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Candida theae sp. nov., a new anamorphic beverage-associated member of the *Lodderomyces* clade

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ABSTRACT

Four strains representing a novel yeast species belonging to the genus *Candida* were independently isolated in Taiwan and Ecuador. Two strains (G17^T and G31) were isolated in Taiwan, by pellet precipitation from plastic-bottled tea drinks produced in Indonesia, while two additional strains (CLQCA 10–049 and CLQCA 10–062) were recovered from ancient chicha fermentation vessels found in tombs in Quito, Ecuador. These four strains were morphologically, and phylogenetically identical to each other. No sexual reproduction was observed on common sporulation media. Large-subunit (LSU) rRNA gene sequence analysis revealed the four strains to belong to the *Lodderomyces* clade, closely related to members of the *Candida parapsilosis* species complex. The four strains, which have identical LSU D1/D2 sequences, differ from their closest phylogenetic neighbors, *Candida orthopsilosis* and Candida *parapsilosis*, by 6–9 nt substitutions, respectively. Physiologically, the four strains are similar to *Candida parapsilosis*, although they can be distinguished from their closest relative by the assimilation of arbutin, nitrite, and creatine. The Indonesian and Ecuadorian strain sets can also be distinguished from one another based on ITS sequencing, differing by 4 substitutions in ITS1 and 1 single nucleotide indel in ITS2. Collectively, the results indicate that the four strains represent a previously unrecognized species of *Candida*. The name *Candida theae* sp. nov. is proposed to accommodate these strains, with G-17^T (BCRC 23242^T = CBS 12239^T = ATCC MYA-4746^T) designated as the type strain.

1. Introduction

Over the past two decades, there has been a considerable increase in the consumption of bottled tea drinks worldwide (Komatsu et al., 1991), which has been attributed to potential benefits such as convenience, a good taste, reduction in body fat (Nagao et al., 2005), and antioxidant and antimutagenic properties (Wang et al., 1989). Therefore, a number of commercial bottled tea products are currently on the market. To maintain the flavor and color and to avoid microbial contamination, control of the processing and storage stages of bottled tea drink production is important in food factories. Despite aseptic manipulation being commonly used in tea production, undesirable pathogenic and saprophytic microorganisms may still be found in the tea infusion (Wilson et al., 2004). Not only does this pose a potential health hazard to consumers, (Iacumin et al., 2009; Monaci et al., 2005; Toscani et al., 2007), but it can also have an economic impact on producers by increasing the cost of production as well as product loss.

During storage tests of a bottled tea drink in an Indonesian food factory in 2009, two contamination cases were identified. One occurred in a bottle of no-sugar green tea, and the other in honey-added green tea. A uniform colony morphology appeared on an isolation agar plate in each case, and two strains, G-17^T and G-31, were isolated from the no-sugar green tea and honeyed green tea, respectively.

In contrast, chicha, or corn beer, is the most important fermented ancestral beverage consumed in South America. Chicha is a clear, yellowish, sparkling beverage that was consumed by indigenous Andean populations for thousands of years (Hastorf and Johannessen, 1993; Jennings, 2005). Ancient chicha from Andean peoples was made using corn as a carbohydrate source to which other ingredients such as flowers and/or fruits were sometimes added. Spanish chroniclers from the 16th Century reported the use of animal bones and even human feces to initiate fermentation (Gomes et al., 2009). Nowadays, this fermented beverage is still consumed in a number of South American countries including Ecuador, Colombia, Perú and Bolivia. However, the practice of using animal bones and human feces as a means to initiate fermentation has now virtually disappeared.

During a taxonomic survey on ancient chicha vessels found in deep tombs at the Necropolis La Florida in Quito, Ecuador, a number of

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ascomycetous and basidiomycetous yeasts were found, including the novel species described in this paper. Two isolates, CLQCA 10–049 and CLQCA 10–062, were recovered from two different clay chicha vessels dating from around 680 A.C. (Gomes et al., 2009) by means of a novel yeast resuscitation method developed by researchers at the Pontificia Universidad Católica del Ecuador. The method, termed the HRM method, involves the hydration, restoration and metabolic activation of dormant yeast isolates.

Collectively, these four strains were found to represent a novel *Candida* species based on sequence analyses of the large subunit (LSU) rDNA D1/D2 domain and the internal transcribed spacer (ITS) region. The novel species is described as *Candida theae* sp. nov. in this paper.

2. Materials and methods

2.1. Contaminated tea sample, microscopic analysis and used strains

Two contaminated bottled tea drinks, green tea without sugar and honeyed green tea, produced in May and June 2009, respectively, were examined during the storage period at room temperature in a food factory in Indonesia. Visible sediment was noted, and the drinks turned cloudy and turbid after the sediment was suspended. The morphological characteristics of the contaminants were examined using a light microscope (BH-2, Nikon, Tokyo, Japan) attached to a digital camera. Yeasts were identified as the cause of contamination and were isolated by the methods described below.

2.2. Archeological chicha vessels sampled

Two ancient chicha fermentation vessels from 680 A.C. belonging to the Sierra Norte culture were sampled in 2008 in Quito, Ecuador. The yeast strains herein reported were isolated along with a number of other ascomycetous and basidiomycetous species (Carvajal et al., unpublished).

2.3. Yeast isolation and characterization

Yeasts were isolated from the contaminated tea drink using yeast extract-malt extract agar (YMA; 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% agar) supplemented with 0.02% chloramphenicol. A 1 ml sample from each contaminated tea drink was diluted with 9 ml of sterilized saline solution (0.9% w/v; NaCl) and then vortex-mixed. Subsamples of 100 μl were spread on YMA plates and then incubated at 25 °C in the dark until yeast colonies developed. The yeast colonies were purified by repeated streaking on YMA plates followed by incubation at 25 °C. Thereafter, the strains were maintained at 4 °C for short-term preservation and at −80 °C with 30% glycerol (w/v) added as a cryoprotectant for long-term preservation. The morphological, physiological and biochemical characteristics were examined according to the standard methods routinely used in yeast taxonomy (Yarrow, 1998). Sporulation tests were performed on McClary's acetate agar, corn meal agar, malt extract agar and V8 agar (Yarrow, 1998), and plates were incubated at 18 °C for 1 month in pure and mixed cultures.

As for the chicha yeasts, the isolation was performed by sampling the vessels in sterile conditions: within a laminar flow chamber and by using sterile stainless steel spatulas to scratch the clay material of the vessels. Three different zones were selected to sample for each vessel: deep, middle and mouth. From each zone an area of approximately 1 cm^2 was sampled by scratching. The clay powder obtained was recovered with sterile cotton swabs that were soaked in resuscitation medium. The powder produced from five 1 mm deep layers was recovered from each sampling zone. Every single sample was subjected to the HRM method. After 3 days, the samples were plated on YPD ampicilin (100 ug/ml) agar and incubated at 25 °C for 3 weeks. Colonies were repeatedly subcultured until pure cultures were obtained and these were characterized morphologically, biochemically and physiologically according to standard methods (Yarrow, 1998).

2.4. Grouping of Indonesian and Ecuadorian isolates

Grouping of isolates was assessed by random amplification of polymorphic DNA (RAPD) analysis using the primers $(GTG)_5$ (5′-GTG GTGGTGGTGGTG-3′) and M13 (Naumov et al., 2006). Amplification was performed for 32 cycles with denaturation at 95 °C for 3 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. Successful amplification was confirmed by agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed under UV light.

2.5. DNA sequencing and phylogenetic analyses

The D1/D2 LSU rDNA and ITS regions were amplified by PCR and sequenced using the primer pairs NL1 and NL4 (Kurtzman and Robnett, 1998), and ITS1 and ITS4 (White et al., 1990), respectively. The resulting sequences were compared with those of reference organisms that were retrieved from the GenBank database. The sequences for phylogenetic analysis were aligned automatically using the multiple sequences alignment program, CLUSTAL_X, version 1.83 (Thompson et al., 1997). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 4.0 software package (Kumar et al., 2004), and evolutionary distances were calculated using the neighbor-joining method with the Kimura two-parameter distance measure. Confidence values were estimated from bootstrap analyses of 1000 replicates (Felsenstein, 1985). *Saccharomyces cerevisiae* NRRL Y-12632^{NT} (AY048154) was the designated outgroup in the analyses; other related sequences listed in Fig. 1 were retrieved from GenBank. Strains G17^T and G31 were deposited in the Bioresource Collection and Research Center (BCRC), Food Industry Development and Research Institute, Hsinchu, Taiwan, the American Type Culture Collection (ATCC), Virginia, United States of American, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Strains CLQCA 10–049 and CLQCA 10–062 were deposited in the Colección de Levaduras Quito Católica (CLQCA), Quito, Ecuador, as well as in the National Collection of Yeast Cultures (NCYC), Norwich, UK (as NCYC 3732 and NCYC 3733, respectively). The GenBank accession numbers for the sequence data are: D1/D2, HM461739 for G17^T, HM461740 for G31, FN706523 for CLQCA 10–049, and FN706524 for CLQCA 10–062; ITS-5.8S, HM461737 for G17^T, HM461738 for G31, FN706517 for CLQCA 10–049, and FN706518 for CLQCA 10–062.

3. Results and discussion

3.1. Yeast isolation, identification and novel species delineation

The precipitated contaminants in the bottled tea drinks were identified as yeasts by microscopic analysis, and uniform yeast colonies appeared when the contaminated bottled tea drinks were plated on YMA plates. All colonies were butyrous, cream colored, with an entire margin and ten colonies were randomly picked from each sample. RAPD analysis demonstrated that the DNA banding patterns of all the isolates were identical (data not shown). Therefore, all isolates were regarded as representative of a single species, and two representative strains from different isolation batches, namely strains G-17^T and G-31, were selected for further analysis. The chicha yeast strains CLQCA 10–049 and CLQCA 10–062 passed through sequential purification steps prior to characterization, to separate them from other yeast species that were also recovered from the clay vessels, including *Rhodotorula mucilaginosa, Rhod. sloffiae, Candida tropicalis, C. parapsilosis, C. lipolytica, Cryptococcus saitoi, and Crypt. laurentii.* The occurrence of such a diverse variety of yeast species in chicha undoubtedly reflects the spontaneous fermentation characteristic of this beverage, as well as the use of different flavoring substrates such as flowers and/or fruits. The presence of

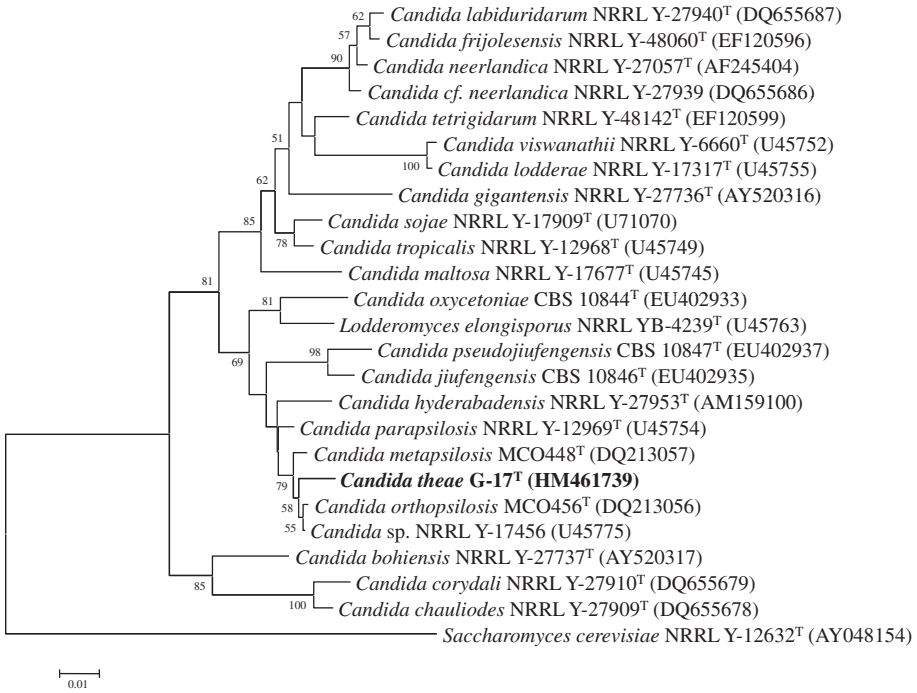

Fig. 1. Neighbor-joining tree based on sequences of the D1/D2 divergent domains of the large-subunit rRNA gene of *Candida theae* sp. nov. G17^T and related species. Bootstrap values (%) above 50 % from 1000 samples are shown. Accession numbers are given in parentheses. Scale bar = 0.01 nt substitutions per site. *Saccharomyces cerevisiae* NRRL Y-12632^{NT} (AY048154) was used as the outgroup.

human-associated species such as *C. parapsilosis* and *C. tropicalis* is possible evidence for the use of human saliva to hydrolyze corn starch and human feces to ferment the resulting wort (Gomes et al., 2009). These ancient yeast isolations as well as its significance in the historical context have been analyzed at the light of the "Microbial Archaeology" approach.

The D1/D2 domain of the LSU rRNA gene as well as the ITS-5.8S region for all four strains was PCR-amplified and sequenced. Phylogenetic analysis based on LSU D1/D2 sequences showed that these four strains had identical sequences and belong to the *Lodderomyces–Spathaspora* clade (Kurtzman and Robnett, 1998; Lachance et al., 2011). It also revealed that the closest known relative is *Candida orthopsilosis* (Fig. 1) which belongs to the *Candida parapsilosis* species group (Lachance et al., 2011). The LSU D1/D2 sequence of strain G17^T (HM461739) differed by 1.1% (6 substitutions) from *C. orthopsilosis* MCO456^T (DQ213056), 1.3% (7 substitutions) from *C. metapsilosis* MCO448^T (DQ213057), and 1.7% (9 substitutions) from *C. parapsilosis* MCO456^T (U45754). Databases containing the sequences of the D1/D2 domain of the LSU rRNA gene are now available for all currently-recognized yeast species (Fell et al., 2000; Kurtzman and Robnett, 1998), resulting in increasing use of this ribosomal DNA (rDNA) region for yeast species identification. Studies have shown that strains belonging to separate species generally exhibit a greater than 1% sequence divergence (Kurtzman and Robnett, 1998; Kurtzman et al., 2011). For further confirmation of the novelty of the species under study, the ITS region has also been proven to be useful in yeast taxonomy, with a similar amount of inter-specific variation (Kurtzman and Robnett, 2003; Kurtzman et al., 2011; Scorzetti et al., 2002). The ITS-5.8S sequence of G17^T differed from *C. metapsilosis* (U10989) by 14 base substitutions, from *C. orthopsilosis* (U10988) by 15 base substitutions, and from *C. parapsilosis* (U10987) by 19 base substitutions. Although the Ecuadorian and Indonesian strains have identical LSU D1/D2 sequences, they can be distinguished from one another based on minor sequence variation found in the ITS1 (4 substitutions) and ITS2 (1 indel) regions (Fig. S1, supplementary data). Collectively, sequence analyses of the D1/D2 and ITS regions indicated that these two strains are members of the same species, a new species distinct from *C. orthopsilosis*, *C. metapsilosis*, and *C. parapsilosis*. These four strains were found to be closely related to the *C. parapsilosis* complex, but sporulation and conjugation were not observed in single-strain or mating cultures on sporulation media. Based on this evidence, the four strains were assigned the status of a new species of *Candida*, for which the name *C. theae* sp. nov. was proposed, with strain G17^T designated as the type strain.

Physiologically, the three current member species of the *C. parapsilosis* complex are very similar to each other (Tavanti et al., 2005; Lachance et al., 2011). The only differences between the three species are that *C. metapsilosis* assimilates D-xylitol, *C. parapsilosis* exhibits proteolytic activity, and *C. orthopsilosis* and *C. metapsilosis* vary in their sensitivity to 5-fluorocytosine (Lin et al., 1995). In view of the fact that the three species are phenotypically very similar, phenotypic differences were compared only between G17^T and *C. parapsilosis* NRRL Y-12969^T. *C. theae* G17^T can be differentiated from *C. parapsilosis* NRRL Y-12969^T by its ability to assimilate arbutin, nitrite, and creatine, and its ability to grow without vitamins (Table 1). Likewise, the Ecuadorian and Indonesian strains can be distinguished from one another based on their differing abilities to assimilate L-arabinose, D-arabinose and D-ribose.

Table 1
Growth characteristics that distinguish *Candida theae* (G17^T) from *Candida parapsilosis* (NRRL Y-12969^T).

Characteristics	G17^T	NRRL Y-12969^T
Arbutin	w	–
Nitrite	w	–
Creatine	w	–
Growth without vitamins	+	–

+, positive; –, negative; w, weak.
Data from reference: *Candida theae* G17^T, this study; *C. parapsilosis* NRRL Y-12969^T, Kurtzman and Robnett (1998).

Food safety and its connection with diet and health are important issues of major concern to consumers. So, tea drinks are not only drunk to quench thirst, but also as a potential functional drink. An aseptic production process is usually emphasized in the production of bottled tea drinks, to lower microorganism contamination and to ensure the health and safety of consumers. This contamination event was proposed to be caused by *C. theae* nov. sp. in this study. This new species is a member of the *Lodderomyces* clade (Lachance et al., 2011), and is phylogenetically closely related to the *C. parapsilosis* complex. Over the past decade, the incidence of *C. parapsilosis* has dramatically increased, and reports indicate that *C. parapsilosis* is the second most commonly isolated pathogenic *Candida* species (Trofa et al., 2008). *C. parapsilosis* was divided into three groups, I to III, before 2005; however, significant differences among these groups observed in genetic studies led to their subsequent separation into three distinct species: *C. parapsilosis* (group I), *Candida orthopsilosis* (group II), and *Candida metapsilosis* (group III) (Tavanti et al., 2005). Most medical microbiology laboratories do not distinguish between these species, as commercial yeast identification systems cannot differentiate these species from each other (Trofa et al., 2008), and so few studies in the literature have made this discrimination.

In the *Lodderomyces* clade (Kurtzman and Robnett, 2003; Lachance et al., 2011), *C. parapsilosis*, *C. albicans*, and *C. tropicalis* are common human pathogens, with the ability to grow at temperatures of 37 °C and above. Likewise, *C. theae* sp. nov. can also grow at temperatures up to and including 42 °C. Therefore, we cannot exclude the possibility that this species represents another example of an emerging fungal pathogen and a possible foodborne threat to immunocompromised populations (Mavor et al., 2005). However, the possible pathogenicity (to humans) of this newly described *Candida* species is yet to be determined.

Species in the *Lodderomyces* clade can be isolated from a diverse variety of different habitats, including human sources such as skin, nails, and blood (Weems, 1992; Lachance et al., 2011), and nonhuman sources such as domestic animals, insects, soil, marine environments (Fell and Meyer, 1967; Ji et al., 2009; Lachance et al., 2011; Suh et al., 2008), and the surfaces of medical plastic and prosthetic devices (Weems, 1992). These potentially opportunistic yeasts, which are extensively distributed in nature, are dispersed by insects, animals, and human beings, and are potential causes of food and beverage contamination as well as infectious disease.

The occurrence of *C. theae* in tea bottles in Indonesia, a large collection of islands in Southeastern Asia, and in ancient chicha pots found in Ecuador, a country in Northwestern mainland South America, is perhaps not surprising given that yeast evolved long before humans and the ancestors of the new species, like its close relative *C. parapsilosis*, probably achieved a global distribution in association with insects and warm blooded animals millions of years before becoming associated with human beverages. Nonetheless, it is interesting to note that a human migration into the New World departed from Asia and from a relatively small group of hunter-gatherers about 17.000 years ago (Lewis et al., 2004). This issue could probably point to a possible human dispersion of *C. theae*, inasmuch as yeasts cannot only be carried in the utensils used for the production and/or storage of food and beverages, but are also present within the human gut. Furthermore, a remarkable human migration departing from Taiwan and Southern China occurred about 6000 years ago in the direction of Indonesia, Melanesia and Micronesia. This historical migration is known by anthropologists as the "out of Taiwan" model and popularly referred to as the "express train to Polynesia" (Oppenheimer and Richards, 2001). There is strong evidence to indicate that Polynesian sailors reached Ecuador sometime during the first millenium A.C. (Scaglion and Cordero, 2011). Thus, there are at least two plausible explanations for the occurrence of the same microbial species (*Candida theae*) on two distant continents, separated by the Pacific Ocean.

3.2. Latin diagnosis of Candida theae Lee sp. nov

In medio agaro YM post dies 3 ad 25 °C, cellulae vegetative ellipsoideae vel ovoideae (1.4–4.2 μm × 1.4–4.1 μm), singulae aut binae. Cultura in agaro YM post dies 3 (25 °C) butyrosa, cremea, pauro hebia, glabra et margine glabra. Hyphae et pseudohyphae non formantur. Asci non formantur. Glucosum, galactosum et α,α-trehalosum fermentantur, at non maltosum, methyl α-D-glucosidum, sucrosum, melibiosum, lactosum, cellobiosum, melezitosum raffinosum, inulinum, amylum, nec D-xylosum. Assimilantur D-glucosum, D-galactosum, L-sorbosum, D-xylosum, D-arabinosum (variabile), L-arabinosum (variabile), D-ribosum (variabile), sucrosum, maltosum, trehalosum, methyl α-D-glucosidum, arbutinum, melezitosum, glycerolum, ribitolum, D-glucitolum, D-mannitolum, D-glucono-1,5-lactonum, 2-ketogluconatum, acidum D-gluconicum, succinatum, citratum, et N-acetyl-D-glucosaminum. Non assimilantur D-glucosaminum, L-rhamnosum, cellobiosum, salicinum, melibiosum, lactosum, raffinosum, inulinum, amylum solubile, erythritolum, xylitolum, L-arabitolum, galactitolum, myo-inositolum, 5-ketogluconatum, D-glucuronatum, acidum galacturonicum, DL-lactatum, methanolum, ethanolum, propane-1,2-diolum, et butane-2,3-diolum. Assimilantur natrium nitrosum, ethylaminum, L-lysinum, cadaverinum nec creatinum. Non assimilantur kalii nitratum. 0.01% cycloheximido vel 10% NaCl addito crescere potest. Non crescit in medio 1% acido acetico addito. Non crescere potest in medio cum 50% glucose. In 35, 40 et 42 °C crescit. Vitamina externa ad crescentiam necessaria non sunt. Ureum haud hydrolysatum, reactio Diazonium blue B infitialis. Amylum et acidum non formatur.

Typus stirps G17^T (BCRC 23242^T = CBS 12239^T = ATCC MYA-4746^T) isolatus ex theae bibere.. Conservatur in Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan; American Type Collection Center (ATCC), Manassas, Virginia, USA et Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

3.3. Description of Candida theae Lee sp. nov

Candida theae (theae adj. derived from Gr. n. tea, where the strains were isolated).

In YM agar medium after 3 days at 25 °C, cells are ellipsoidal to ovoid, 1.4–4.2 μm long by 1.4–4.1 μm wide, and occur singly, or in pairs (Fig. 2). On YM agar after 3 days at 25 °C, the colonies are butyrous, cream-colored, dull, with a smooth surface, and has an entire margin. No hyphae or pseudohyphae formed in Dalmau plate cultures on corn meal agar after 2 weeks. Ascospore production have not been observed on YM agar, Fowell's acetate agar, CMA, or malt extract agar after incubation at 25 and 18° 1 C for 1 month. Glucose, galactose, and trehalose are fermented, but maltose, methyl α-D-glucoside, sucrose, melibiose, lactose, cellobiose, melezitose, raffinose, inulin, starch, and

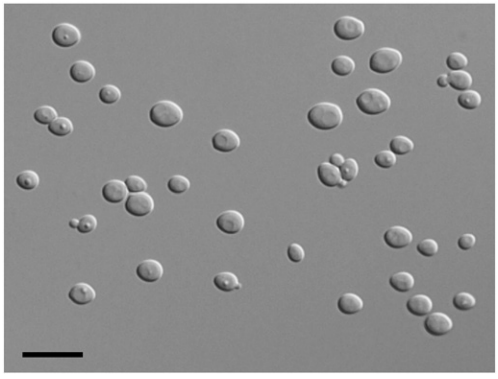

Fig. 2. Morphology of *Candida theae* sp. nov. vegetative cells grown on YM agar for 3 days at 25 °C. Scale bar = 10 μm.

xylose are not fermented. D-Glucose, D-galactose, L-sorbose, D-xylose, D-arabinose (variable), L-arabinose (variable), D-ribose (variable), sucrose, maltose, trehalose, methyl α-D-glucoside, arbutin, melezitose, glycerol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, succinate, citrate, and N-acetyl-D-glucosamine are assimilated. D-glucosamine, D-arabinose, L-rhamnose, cellobiose, salicin, melibiose, lactose, raffinose, inulin, soluble starch, erythritol, xylitol, L-arabinitol, galactitol, myo-inositol, 5-keto-D-gluconate, D-glucuronate, D-galacturonic acid, DL-lactate, methanol, ethanol, propane-1,2-diol, and butane-2,3-diol are not assimilated. Sodium nitrite, ethylamine, L-lysine, cadaverine and creatine are utilized. Potassium nitrate is not utilized. Growth in the presence of 0.01% cycloheximide and 10% sodium chloride is positive. No growth in the presence of 1% acetic acid, and 50% glucose/yeast extract agar. Growth occurs at 35, 40 and 42 °C. Growth does occur in vitamin-free medium. The production of starch-like compounds and acid formation on chalk agar is negative. Reaction to Diazonium Blue B and urease is negative.

The type strain is strain G17^T (BCRC 23242^T = CBS 12239^T = ATCC MYA-4746^T), isolated from tea drink and deposited in the Bioresource Collection and Research Center, Hsinchu, Taiwan; American Type Collection Center (ATCC), Manassas, Virginia, USA and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

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