alignment blocks for each gene region were then concatenated (552 positions for ITS, 637 positions for LSU, and 476 positions for *tef1*, resulting in 1665 positions in the final data set) and compared using the partition model in Kakusan4 (released on 4.0.2015.01.23; Tanabe 2011). All ML phylogenetic analyses were performed using RAxML version 8.1.5 (Stamatakis 2014) with a 1,000 bootstrap analysis. For the concatenated dataset, the partition model was set as the "separate" model selected (lowest AIC) by Kakusan4. The nucleotide substitution model for the concatenated dataset was set as the GTRGAMMA model following the default setting of Kakusan4. The nucleotide substitution model for each region was set as the GTRGAMMAI model selected in MEGA 6.06. *Apophysomyces elegans* was used as the outgroup.

Table 1
Newly determined sequence of *Saksenaea boninensis* (bold) and sequences retrieved from GenBank for phylogenetic analyses.

| Taxon | Strain No. | | ITS | LSU | *tef1* |
|---|---|---|---|---|---|
| *Apophysomyces elegans* | CBS 476.78, NRRL22325 | ex-type | FN556440 | FN554249 | AF157231 |
| *Saksenaea boninensis* | Sak3 | | **MK757862** | **MK757858** | **LC474956** |
| *Saksenaea boninensis* | Sak4, JCM 39173, NBRC 114970, CBS 147591 | ex-type | **MK757863** | **MK757859** | **LC474957** |
| *Saksenaea boninensis* | Sak5 | | **MK757864** | **MK757860** | **LC474958** |
| *Saksenaea boninensis* | Sak6 | | **MK757865** | **MK757861** | **LC474959** |
| *Saksenaea dorisiae* | BiMM-F232 | ex-type | MK559697 | MK570305 | MK569515 |
| *Saksenaea erythrospora* | CBS 138279 | | KM102733 | KM102734 | KM102735 |
| *Saksenaea erythrospora* | CIMCE001 | | KU951560 | - | - |
| *Saksenaea erythrospora* | FMR 13392 | | KR527481 | - | - |
| *Saksenaea erythrospora* | FMR 13516 | | KR527482 | - | - |
| *Saksenaea erythrospora* | FMR 13880 | | KR527483 | - | - |
| *Saksenaea erythrospora* | M-1024/14 | | - | KR527484 | - |
| *Saksenaea erythrospora* | M-340/14 | | - | KR527485 | - |
| *Saksenaea erythrospora* | RTCC239110910 | | JF433911 | JF433912 | - |
| *Saksenaea erythrospora* | UTHSC 06-576 | | FR687331 | HM776683 | HM776694 |
| *Saksenaea erythrospora* | UTHSC 08-3606 | ex-type | NR_149333 | NG_059935 | HM776691 |
| *Saksenaea erythrospora* | UZ1908 15 | | KU321692 | KU321691 | - |
| *Saksenaea loutrophoriformis* | M-1012/15 | | LT796164 | LT796165 | LT796166 |
| *Saksenaea loutrophoriformis* | UTHSC 08-379 | ex-type | FR687330 | HM776682 | HM776693 |
| *Saksenaea oblongispora* | CBS 133.90 | ex-type | NR_137569 | NG_057868 | HM776687 |
| *Saksenaea trapezispora* | UTHSC DI 15 – 1, CBS 141687 | ex-type | NR_147690 | LT607407 | LT607408 |
| *Saksenaea vasiformis* | AJ1-1 | | MH059541 | - | - |
| *Saksenaea vasiformis* | ATCC 28740 | | FR687322 | HM776674 | HM776685 |
| *Saksenaea vasiformis* | ATCC 60625 | | FR687323 | HM776675 | HM776686 |
| *Saksenaea vasiformis* | CNRMA 05.1337 | | EU182902 | - | - |
| *Saksenaea vasiformis* | CNRMA 07.577 | | EU644757 | EU644756 | - |
| *Saksenaea vasiformis* | CNRMA 08 1143 | | KP132600 | - | - |
| *Saksenaea vasiformis* | CNRMA F/9–83 | | FR687325 | HM776677 | HM776688 |
| *Saksenaea vasiformis* | F5 | | MF187627 | - | - |
| *Saksenaea vasiformis* | FMR 10131 | | FR687326 | HM776678 | HM776689 |
| *Saksenaea vasiformis* | NRRL 2443 | ex-type | FR687327 | HM776679 | AF157291 |
| *Saksenaea vasiformis* | PHF-MC2 | | MK346253 | - | - |
| *Saksenaea vasiformis* | PHF-MC200 | | MK501620 | - | - |
| *Saksenaea vasiformis* | PHF-MC201 | | MK499472 | - | - |
| *Saksenaea vasiformis* | PWQ2338 | | KP132601 | - | - |
| *Saksenaea vasiformis* | TN254AU15 | | KU314816 | - | - |
| *Saksenaea vasiformis* | UTHSC 09-528 | | FR687329 | HM776681 | HM776692 |
| *Saksenaea vasiformis* | UTHSC R-2974 | | FR687332 | HM776684 | HM776695 |

For the endofungal bacterium, 16S rRNA gene sequences of *Burkholderiaceae*-related endobacteria and other sequences related to the family *Burkholderiaceae* were obtained from GenBank with the accession numbers shown beside each taxon name in Additional file 1: Fig. S1. These sequences and 16S rRNA gene sequences of the endofungal bacterium obtained from fungal mycelia and pure cultures as DNA templates were aligned, and poorly aligned positions in the alignment block were removed automatically and manually as described above. Subsequently, the alignment block (1329 positions) was used for model selection for ML phylogeny using MEGA 6.06. The ML phylogenetic analysis was performed using RAxML version 8.1.5 with a 1,000 bootstrap analysis. The nucleotide substitution model was set as the GTRGAMMAI model selected in MEGA 6.06.

## Results

# Identity of the fungal host

The representative isolate Sak4 obtained from litter collected from Haha-jima Island located in the Ogasawara Islands showed the unique morphology of sporangium having a long neck (Fig. 1), which is one of the characteristic morphologies of the genus *Saksenaea* in *Mucorales* (Saksena 1953). Six species (*S. dorisiae*, *S. erythrospora*, *S. loutrophoriformis*, *S. oblongispora*, *S. trapezispora*, and *S. vasiformis*) are currently recognized in this genus (Alvarez et al. 2010; Crous et al. 2016; Crous et al. 2017; Labuda et al. 2019; Saksena 1953). The genus *Saksenaea* is also known as one of the thermophilic fungi in *Mucorales* and the maximum growth temperature exceeds over 40°C in four species (Alvarez et al. 2010; Baijal 1965; Crous et al. 2017). The growth range of the representative isolate was determined as 13–37°C on CZA (Fig. 2). The growth at the threshold low and high temperatures (15 and 37°C, respectively) of the genus was ca. 6.0 mm/d (ca. 22 mm after 4 d on CZA) and ca. 3.7 mm/d (ca. 20 mm after 4 d on CZA), respectively. The lower maximum growth temperature (less than 40°C) was similar to that of *S. dorisiae* and *S. trapezispora* (Crous et al. 2016; Labuda et al. 2019). The minimum growth temperature of the representative isolate (13°C) was closer to that of *S. dorisiae* (12°C) than that of *S. trapezispora* (15°C). The growth of the representative isolate at the higher temperature (37°C) was 19–23 mm on CZA for 4 d, which was slightly slower than that of *S. dorisiae* (25–30 mm). Detailed morphological comparisons among the representative isolate and these physiologically related species showed that the isolate closely resembled these two species morphologically. However, the morphologies can be distinguished from those of *S. trapezispora* by the longer sporangium and neck (59.6–193.9 vs. 50–140 µm, respectively), and ellipsoidal to cylindrical sporangiospores (4.6–9.7 × 2.7–5.6 vs. 5.5–7.5 × 3.5–4 µm, respectively). The morphologies can be distinguished from those of *S. dorisiae* by having the longer neck (38.0–165.1 vs. 70–100 µm, respectively) and the slightly longer and wider sporangiospores (4.6–9.7 × 2.7–5.6 vs. 5.0–5.5 × 2.5–3.0 µm, respectively).

The Maximum likelihood (ML) phylogenetic trees showed that the isolates were phylogenetically identical to each other (Fig. 3, Additional file 2–4: Fig. S2–4). The isolates were located in a highly supported clade, "clade 3" defined by Alvarez et al. (2010), containing isolates of *S. dorisiae*, *S. oblongispora*, and *S. trapezispora*. This result suggests that the present isolates were phylogenetically close to these species. However, the present isolates were also distinct from these species. Therefore, the present isolates were morphologically, physiology, and phylogenetically distinguishable from known species and proposed as a new species, *S. boninensis*.

# Taxonomy

Saksenaea boninensis Y. Takash., K. Narisawa, sp. nov.

MycoBank no.: MB 843122

Figures 1, 2.

Diagnosis: The longer neck of sporangia, longer and wider sporangiospores, and slightly more sensitive growth at the threshold high temperature (37°C) are distinctive characters of this species compared with *S. dorisiae*.

Type: Japan, Tokyo, Ogasawara Islands, Haha-jima Island, Ogasawara-mura, Higashidai, near the north port, isolated from a culture plate ($_{LC}$A medium) directly inoculated with litter by Y. Takashima on 1 Dec 2018; dried fungal materials on CZA (Holotype, KPM-NC0028612), ex-holotype strain (Sak4 = JCM 39173 = NBRC 114970 = CBS 147591).

Gene sequence ex-holotype: MK757863 (ITS), MK757859 (LSU), LC474957 (*tef1*).

Etymology: boninensis, referring to the Bonin Islands (Ogasawara Islands), the geographic origin of the type culture.

Colonies fast growing, filling the 90-mm-diameter petri dish after 4 d of incubation (ca. 20 mm/d) on CZA at the optimum growth temperatures (28 and 30°C), hyaline, with scarce aerial mycelia. The minimum growth observed at 13°C (ca. 3.3 mm/d, ca. 15 mm after 4 d on CZA). No growth observed above 40°C. Mycelia 1.9–10.3 (Mean ± SD = 4.7 ± 2.0) µm wide. Sporulation abundant on CZA at 23°C. Sporangia generally single, rarely in twos, erect, developed at the end of a hyphal branch with a dichotomously branched rhizoidal structure below, rhizoids, hyaline, 2.9–5.8 (Mean ± SD = 4.3 ± 0.6) µm wide, stalk, brownish, 4.9–13.3 (Mean ± SD = 8.2 ± 2.2) × 44.9–89.4 (Mean ± SD = 68.0 ± 11.2) µm, flask-shaped with a brownish spherical venter, 18.8–41.3 (Mean ± SD = 28.9 ± 5.7) × 21.4–50.4 (Mean ± SD = 34.4 ± 6.8) µm, with a distinct dome-shaped columella; venter surmounted by a brownish long neck, 5.5–11.9 (Mean ± SD = 8.9 ± 1.3) × 38.0–165.1 (Mean ± SD = 93.2 ± 26.5) µm (venter + neck: 59.6–193.9 (Mean ± SD = 124.0 ± 31.2) µm long), apex of the neck slightly broader, 8.2–15.2 (Mean ± SD = 12.0 ± 1.8) µm in diameter, closed with a mucilaginous plug, which is gradually dissolved when mature. Surface of brownish parts of sporangia (stalk, venter, and neck) asperulate, ornamented with fine spines, dissolved in lactic acid within 12 h. Sporangiospores hyaline, ellipsoidal to cylindrical, 4.6–9.7 (Mean ± SD = 6.8 ± 1.1) × 2.7–5.6 (Mean ± SD = 3.5 ± 0.5) μm [Q (quotients of spore length and width) = 1.4–2.7, $Q_m$ (the mean Q value) = 1.9]. Zygospores unknown.

**Note** A *Burkholderiaceae*-related endobacterium has been associated with isolates of this species since the isolates were obtained. However, the morphological differences between isolates with/without the endofungal bacterium, such as sporulation of sporangiospores observed in *R. microsporus* (Lackner et al. 2011), were not examined in this study.

**Discovery of new BRE lineage from isolates of** Saksenaea

As the survey for the endofungal bacteria associated with *Mucoromycota*, all isolates obtained from different sporangia were subjected to PCR amplification of 16S rRNA using the DNA templates prepared from fungal mycelia. Since positive amplification was confirmed in all isolates, fluorescence microscopic observations were conducted to confirm the presence of endofungal bacteria. FISH observation of the representative isolate using the Cy3-labeled bacterial universal probe showed the presence of the endofungal bacterium within hypha (Fig. 4). The observation of the endofungal bacterium using the nucleic staining reagent also showed that the endofungal bacterium was present throughout the asexual stage, such as aerial mycelium, rhizoid, stalk, sporangium, sporangiospore, and geminated sporangiospore (Fig. 5). These observations strongly suggested that the amplification of 16S rRNA was derived from the endofungal bacterium, which was vertically transmitted through asexual sporogenesis of the fungal host. The 16S rRNA gene sequences determined from PCR amplicons with fungal mycelia of each isolate were identical to each other. ML phylogeny of the 16S rRNA gene clearly showed that the endofungal bacterium was phylogenetically related to the family *Burkholderiaceae* (Fig. 6, Additional file 1: Fig. S1). However, the phylogenetic position of the endofungal bacterium was not clustered with either “Glomeribacter-Mycoavidus clade” or *Mycetohabitans* spp. (Fig. 6). This result indicates that the endofungal bacterium, which thrived within isolates of *S. boninensis* was the new BRE lineage (hereafter, named “SakBRE”).

# Culturability of SakBRE

Because *Mycetohabitans* spp. associated with *R. microsporus* are known to be culturable (Partida-Martinez et al. 2007), SakBRE has the potential to be culturable. To attempt bacterial isolation, a filtrated bacterial suspension of SakBRE obtained from each isolate was inoculated onto BCYEα medium. As expected, bacterial growth by visually increasing the bacterial density in the filtrates was confirmed after 3 to 4 d at 30°C and after 8 d at 23°C, respectively. The clouded filtrates were streaked onto fresh BCYEα medium and incubated at 30°C, and then pure cultures were successfully established from single colonies obtained after incubation for at least 5 d (Fig. 7). The 16S rRNA gene sequences obtained from the pure cultures were identical to the sequences detected from fungal mycelia of the host (Fig. 6). We also checked the culturability in several media by streaking the obtained pure cultures on BCYEα medium, LB agar, 1/10 nutrient broth agar, and R2A agar media, and incubating at 30°C. Bacterial growth was confirmed after at least 5d on BCYEα medium (Fig. 7), while growth was not observed on the other three media even after 14 d.

## Discussion

The number of species of the genus *Saksenaea* has recently increased; however, its diversity is not fully understood. In Japan, the occurrence of *Saksenaea* was very rarely reported, while no clinical cases of mucormycosis caused by *Saksenaea* spp. have been reported (Mori et al. 2011). *Saksenaea vasiformis* was only found in soil from the Ryukyu Islands previously (Watanabe 1971; Tubaki et al. 1990). The isolate 69–323 obtained by Watanabe (1971) was identified as *S. vasiformis*, and morphologies such as the length of the neck (ca. 32.5 μm) and size of sporangiospores (up to 5.0 × 2–2.5 μm) were not similar to the present species (Watanabe 2010). Chien et al. (1992) isolated *S. vasiformis* from sources near marine environments such as soil around the seashore in Taiwan and unidentified intertidal driftwoods along the seacoast in Ethiopia. Our isolation of *Saksenaea* from the north port of Haha-jima Island may indicate that this genus is likely to prefer places with a subtropical oceanic climate in Japan and has an affinity for marine environments as one of its natural habitats.

To date, screenings of endofungal bacteria with broad-ranging fungal hosts at the generic level in *Mucorales* have been conducted in few studies. Schmitt et al. (2008) screened over 300 zygomycete strains other than *R. microsporus* (the exact number of genera used was not shown in the article) and they concluded that endofungal bacteria were limited to detection in *R. microsporus*. On the other hand, Okrasińska et al. (2021) screened 71 strains containing 10 genera in *Mucorales*, and they detected *Mycetohabitans* spp. and *Paraburkholderia* sp. from 3 of *Rhizopus* and 1 of *Mucor* strains, respectively. The comprehensive screening of bacteria in 64 strains of *Rhizopus* spp. resulted in the detection of *Mycetohabitans* spp. and *Burkholderia* sp. (Dolatabadi et al. 2016). In a recent comprehensive study of bacteriomes of fungi, 4 strains (including 2 genera, *Mucor* and *Rhizopus*) and 8 species (including 8 genera) of *Mucorales* genomes were screened by a 16S rRNA gene amplicon sequencing and bioinformatic screening, respectively, and numerous bacterial lineages were detected by both analyses (Robinson et al. 2021). However, one genus (*Cupriavidus*) from a strain of *Mucor* sp. was only listed as the detected bacteria in the family *Burkholderiaceae* (Robinson et al. 2021). These screening results suggest that the bacterial lineage regarded as BRE, strictly comprised of endofungal bacteria such as *Mycetohabitans*, are only detected from *Rhizopus* spp. Even though the genus *Saksenaea* is known to be thermophilic, similar to *Rhizopus*, this genus is completely overlooked as fungal hosts. Therefore, the finding of the phylogenetically distinct lineage, SakBRE, from the genus *Saksenaea* shown in the present study is notable. The origin and evolution of BRE associated with *Mucoromycota* remain unclear. Bonfante and Desirò (2017) proposed two hypothetical scenarios of bacterial invasion (early vs. late) on the basis of the origin of the associations between BRE and *Mucoromycota*. Our discovery of SakBRE may support the late bacterial invasion scenario of BRE among members of *Mucoromycota*. Our results expand known hosts of BRE in *Mucorales* into the two genera *Rhizopus* and *Saksenaea*, which are classified as different fungal families (*Rhizopodaceae* and *Saksenaeaceae*) and phylogenetically distinct (Hoffmann et al. 2013). This distinct host range adds to the enigma of the origin and evolution of BRE associated with *Mucorales*. Further studies such as a comparative genomics study of SakBRE and *Mycetohabitans* spp. are suggested by this study, which will add new insight into whether these BRE have a single common evolutionary ancestor.

Currently, four BRE species including two species each of the genera *Mycoavidus* and *Mycetohabitans*, respectively, are known to be culturable (Partida-Martinez et al. 2007; Ohshima et al. 2016; Guo et al. 2020). *Mycoavidus* spp. require cysteine for growth and can only be cultured on BCYEα medium (Ohshima et al. 2016; Guo et al. 2020). For its isolation, incubation for 7 d at 30°C and 30 d at 23°C is required for *M. cysteinexigens* B1-EB$^T$ and *Mycoavidus* sp. B2-EB on the medium, respectively (Ohshima et al. 2016; Guo et al. 2020). On the other hand, the growth of *Mycetohabitans* spp. was faster than that of *Mycoavidus* spp. and these can be cultured within 2–3 days (Rohm et al. 2010) or several days (Scherlach et al. 2006) on conventionally used agar media such as nutrient agar, LB agar, tryptic soy agar, and potato dextrose agar (Partida-Martinez and Hertweck 2005; Scherlach et al. 2006; Partida-Martinez et al. 2007; Rohm et al. 2010). In comparison with these incubation conditions of BRE, the growth of SakBRE in the isolation step (3 to 4 d within filtrates and 5 d on plate at 30°C) is faster than that of *M. cysteinexigens* on BCYEα medium but is limited and slower than that of *Mycetohabitans* spp. on the other media. Further analysis such as whole-genome sequencing of SakBRE will be needed to clarify whether the fastidious character of SakBRE comes from a nutrient deficiency or other factors such as hydrogen peroxide generated within media, which is known to be decomposed by activated charcoal in BCYEα medium (Hoffman et al. 1983).

Mucormycosis has received much attention as “Black fungi” due to the increasing number of infections among patients affected by COVID-19 (Chavda and Apostolopoulos 2021; Singh et al. 2021). Thermophilic or thermotolerant mucoralean fungi including the eleven genera: *Actinomucor*, *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Rhizomucor*, *Rhizopus*, *Saksenaea*, *Syncephalastrum*, and *Thamnostylum* are known as causative agents of mucormycosis (Ajello et al. 1976; Chakrabarti and Singh 2014; Chander et al. 2018; Roden et al. 2005). In the present study, most species in “clade 3” including *S. boninensis* were isolated from non-animal substrates (litter, soil, and water) and sensitive to high temperatures except for *S. oblongispora*, and there have been no clinical cases in humans to date (Alvarez et al. 2010; Crous et al. 2016; Labuda et al. 2019). However, it remains a possibility that this species is an animal pathogen because its close relative, *S. trapezispora*, was isolated from the knee wound of a soldier (Crous et al. 2016).

*Mycetohabitans* spp. were previously detected from clinical isolates of *Rhizopus* spp. (Ibrahim et al. 2008; Partida-Martinez et al. 2008) and directly isolated from clinical specimens (Gee et al. 2011). The contributions of rhizoxin-producing *Mycetohabitans* spp. to *Rhizopus* virulence were debated previously (Ibrahim et al. 2008; Partida-Martinez et al. 2008), and it was demonstrated that such endobacteria are not essential for *Rhizopus* infection by comparing *Mycetohabitans*-harboring and -free strains in infection models, such as human endothelial cell, fly, and mouse models. On the other hand, recently in *R. microsporus*, *Ralstonia pickettii* was discovered as an endofungal bacterium (Itabangi et al. 2022). This bacterium is not capable of synthesizing rhizoxin but is required for *Rhizopus* virulence in both zebrafish and mouse models and reduces the sensitivity of the fungal host to the antifungal treatment Amphotericin B (Itabangi et al. 2022). Although it is still unclear whether SakBRE can produce harmful toxins and is involved in host virulence in mucormycosis, our results emphasize the importance of careful screening of endofungal bacteria in clinical isolates, especially *Rhizopus* and *Saksenaea* spp. and the related genus *Apophysomyces*, and other thermophilic mucoralean environmental isolates that are potentially causative agents of mucormycosis should be monitored.

## Conclusion

In the present study, we described a new species, *Saksenaea boninensis*, which was morphologically, physiology, and phylogenetically distinguishable from known species. In Japan, species of the genus *Saksenaea* was firstly isolated from the Ogasawara (Bonin) Islands other than the Ryukyu Islands. We showed the presence of a new BRE lineage (SakBRE) within the hyphae in this species, the vertical transmission through asexual sporogenesis by SakBRE, and the growth of SakBRE on the artificial media which was less fastidious than *Mycoavidus* spp. The discovery of new culturable BRE from *Mucorales* as well as *Rhizopus* spp. will add new insight into the evolutional origin of mucoralean fungus-BRE associations and emphasize the need to pay more attention to endofungal bacteria potentially associated with isolates of thermophilic mucoralean fungi causing mucormycosis.

## Abbreviations

1/2 CMMY
Half-strength cornmeal-malt-yeast agar
BCYEα
Buffered charcoal yeast extract with 0.1% α-ketoglutalate
BRE
*Burkholderiaceae*-related endobacteria
CZA
Czapek-Dox agar medium
ITS
Internal transcribed spacer region
LB
Luria-Bertani
LSU
Large subunit (28S) rRNA gene region
ML
Maximum-likelihood
SakBRE

A BRE lineage thrived within isolates of *Saksenaea boninensis*
*tef1*
Translation elongation factor 1-α gene.

## Declarations

**Ethics approval and consent to participate**

Not applicable.

**Adherence to national and international regulations**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and material**

Nucleotide sequences of the fungal isolates generated during this study were deposited in GenBank [MK757862–MK757865 (ITS), MK757858–MK757861 (LSU), LC474956–LC474959 (*tef1*)]. Nucleotide sequences of the 16S rRNA gene detected from fungal mycelia of each isolate and from pure cultures were deposited in GenBank (MK757866–MK757869 and MK761202–MK761205, respectively). Multiple sequence alignments for the phylogenetic analyses of *Saksenaea* spp. and the family *Burkholderiaceae* generated during this study are available in the Zenodo repository, https://doi.org/10.5281/zenodo.6303649.

The doi link is currently restricted for access and will be available after the acceptance. Please use a private link below for review process (This private link will be deleted in the final version for publication). https://zenodo.org/record/6303649?token=eyJhbGciOiJIUzUxMiIsImV4cCI6MTY0ODY4MTE5OSwiaWF0IjoxNjQ2MDkxNTIwfQ.eyJkYXRhIjp7InJlY2lkIjo2MzAzNjQ5fSwiaWQiOjIwOTI4LCJybmQiOiIxNzJjMWMxNiJ9.jD8RMQBpaZTVr8mLzctXlPdlI2dM5bvUqk6wF5uBfTEDfLENAi7a1hLox7X8J95-QzEFJine1HKfq8tCTb4oNA

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

Sampling: KY; experimental designs, fungal and bacterial isolations, culturing, microscopic observations, and phylogenetic analyses: YT; writing—fungal description: YT, KY, YD, and KN; writing—original draft preparation: YT and KN; writing—review and editing, YT, KY, YD, YG, TN, HO, and KN. All authors read and approved the final manuscript.

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## Figures

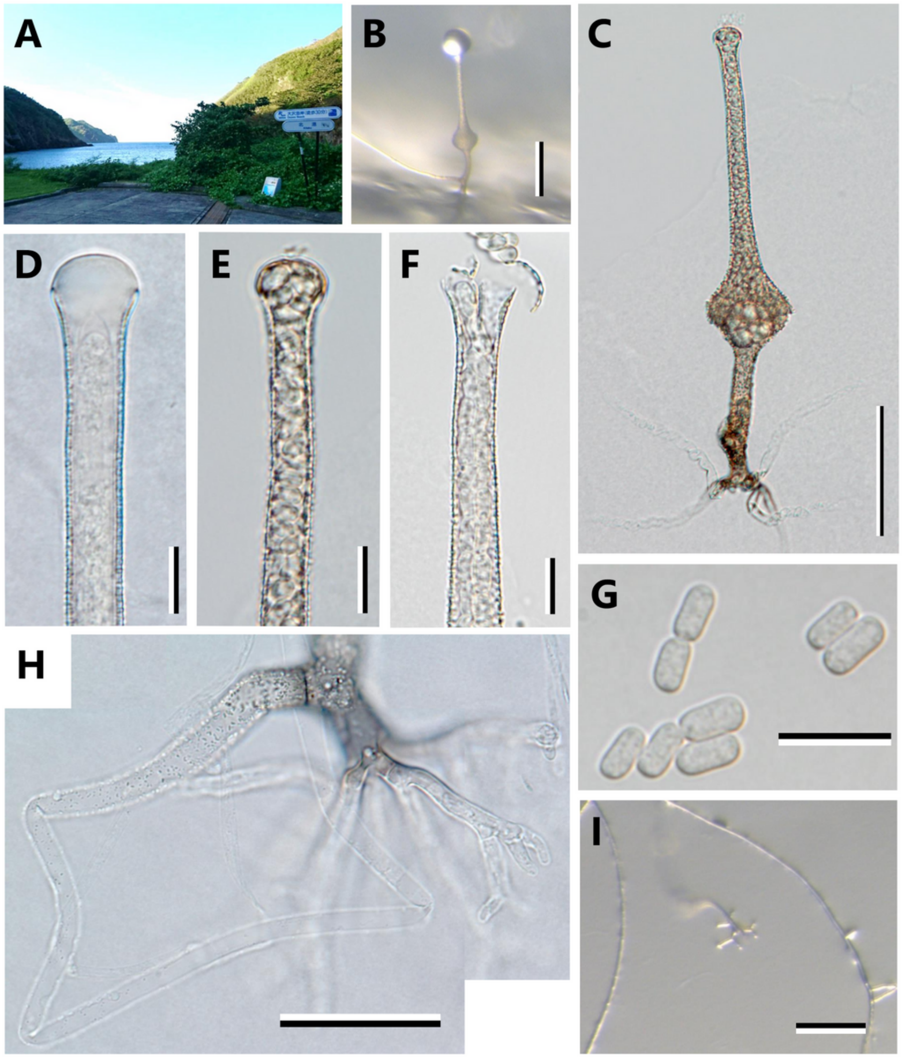


**Figure 1**

The landscape of collection site and microscopic characters of *Saksenaea boninensis* Sak4. The landscape of Haha-jima Island where the litter was collected (A). The morphologies of the isolate were observed after incubation for 1 month on CZA at 23 °C, unless otherwise specified. (B–I). B: A sporangium developed from a stolon. C: A sporangium. D–F: Stages of sporangia showing formation of the apical portion of the neck with a mucilaginous plug after incubation for 7 d (D), the gradual dissolution of apical mucilage (E), and liberation of sporangiospores (F). G: Sporangiospores. H: A dichotomously branched rhizoid partitioned by a septum from a stolon. I: An early stage of a rhizoid formed when the tip of the stolon touched the surface of the medium. Scale bars: B, I 100 μm; C 50 μm; D–G 10 μm; H 30 μm.

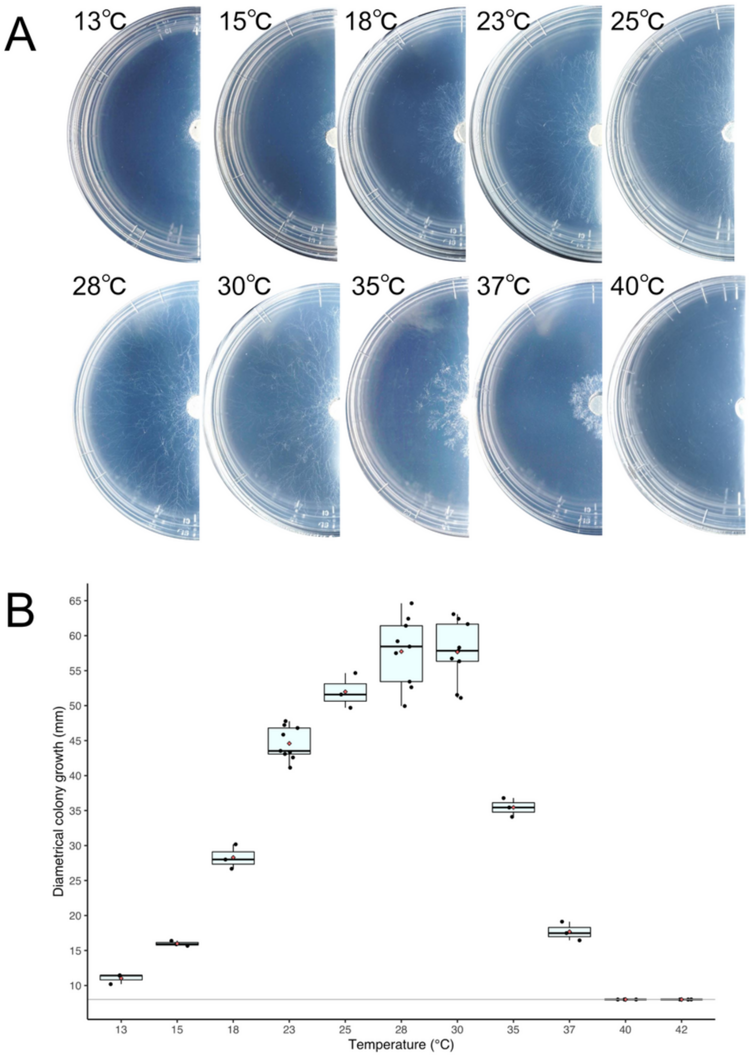


**Figure 2**

Colony growth of *Saksenaea boninensis* Sak4 incubated on CZA at different temperatures. A: Colony appearance of *Saksenaea boninensis* Sak4 incubated on CZA for 4 d at different temperatures. B: Diametrical colony growth of *Saksenaea boninensis* Sak4 incubated on CZA for 3 d at different temperatures. Optimum growth was observed around 28–30 °C. No growth was observed above 40 °C. Three replicates (N=3) were measured for each temperature except for 23, 28, and 30 °C (N = 9). Pinkish diamonds indicate the mean value.

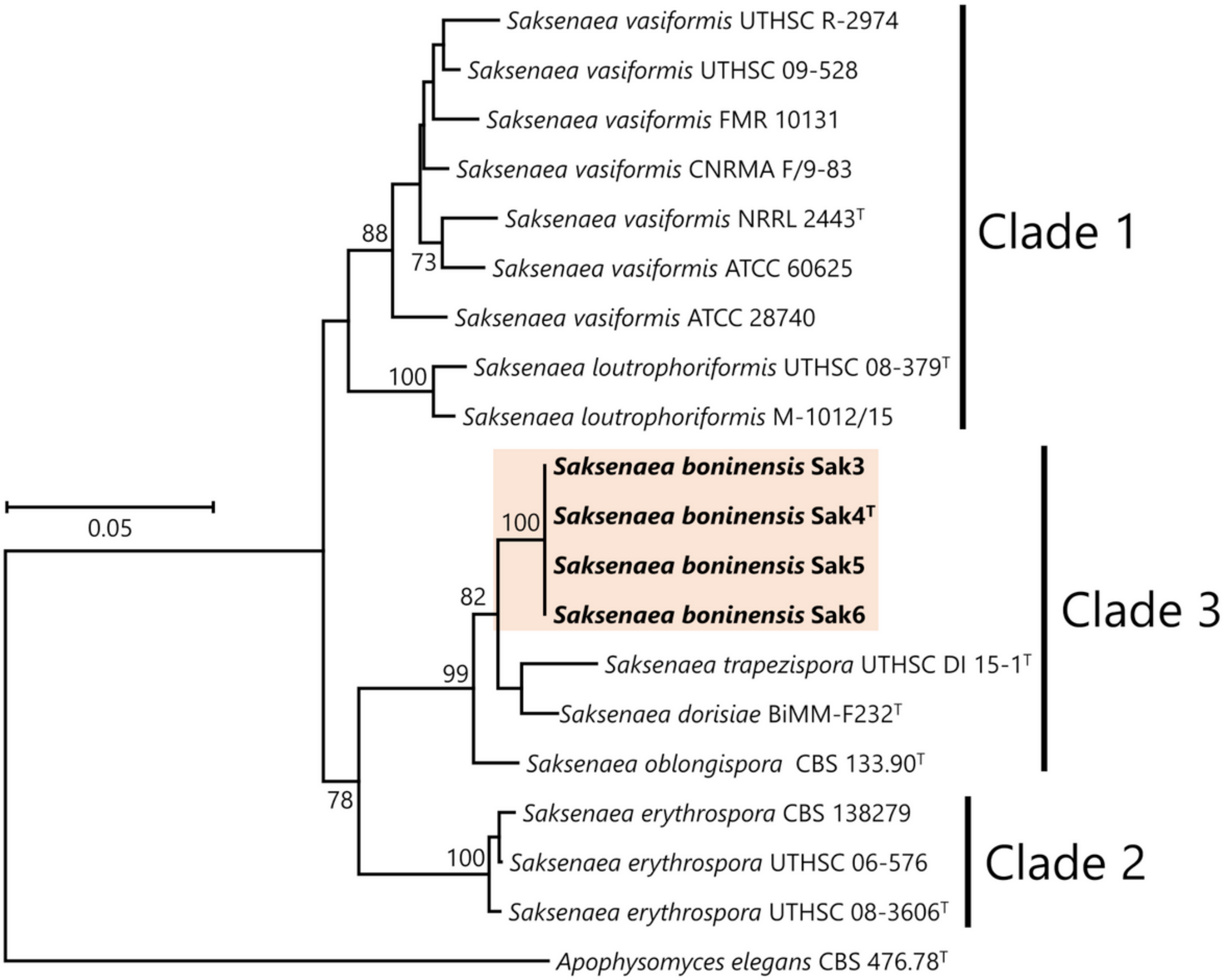


Figure 3

Maximum likelihood (ML) tree of *Saksenaea* spp. based on the concatenated sequences. Concatenated sequences (1,665 positions in total) consisted of the ITS region (552 positions), the partial LSU region (637 positions), and the partial *tef1* gene (476 positions). Bootstrap values ≥70% are shown at nodes. The value of the log likelihood was −5064.862701. *Apophysomyces elegans* was used as the outgroup. *Saksenaea* spp. were clustered into three clades as defined by Alvarez et al. (2010). “T” beside each strain name indicates the strains as ex-type strains.

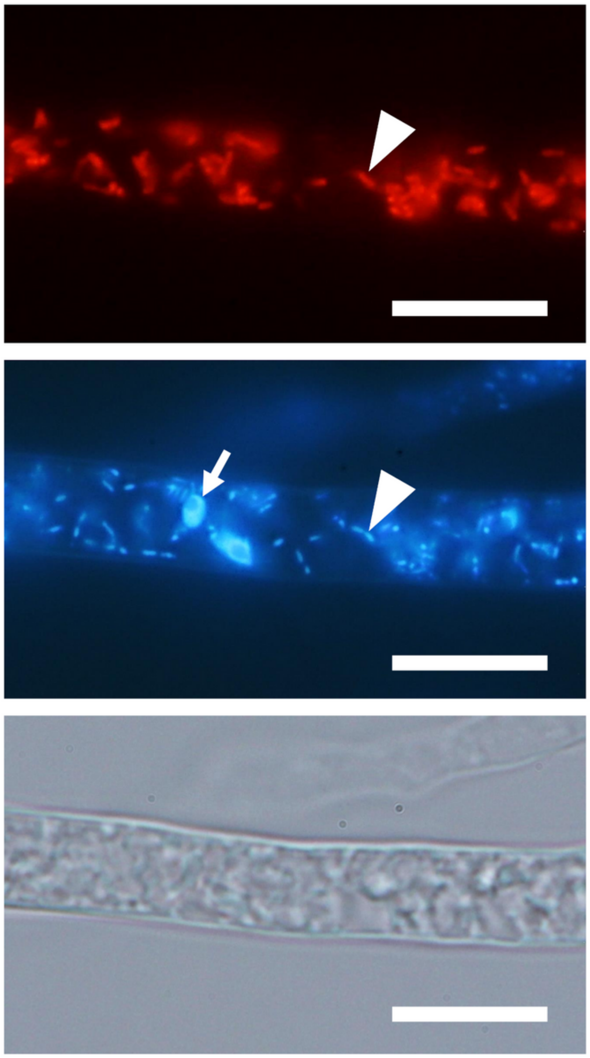

Figure 4

FISH image of SakBRE associated with *Saksenaea boninensis* Sak4. FISH was performed using the Cy3-labeled (Red) EUB338 probe (top). DAPI (center) and bright field (bottom) images are also shown. A representative host nucleus and bacterial cell within a hypha are indicated by an arrow and arrowhead, respectively. Scale bars: 10 μm.

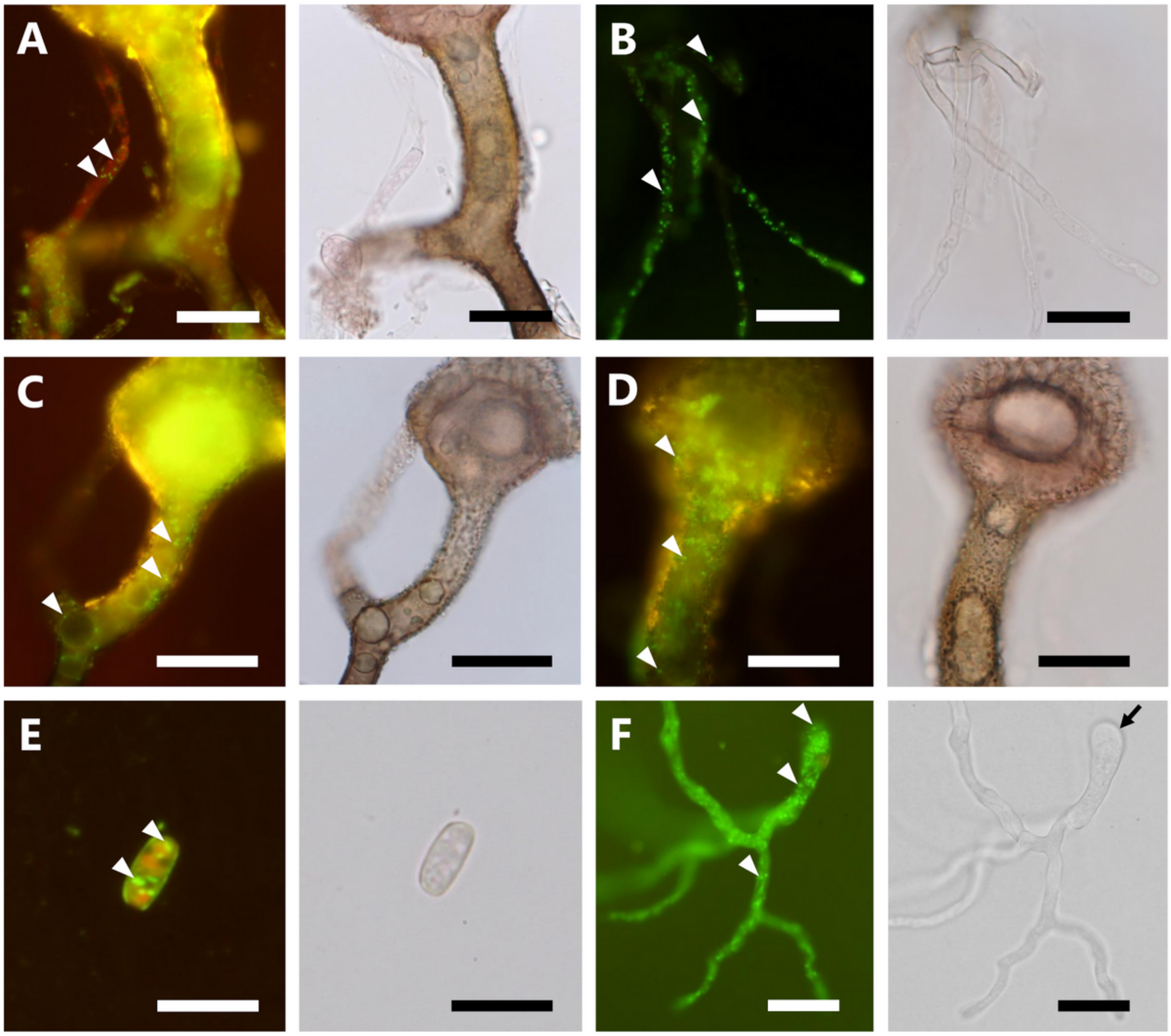


**Figure 5**

LIVE/DEAD stained fluorescence images of SakBRE associated with *Saksenaea boninensis* Sak4. Bright field images are shown beside each fluorescence image. Bacterial cells are indicated by arrowheads. Rod-shaped endofungal bacterium-like cells were localized within asexual stages such as aerial hypha (A), rhizoid (B), stalk (C), sporangium (D), sporangiospore (E), and germinated sporangiospore incubated for 12 h on $_{LC}$A (F). Scale bars: A–D 20 μm; E, F 10 μm.

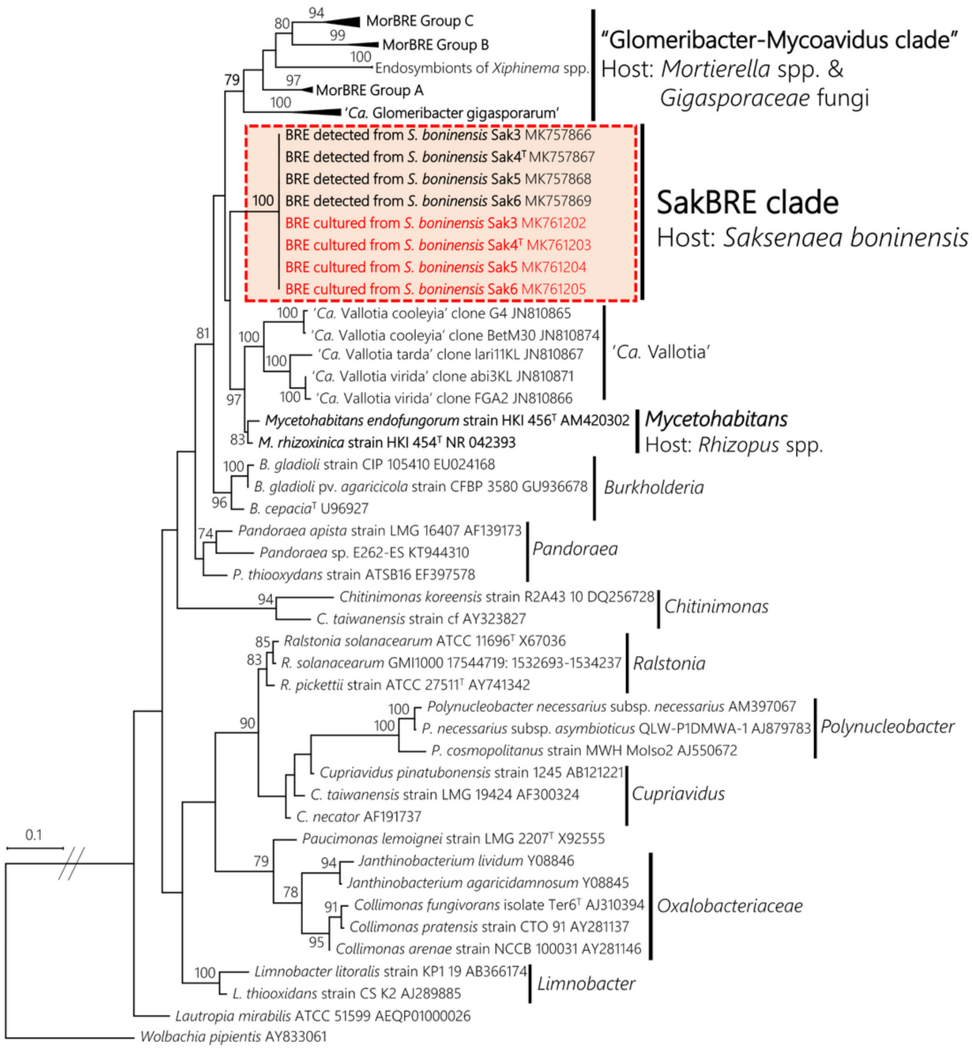


**Figure 6**

Maximum likelihood (ML) tree of *Burkholderiaceae* based on 16S rRNA gene sequences (1329 positions). Bootstrap values ≥70% are shown at nodes. The value of the log likelihood was −12214.778642. *Wolbachia pipientis* was used as the outgroup. "T" beside each strain name indicates the strains as ex-type strains. The SakBRE clade consisted of the obtained sequences in this study, indicated by a square with dashed red lines. In the square,16S rRNA gene sequences determined from the DNA templates prepared from BRE-harboring mycelia and pure cultures of the BRE are shown in black and red letters, respectively.

**Figure 7**

Pure culture of SakBRE grown on BCYEα medium after 14-d incubation at 30 °C.

## Supplementary Files

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

- SakBREAdditionalfile1FigS1.tiff
- SakBREAdditionalfile2FigS2.tiff
- SakBREAdditionalfile3FigS3.tiff
- SakBREAdditionalfile4FigS4.tiff