Journal of Medical Microbiology

Prototheca bovis, a unicellular achlorophyllous trebouxiophyte green alga in the healthy human intestine

--Manuscript Draft--

Manuscript Number:	JMM-D-20-00707R3
Full Title:	Prototheca bovis, a unicellular achlorophyllous trebouxiophyte green alga in the healthy human intestine
Article Type:	Research Article
Section/Category:	Disease, Diagnosis and Diagnostics
Keywords:	Gut protists; Imaging; Infection; Phylogeny; Prototheca; Zoonosis
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Manuscript Region of Origin:	THAILAND
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July 7th 2021

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Journal of Medical Microbiology

Dear Editors,

Please accept the revised version of our manuscript entitled: "***Prototheca bovis,* a unicellular achlorophyllous trebouxiophyte green alga in the healthy human intestine"** for consideration as an article in the *Journal of Medical Microbiology*.

In this version, we have addressed all of the editor's concerns/comment.

Please note that both Dr. Tsaousis and Dr. Gentekaki are corresponding authors.

We look forward to hearing from you regarding our manuscript.

Sincerely,

Dr. Anastasios Tsaousis and Dr. Eleni Gentekaki

Prototheca bovis, a unicellular achlorophyllous trebouxiophyte green alga in the healthy human intestine

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Short running title: *Prototheca bovis* in humans

List of Abbreviations: *cytb* (cytochrome b); LV (low viscocity); MEA (malt extract agar); NA (nutrient agar); PDA (potato dextrose agar); PDB (potato dextrose broth); SSU rRNA (small subunit ribosomal RNA); TEM (transmission electron microscopy)

Abstract

Introduction. *Prototheca* species are non-photosynthetic trebouxiophyte algae ubiquitously distributed in nature and can be found in sewage and soil. This microbial eukaryote causes human protothecosis in immunocompromised individuals. Thus, *Prototheca* presence in the stool of individuals without gastrointestinal symptoms has been reported only rarely.

Hypothesis/Gap statement. There is an absence of detailed characterization of human *Prototheca* isolates.

Aim. The aim of this study was to perform morphological and molecular characterization of *Prototheca* isolates obtained from human stool.

Methodology. *Prototheca* was isolated from fecal samples of four individuals living in a rural area in Thailand. A combination of bioimaging along with molecular and bioinformatics tools was used to characterize the four strains. The growth rate was tested using four media and three temperature conditions. Phylogenetic analysis using the small subunit ribosomal RNA (SSU rRNA) and cytochrome b (*cytb*) was also performed.

Results. Static and live microscopy demonstrated the various life stages of *Prototheca* and its major defining cellular characteristics. An optimized DNA extraction methodology that improves DNA yield is provided. Partial fragments of the SSU rRNA and *cytb* genes were obtained. Phylogenetic analysis placed all four strains in the clade with *Prototheca bovis*. More broadly, *Prototheca* was not monophyletic but split into at least two distinct clades instead.

Conclusion. The results represent the first molecular characterization of *Prototheca* in Thailand. The study provides insight into transmission dynamics of the organism and potential caveats in estimating the global prevalence of *Prototheca*. These will spearhead further investigations on *Prototheca* occurrence in rural areas of both industrialized and developing nations.

Keywords: Gut protists; Imaging; Infection; Phylogeny; *Prototheca*; Zoonosis

Introduction

Studies on human enteric organisms in Thailand have focused primarily on viral and bacterial pathogens and parasitic worms, such as *Opisthorchis, Taenia,* and *Enterobius* (1–3). With the exception of *Blastocystis*, *Cryptosporidium*, *Entamoeba*, and *Giardia*, other microbial eukaryotes are usually overlooked (4–7). One such example is *Prototheca*, a unicellular achlorophyllous trebouxiophyte green alga (8). *Prototheca* has been isolated from a broad range of animal species, including cats, dairy cattle, rats, and swine (9,10). In dairy cattle, the organism causes mastitis (11–13). Skin and soft tissue infections are seen in dogs and cats (14,15). Five species of *Prototheca* are known to infect humans and/or other animals: *P. blaschkeae*, *P. cutis*, *P. wickerhamii*, *P. ciferrii* (previously known as *P. zopfii* genotype 1) and *P. bovis* (previously known as *P. zopfii* genotype 2) (16, 17, 18). The latter is of special interest as it has been found in both animals and humans. The disease caused by *Prototheca* is known as protothecosis (18, 19, 20).

Prototheca bovis and *P. wickerhamii* are currently the two most common etiological agents of the disease (19, 21, 22). *Prototheca* infections have been reported in Europe, Asia, North America, and Africa (23). Nonetheless, the number of human cases is sparse: less than 200 cases of human protothecosis have been reported worldwide (20, 24). Human protothecosis has three main manifestations: cutaneous lesions, olecranon bursitis, and disseminated or systemic infection accompanied by varying symptoms (25). Cutaneous lesions are the most common manifestation of human protothecosis. *Prototheca* has only very rarely been isolated from the human intestine (26).

While performing a survey for eukaryotic microbes in a rural area of Thailand, we identified four potential cases of human intestinal protothecosis. Using a combination of tools from culturomics, cellular and molecular biology, and phylogenetics we characterized the isolates as *Prototheca bovis*.

Methods

Ethics statement

The Human Ethics Committee of Phramongkutklao College of Medicine approved the collection of fecal samples from Thai volunteers (License approval number S053q/58).

Human subjects and sample collection

Human volunteers were recruited as part of a large-scale parasitological survey in Chachoengsao Province, Thailand. All volunteers were Thai nationals and lived in the province at the time of collection (**Fig. 1**). Fecal samples were randomly collected from 98 volunteers living in three villages (villages 11, 16, and 18), who did not have diarrhea at the time of collection. Sterile collection kits containing a plastic container, gauze, and spatula were distributed to all volunteers. Small amounts of each fecal sample were introduced in two separate tubes containing HL-5 and LYSGM (27) media (http://entamoeba.lshtm.ac.uk/xenic.htm media). Upon transfer to the laboratory, all samples (98 tubes of HL-5 and 98 tubes of LYSGM) were incubated at 37°C.

Culture conditions

The cultures in HL-5 media and LYSGM were monitored daily using light microscopy starting from Day five post-inoculation. *Prototheca* cells were observed in samples from four volunteers in both media. One ml of *Prototheca* positive samples was sub-cultured in fresh

media. After 24 hours, 10 µl of each *Prototheca* culture were streaked on Petri plates containing potato dextrose agar (PDA) and sealed with parafilm. Colonies appeared within 24 hours. A single colony was then picked from each of the four cultures and transferred into fresh PDA media. Cultures were also established in potato dextrose broth (PDB). Pure cultures of all four isolates were also established in blood agar, nutrient agar (NA), and malt extract agar (MEA) to examine macroscopic colony characteristics. The PDA plates were incubated at 25°C, 37°C, and 40°C for three days to observe the growth pattern of *Prototheca* isolates. Photographs were taken on Day three.

Prototheca cells were counted using a haemocytometer and seeded at a density of 5000 cells per well of a 12 well plate (Greiner) containing 1 ml of PDB to generate video stills. The plate was mounted on a JuLi™Stage Real-Time Cell History Recorder, inside an incubator at 37°C. The JuLi™Stage system was set to image the wells every minute until the cells in the field of view reached confluency using a 10X zoom lens. Image captures were then assembled into a video using the JuLi™Stage proprietary software. Image stills were taken using the snapshot function on the VLC media player (VideoLAN). Image editing was done using the Graphical Image Manipulation Program.

Microscopy

Fecal smears were prepared by diluting fecal matter with sterilized bottled drinking water. Micromorphological characteristics of *Prototheca* cells were observed by diluting PDB culture with bottled water. A Nikon 80i compound microscope equipped with a Nikon DS-Ri2 camera was used. Transmission electron microscopy (TEM) was carried out to characterize key morphological features in detail. Briefly, cells of *Prototheca* were centrifuged at 300 x g and resuspended in 2.5% glutaraldehyde, 100 mM sodium cacodylate buffer (pH 7.2), and fixed overnight at 4°C. Samples were washed with cacodylate buffer twice for 10 minutes and then

post-fixed in 1% osmium tetroxide in cacodylate buffer for one hour at room temperature. Samples were then washed twice in distilled water for 10 minutes and then dehydrated through a graded ethanol series of 50%, 70%, 90% at 10 minutes per step and then three times for 10 minutes in 100% ethanol. This was followed by two 10 minute incubations in propylene oxide, followed by 30 minutes in 50:50 propylene oxide:agar low viscosity (LV) resin. Samples were incubated twice for two hours in freshly prepared agar LV resin and then spun down in BEEM® capsules. The resin infiltrated samples were polymerised at 60°C for 24 hours. Sections of 70 nm were cut on a Leica EM UC7 ultramicrotome and collected on 400 mesh copper grids. Sections were counterstained in 4.5% uranyl acetate in 1% acetic acid for 45 minutes and Reynolds lead citrate for seven minutes. Sections were imaged in a JEOL 1230 Transmission Electron Microscope operated at 80 kV, and images were captured with a Gatan One view digital camera.

DNA extraction, PCR and Sequencing

Genomic DNA was extracted from pure cultures using a Qiagen DNA stool mini kit (Qiagen, Hilden, Germany) with the following modification. Briefly, during the lysis step, 250mg of 0.5 mm zirconia beads and 20 µl of 10% SDS were added along with the lysis buffer provided by the kit. Polymerase chain reaction (PCR) was performed using EmeraldAmp GT PCR Master Mix (TaKaRa Bio USA, Inc.). Genomic DNA was also extracted from the faecal samples of the four *Prototheca* positive individuals. A fragment of 1550 bp of the small subunit ribosomal RNA (SSU rRNA) gene was amplified using DNA extracted from pure cultures with the NS1F: 5'-GTAGTCATATGCTTGTCTC-3' (28) and proto18S-4r: 5'-AGCACACCCAATCGGTAGGA-3' primers (17). The PCR conditions were as follows: denaturation at 95°C for 3 min, 35 cycles with denaturation at 95°C for 2 min, annealing at 55°C for 90 sec, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes.

DNA extracted from the stool of *Prototheca* positive individuals was used in an attempt to amplify a fragment of 430 bp of the SSU rRNA using the proto18S-4f: 5'-GACATGGCGAGGATTGACAGA-3' and proto18S-4r primers. The PCR conditions were as follows: denaturation at 95°C for 3 min, 35 cycles with denaturation at 95°C for 90 sec, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 minutes. All amplified products were purified with GeneJET Gel Extraction Kit (Thermo Scientific) and sent for bidirectional sequencing at U2Bio (Korea). A fragment of 600 bp of the *cytb* gene was amplified from DNA obtained from pure cultures using the cytb-F1: 5′-GyGTwGAACAyATTATGAGAG-3′ and cytb-R2: 5′-wACCCATAArAArTACCATTCwGG-3′ primers (29). Sequences have been deposited in GenBank under the accession numbers MT920919, MW228085-87 (SSU rRNA), and MZ209407-MZ209410 (*cytb*).

Phylogenetic analysis

The forward and reverse sequencing chromatograms were combined into contigs using CLC Main Workbench v.8 (Qiagen). The assembled sequences of SSU rRNA and *cytb* genes were used as queries to perform BLAST searches against GenBank to exclude contamination and collect additional sequences of *Prototheca* and other green algae. Two datasets were assembled, one for SSU rRNA and one for *cytb*. The SSU rRNA dataset contained 203 sequences spanning the diversity of trebouxiophyte algae, and including reference and type sequences of *Prototheca* was assembled and aligned using MAFFT v.7.394 (30). The *cytb* dataset contained 108 sequences. Ambiguous and poorly aligned regions were removed using Trimal v.1.3 (31) available on the online platform Phylemon 2.0 (http://phylemon.bioinfo.cipf.es). Following trimming, 1117 and 597 sites remained in the SSU rRNA and *cytb* datasets, respectively. Maximum likelihood trees were constructed using

RAxML v.8 (32) available on CIPRES Science Gateway v. 3.3 (http://www.phylo.org/index.php/). The general time-reversible + Γ model of nucleotide substitution was used. Bootstrap support was assessed from 1000 bootstrap replicates.

Results

Isolation and culturing of *Prototheca bovis* from four human stool samples

Culture media containing fecal samples of 98 volunteers were examined using a compound microscope. *Prototheca* was found in the stool of four volunteers. Fecal smears of the *Prototheca* positive stool samples provided additional confirmation of its presence (**Sup. Fig. 1**). Colonies grew on all four media (PDA, MEA, NA, and blood agar). Cells appeared within 12 hours and dense colonies appeared on the nutrient-rich PDA, MEA, and blood agar plates, while colonies were smaller and not as dense in the nutrient-poor NA (**Sup. Fig. 2**). Colonies were yeast-like and with smooth edges. In PDA, MEA and blood agar, colonies were pale white to cream-white, 2 to 3 mm in diameter, while those in NA were one-third of the size at the same time of incubation (Day 3). *Prototheca* colonies were also incubated at different temperatures to observe growth. At 25°C, colonies of *Prototheca* on PDA were smaller than those at 37°C and 40°C on day 3 (**Sup. Fig. 3**).

Prototheca bovis growth pattern

The algal growth, maturation, and division were recorded live. Cells seeded in PDB media were monitored until the field of view was confluent. Upon maturation, cells (sporangia) ruptured to yield eight daughter cells (sporangiospores) (**Sup. File 1**). Under the growth conditions used herein, newly divided daughter cells reached maturity and divided anew in five and a half hours (**Sup. File 1**). A crowded environment appeared to affect on cell size, as when

the field of view reached confluency, most of the newly divided cells were smaller in size compared to cells of previous generations.

Microscopic investigations highlights basic cell features

All four isolates formed sporangia seen at various stages of multiplication containing two, four, or eight sporangiospores. Sporangia with more than eight sporangiospores were not observed at any point. Sporangiospores arranged in morulae (flower petal formation) were commonly observed (**Fig. 2**). Sporangiospores were 7.0-16.0 µm long x 6.0-12.5 µm wide. Thick septa were present between sporangiospores within the sporangia. The thick cell wall characteristic of *Prototheca* was clearly visible.

Transmission electron microscopy was used to obtain high-resolution images of *Prototheca* cells. Various life stages of *Prototheca* and its subcellular organelles were observed (**Fig. 3**). These included a cell membrane surrounded by a thick cell wall, canonical mitochondria (numerous cristae), remnant plastids with double membranes, filled with starch granules, and dense lipid droplets of various sizes dispersed in the cell (**Fig. 3**).

Sequence and phylogenetic analyses

Prototheca sequences were successfully obtained only for the fragments amplified from the DNA obtained from pure cultures. The sequences from DNA extracted from stool samples were from edible plants. The newly generated SSU rRNA sequences were identical to each other. Two separate phylogenetic trees were inferred from SSU rRNA (**Sup. Fig. 4**) and *cytb* (**Fig. 4**) gene sequences. In the SSU rRNA tree, *Prototheca* was not monophyletic, as it split into three clades with *Helicosporidium* grouping within. These clades were: 1) a clade containing most *Prototheca* species; 2) a clade containing *P. miyajii* and *P. cutis*, which grouped as sister to *Helicosporidium*, albeit with low support; and 3) the *P. xanthoriae* clade,

which grouped as sister to *Auxenochlorella*. The latter clade contained two *P. xanthoriae* sequences, which grouped separately from the typed sequence of this species, which groups within clade 1. The four newly identified strains nested within *P. bovis* sequences along with a single sequence of *P. xanthoriae* in clade 1. In the *cytb* gene tree, *Prototheca* was also not monophyletic, with strains of *Chlorella*, *Helicosporidium*, and *Auxenochlorella* grouping within. The *Prototheca* sequences were distributed into the same nine previously defined clusters (I-IX), with each cluster representing a separate species (29). The newly generated sequences grouped with *P. bovis*; thus, we have designated them as such.

Discussion

Prototheca is likely transmitted to humans by an animal or environmental reservoir even though these sources have yet to be identified (16). Human protothecosis manifests as localized infection of the skin, olecranon bursitism and disseminated infection (20, 24, 33). It has been proposed that immunocompromised individuals and those with underlying conditions are more susceptible to the disease (34, 35). The two most common species of *Prototheca* causing the human infection are *P. bovis* and *P. wickerhamii*. The latter is the principal etiological agent of human protothecosis, whereas *P. bovis* commonly causes disease in other animals (10, 25). Workers in rice paddies, fishermen, farmers, handlers of raw seafood, and aquarium staff are at risk of exposure to *Prototheca* (16). Nonetheless, only a few studies from countries where these jobs are common, including South and Southeast Asian countries. In Thailand, *Prototheca* has been reported only rarely (36, 37).

Traditionally, protothecosis diagnosis has focused on microscopic observation and physiological/biochemical tests of the isolated organism or direct examination of affected tissues (24). Nonetheless, neither of these methods can accurately identify *Prototheca* to the species level. Molecular characterization using SSU rRNA and *cytb* genes has greatly

facilitated species identification (29, 38). Herein we isolated four strains of *Prototheca* from fecal samples of Thai volunteers living in the rural area of Chacheongsao. Phylogenetic analysis of the SSU rRNA and *cytb* genes placed all four isolates in the *P. bovis* clade (**Sup. Fig. 4**, **Fig. 4**). This species is associated with bovine mastitis (in the form of persistent udder inflammation) and infections of companion animals (17, 22, 39, 40). Nevertheless, there have been occasional reports of clinical cases of human protothecosis involving *P. bovis* (41-44). This species was previously designated as *P. zopfii* genotype 2 and was recently elevated to species level (29). Given that finding *Prototheca* in human stool is extremely rare, its occurrence in four individuals raises questions about its original source, distribution, and transmission.

Pinpointing the source of *Prototheca* is challenging, as the human-to-human transmission is unknown (19). Herein, volunteers positive for *Prototheca* lived in four separate households distributed in two different villages 15 km apart. Participants in this study spend extended periods in rice fields and have frequent contact with ruminants and poultry, whose dung is used to fertilize their vegetable gardens. The three villages' water source comprises groundwater stored in tanks composed of layers of sand and gravel filters. Rice fields, dung, and groundwater constitute habitats from which *Prototheca* has been previously isolated (16). Hence, the alga likely came from one or all of the aforementioned sources, though it is not possible to confidently identify the source. Given these uncertainties, a comprehensive One Health approach that includes humans, other animals, and the environment in all three villages is needed in the future.

Remarkably, little is known regarding the pathogenesis and virulence of *Prototheca*. Many studies focus on infections of immunocompromised hosts, and discovery of the organism is often incidental (35). Notwithstanding, its ability to survive passage through the gastrointestinal tract has been previously discussed (16). In this study, *P. bovis* was detected in

the stool of four individuals that exhibited no diarrhea. Smears of stored fecal samples from all *Prototheca* positive volunteers revealed the presence of organisms, some of which were dividing (see **Sup. Fig. 1**). This finding suggests that the organism remained in the host long enough to undergo cell division. It is currently not feasible to determine whether there was an established infection as samples were collected during a large-scale survey, where only the diarrhea status was checked. At the same time, one cannot exclude the possibility of *P. bovis* asymptomatically colonizing the hosts.

The rarity of protothecosis is a recurrent theme of literature reviews (20, 45). Our study suggests methodological caveats as a possible cause. At the onset of the experiments, DNA yield from the newly established pure cultures of *P. bovis* was disproportionately low, given the large number of cells used for the extraction. Direct microscopic observation of cells right after lysis revealed that the vast majority of them failed to lyse. This is not surprising given the thick cell wall of *Prototheca* (see **Fig. 3**). After adapting various protocols, we found that using zirconia beads (0.5 mm) along with SDS (10%) improved DNA yield substantially. Microscopic examination showed that more than half of the cells had ruptured after using this lysis method. Given the difficulty of cell rupture, we started to suspect that *Prototheca* cells in the stool might not be disrupted either when using stool DNA extraction methods. To further examine this, we went back to the DNA obtained from the four original stool samples of *Prototheca* positive volunteers. To our surprise, attempts to amplify and sequence SSU rRNA and *cytb* gene fragments from these DNA samples only yielded sequences of edible plants. Collective consideration of these data strongly suggests that the alga remained intact during DNA extraction from stool samples. If this is the case, then it is highly likely that the prevalence of *Prototheca* is consistently underestimated and not considered in surveys of fecal and/or environmental eukaryotic diversity.

Herein, we performed molecular characterization of *Prototheca* from the stool of four volunteers in Thailand. Based on the potential methodological caveats discussed above, we speculate that *Prototheca* prevalence might be underestimated in human hosts. During this study, it became evident that there is a need for further studies to determine whether the organism is transient, temporary/long-term colonizer, or a pathogen. Thus, there is an urgent need to elucidate aspects of the biology and life cycle of *Prototheca*. In that vein, we outline a toolkit of techniques that can be used to study cell biology and characterize specific components of *Prototheca* in the future. Such approaches comprise a significant step towards developing this organism as a model to understand various aspects of its pathogenicity and opportunistic nature.

Author statements

Authors and contributors

Vasana Jinatham: Formal analysis, Methodology, Investigation, Writing-original draft, Writing-review & Editing. **Diego M. Cantoni:** Methodology, Investigation. **Ian R. Brown:** Methodology, Investigation. **Thanakrit Vichaslip:** Investigation. **Picha Suwannahitatorn:** Investigation. **Siam Popluechai:** Investigation. **Anastasios D. Tsaousis:** Methodology, Investigation, Resources, Supervision, Writing-original draft, Writing-review & Editing. **Eleni Gentekaki:** Conceptualization, Funding acquisition, Methodology, Investigation, Project administration, Supervision, Writing-original draft, Writing-review & Editing.

Conflicts of interest

The authors have no known conflict of interest.

Funding information

This project was supported by the Thailand Research Fund (grant no RSA6080048) awarded to EG. Research at Kent was supported by a BBSRC research grant (BB/M009971/1).

Ethical approval

The Human Ethics Committee of Phramongkutklao College of Medicine approved the collection of fecal samples from Thai volunteers (License approval number S053q/58) used in this study.

Acknowledgements

The authors wish to thank Mae Fah Luang University for supporting the Gut Microbiome Research Group. ADT's visits to Thailand were supported by a University of Kent internal GCRF grant, and a Biochemical Society Visiting Fellowship for Developing Countries travel grant as well. The JuLi™Stage system was purchased by ADT's laboratory under the EU Interreg-2-seas H4DC grant.

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Figure legends

Figure 1. Sampling locations in villages 11, 16 and 18, Chachoengsao Province, Thailand.

Figure 2. Details of *Prototheca* sp. cells incubated on PDA medium at 37ºC for 72 hours. *Prototheca* sp. sporangiospores (A); sporangium just before first cell division, granular content clearly visible (B); sporangia at various stages of division and released sporangiospores (C-I); sporangiospore exiting bursting sporangium (J); mature sporangium containing eight sporangiospores (K); Remnant cell wall of a bursting sporangium (L). Scale bar: 10 μm

Figure 3. Transmission electron microscopy depicting structural features commonly associated with *Prototheca* species: lipid droplets (L), nucleus (N), mitochondria (M), starch granules (SG) and a thick cell wall (Panel A). Close up of mitochondria located near the cell walls (Panels B and C).

Figure 4. Maximum likelihood phylogeny of cytochrome b inferred from 108 sequences and 597 sites. Numerical values at nodes represent maximum likelihood bootstrap support. Only values above 70 are shown. New sequences are presented in bold letters. Sequences from type specimens are represented with a (T). Roman numerals (I-IX) represent *Prototheca* species clusters as those are defined in Jaglieski et al. (29).

Supplementary figure legends

Supplementary Figure 1. Fecal smears of four *Prototheca bovis* positive human stool samples.

Supplementary Figure 2. *Prototheca bovis* colonies of four strains (VJ1-VJ4) growing on different media at 37°C 72 hours post-incubation: blood agar (A), PDA (B), MEA (C) and NA (D).

Supplementary Figure 3. *Prototheca bovis* colonies of four strains (VJ1-VJ4) on PDA media at 25°C, 37°C and 40°C, 72 hours post-incubation.

Supplementary Figure 4. Maximum likelihood phylogenetic tree of trebouxiophyte algae inferred from 203 SSU rRNA gene sequences and 1117 sites. Numerical values at nodes represent maximum likelihood bootstrap support. Only values above 70 are shown. New sequences are presented in bold letters. Sequences from type specimens are represented with a (T). Leaves have been collapsed to highlight the *Prototheca* clades.

Figure 1

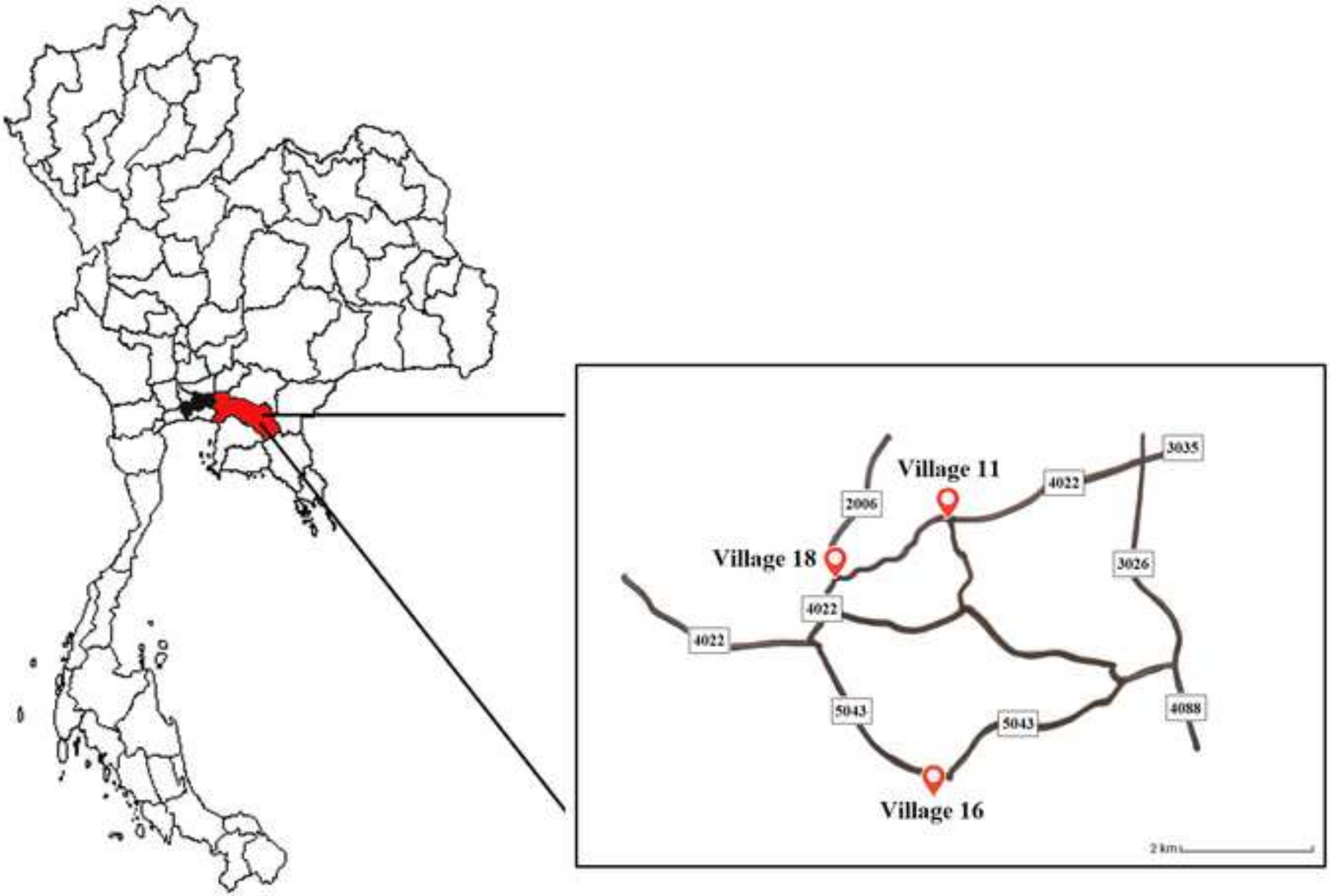

Figure 2

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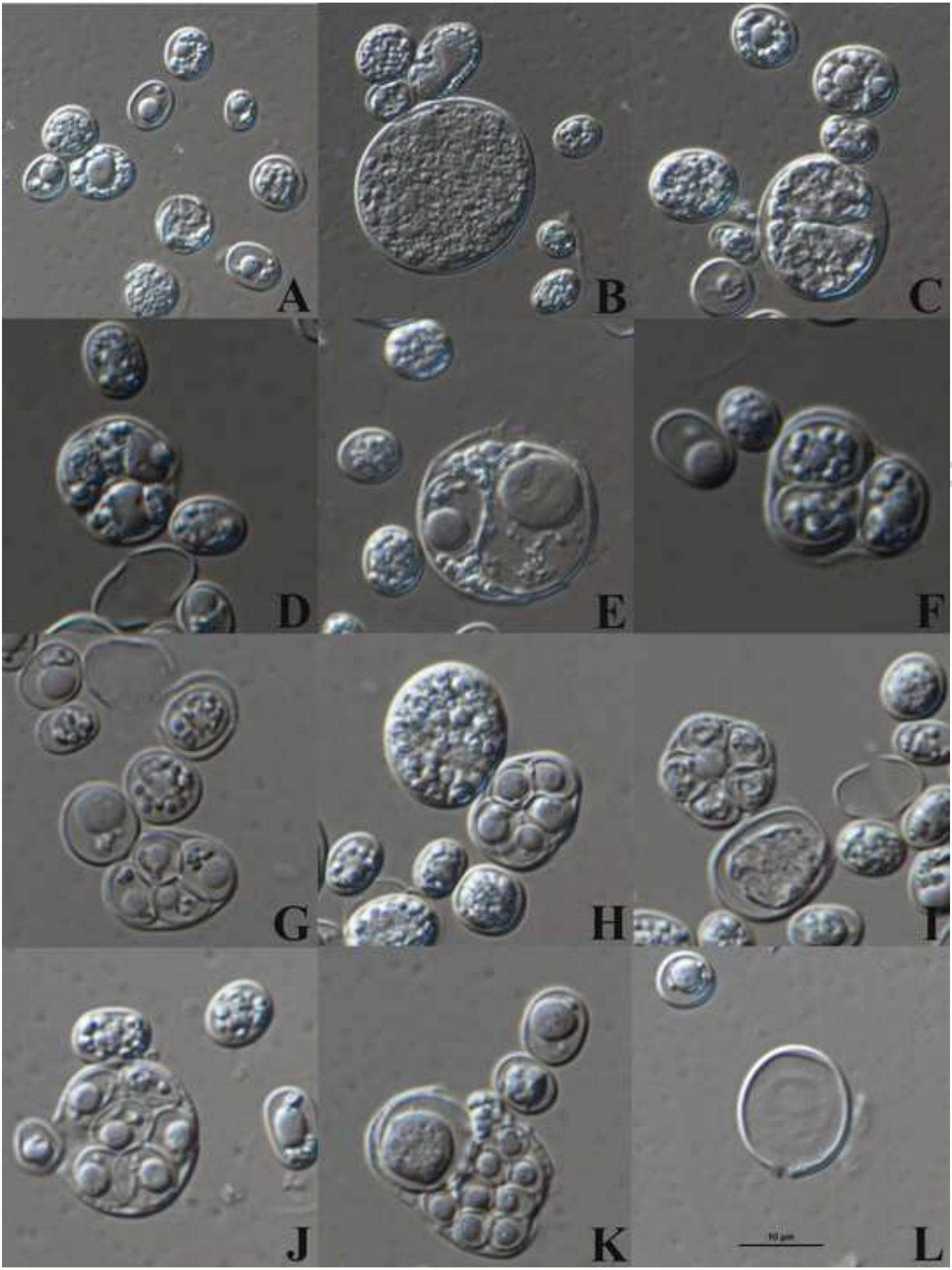
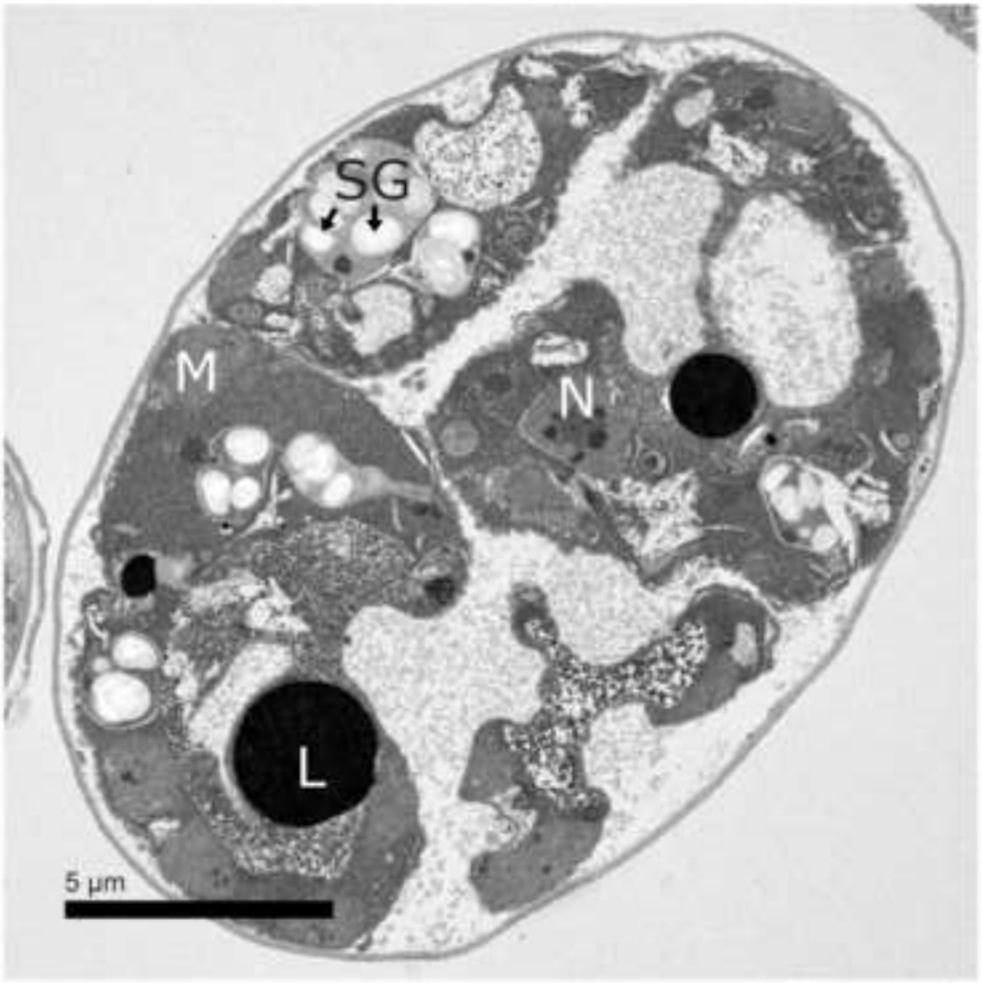
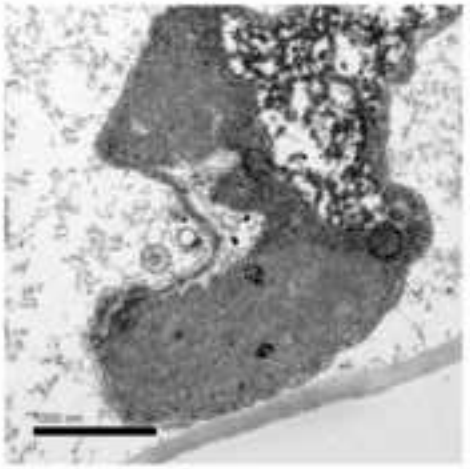


Figure 3





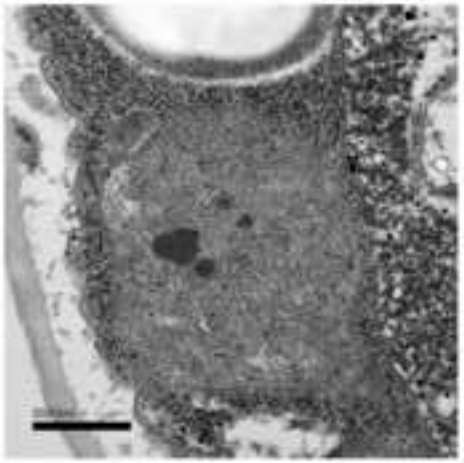


Figure 4 Labelled bigger font.tif

Figure 4

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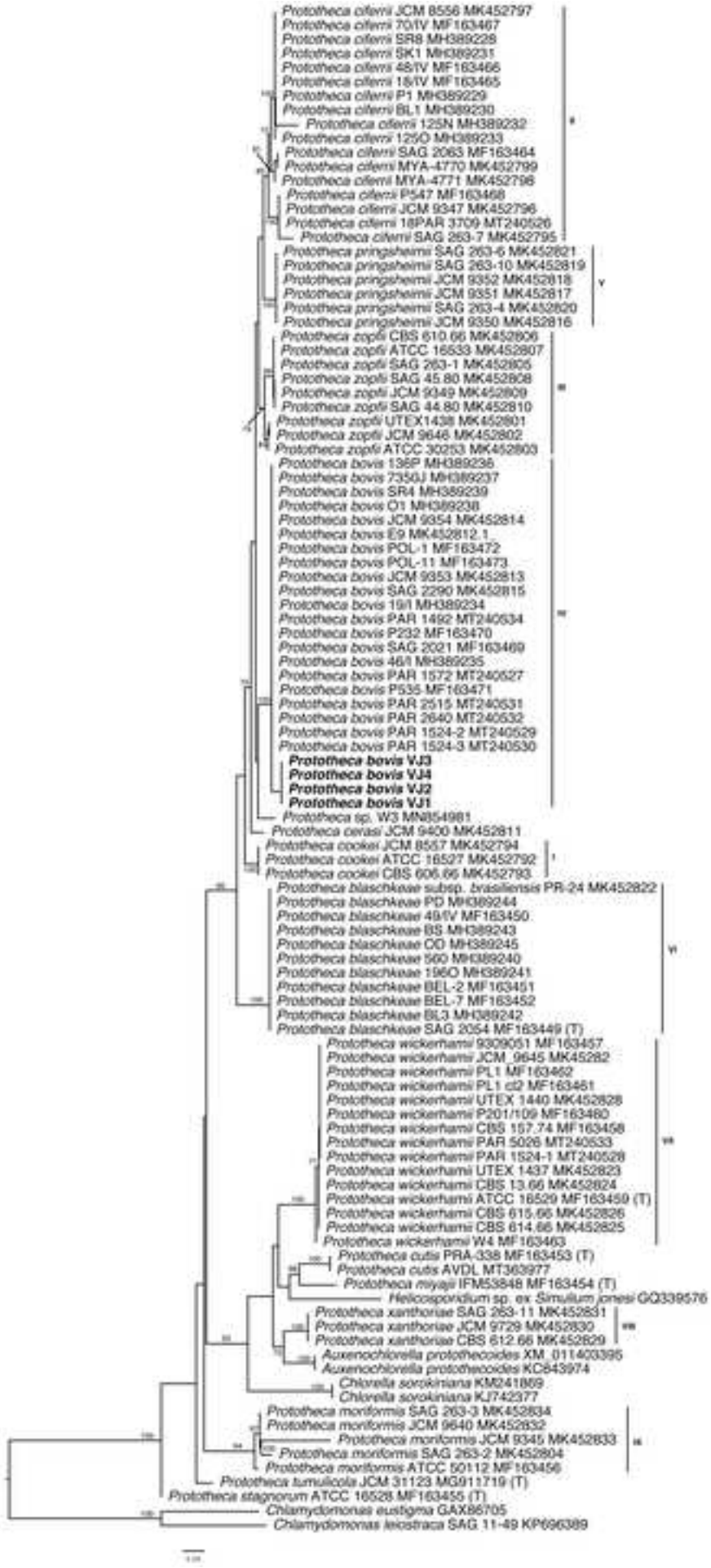

Prototheca in the human intestine: a first step towards showcasing *Prototheca* occurrence in Thailand

Vasana Jinatham[1], Diego M. Cantoni[2], Ian R. Brown[3], Thanakrit Vichaslip[4], Picha Suwannahitatorn[4], Siam Popluechai[1, 5], Anastasios D. Tsaousis[2], and Eleni Gentekaki[1, 5,]

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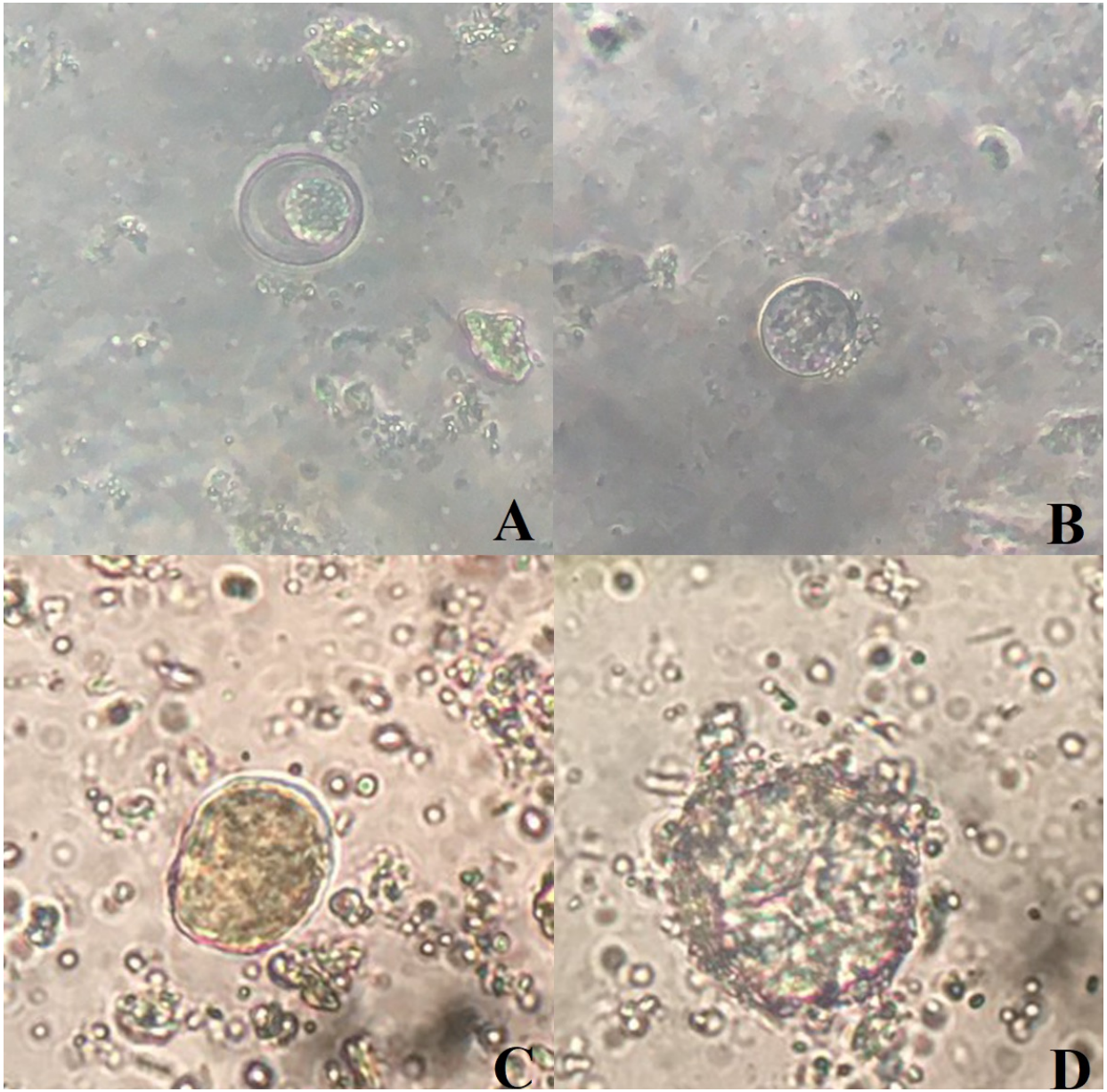
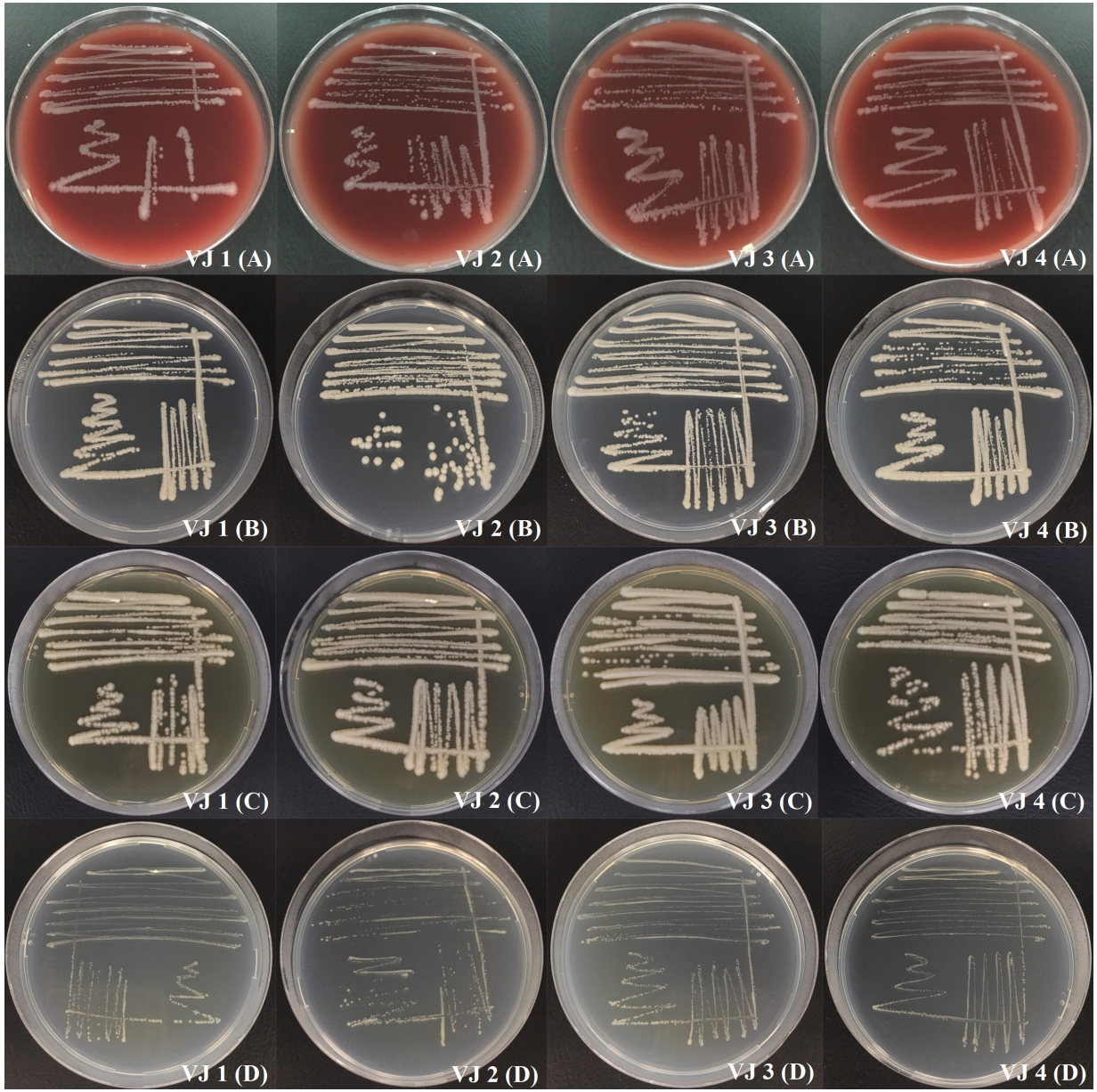
Supplementary Figure 1

Fecal smears of four *Prototheca bovis* positive human stool samples.



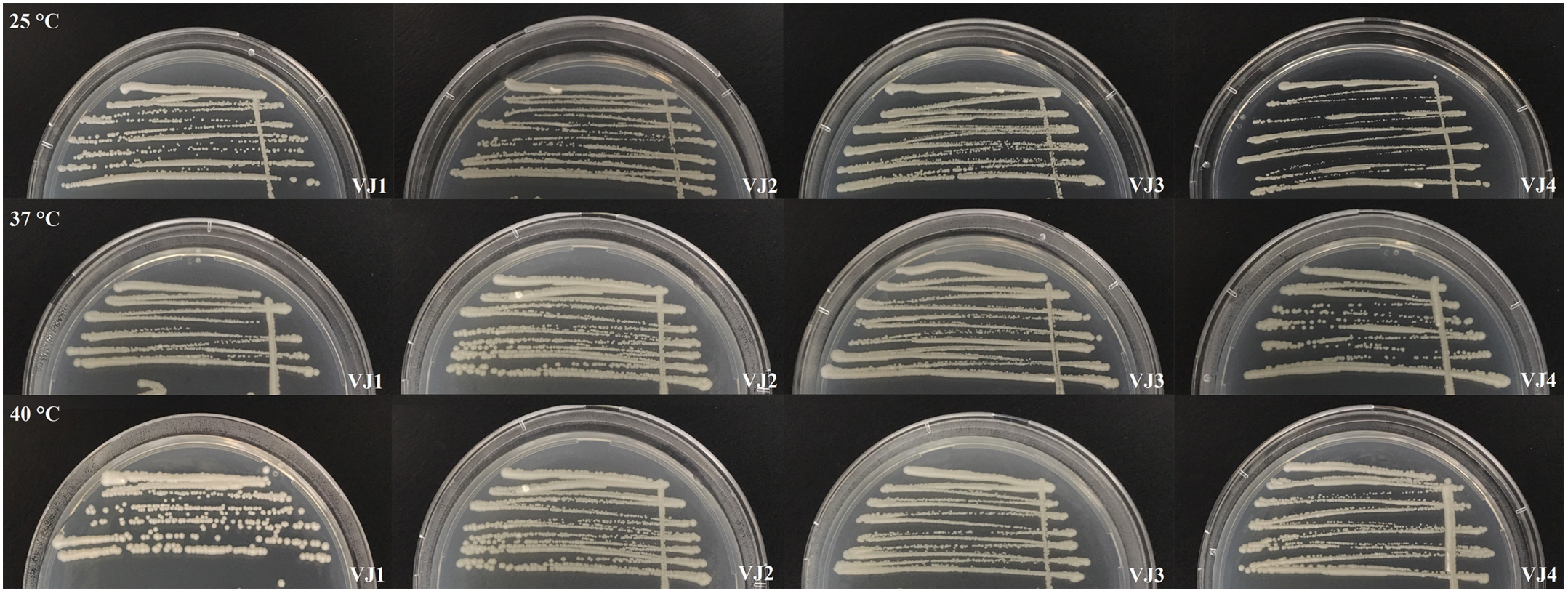
Supplementary Figure 2

Prototheca bovis colonies of four strains (VJ1-VJ4) growing on different media at 37°C 72 hours post-incubation: blood agar (A), PDA (B), MEA (C) and NA (D).



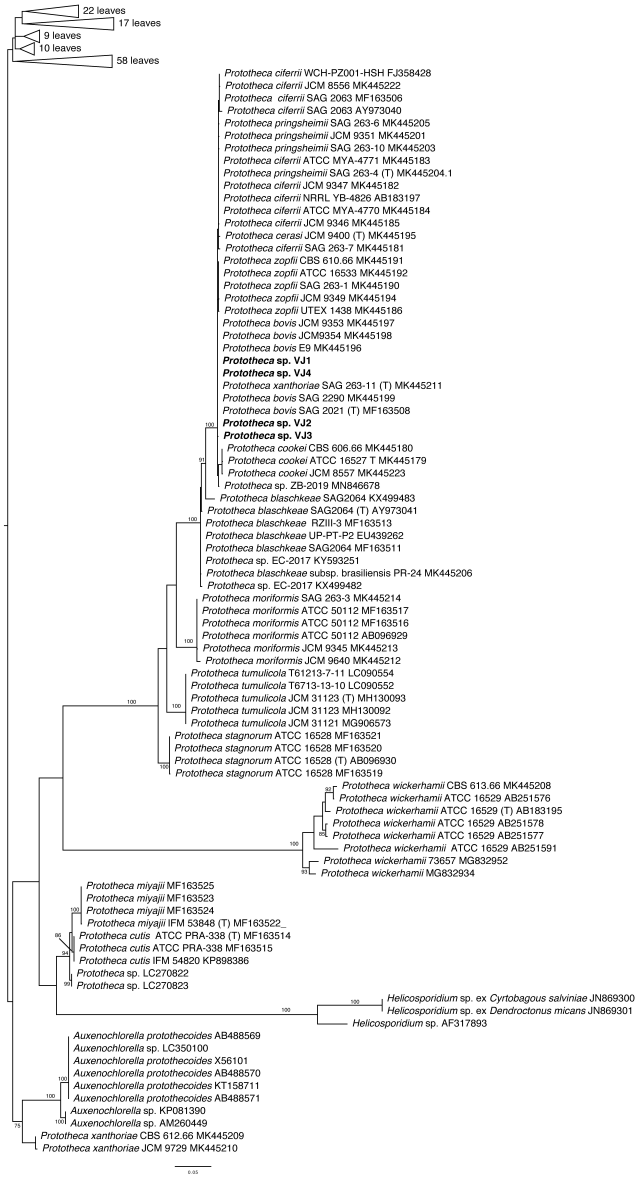
Supplementary Figure 3

Prototheca bovis colonies of four strains (VJ1-VJ4) on PDA media at 25°C, 37°C and 40°C, 72 hours post-incubation.



Supplementary Figure 4

Maximum likelihood phylogenetic tree of trebouxiophyte algae inferred from 203 SSU rRNA gene sequences and 1117 sites. Numerical values at nodes represent maximum likelihood bootstrap support. Only values above 70 are shown. New sequences are presented in bold letters. Sequences from type specimens are represented with a (T). Leaves have been collapsed to highlight the *Prototheca* clades.



Supplementary video 1

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Video file

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