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Kazachstania yasuniensis sp. nov., a novel ascomycetous yeast species found in mainland Ecuador and on the Galápagos

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Abstract:	Seven strains representing a novel yeast species belonging to the genus Kazachstania were found at several collection sites on both mainland Ecuador (Yasuní National Park) and the Galápagos (Santa Cruz Island). Two strains (CLQCA 20-132T and CLQCA 24SC-045) were isolated from rotten wood samples, two further strains (CLQCA 20-280 and CLQCA 20-348) were isolated from soil samples, and three strains (CLQCA 20-198, CLQCA 20-374 and CLQCA 20-431) were isolated from decaying fruits. Sequence analyses of the D1/D2 domains of the large-subunit (LSU) rRNA gene and ribosomal internal transcribed spacer (ITS) region indicated that the novel species is most closely related to Kazachstania servazzii and Kazachstania unispora. Although the strains could not be distinguished from one another based upon their differing geographical origins, they could be differentiated according to their isolation source (fruit, soil or wood) by ITS sequencing. The species name of Kazachstania yasuniensis sp. nov. is proposed to accommodate these strains, with CLQCA 20-132T (=NCYC 4008T) designated as the type strain.

Kazachstania yasuniensis sp. nov., a novel ascomycetous yeast species found in mainland Ecuador and on the Galápagos

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The GenBank/EMBL/DDBJ accession numbers for the LSU D1/D2 and ITS sequences of CLQCA 20-132^T are HG934855 and HG934852, respectively.

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ABSTRACT

Seven strains representing a novel yeast species belonging to the genus *Kazachstania* were found at several collection sites on both mainland Ecuador (Yasuní National Park) and the Galápagos (Santa Cruz Island). Two strains (CLQCA 20-132^T and CLQCA 24SC-045) were isolated from rotten wood samples, two further strains (CLQCA 20-280 and CLQCA 20-348) were isolated from soil samples, and three strains (CLQCA 20-198, CLQCA 20-374 and CLQCA 20-431) were isolated from decaying fruits. Sequence analyses of the D1/D2 domains of the large-subunit (LSU) rRNA gene and ribosomal internal transcribed spacer (ITS) region indicated that the novel species is most closely related to *Kazachstania servazzii* and *Kazachstania unispora*. Although the strains could not be distinguished from one another based upon their differing geographical origins, they could be differentiated according to their isolation source (fruit, soil or wood) by ITS sequencing. The species name of *Kazachstania yasuniensis* sp. nov. is proposed to accommodate these strains, with CLQCA 20-132^T (=CBS 13946^T =NCYC 4008^T) designated as the type strain.

Keywords: Yeast, ascomycota, *Kazachstania*, Yasuní, Galápagos archipelago, novel species

The genus *Kazachstania* was first proposed by Zubkova in 1971 with the description of *Kazachstania viticola*, a yeast isolated from fermenting grapes in Kazachstan (Zubkova, 1971). Later in the same decade, the taxonomic status of *K. viticola* was re-assessed by Von Arx *et al*. (1977), and it was considered to be a synonym of *Saccharomyces dairenensis*. However, the genus was re-introduced and redefined in 2003 (Kurtzman, 2003), to accommodate *K. viticola* (type species) and 19 other species from the genera *Arxiozyma*, *Kluyveromyces*, *Pachyticospora*, and *Saccharomyces* (*sensu lato*). This resulted from a detailed multigene sequence analysis carried out by Kurtzman and Robnett (2003) to examine the phylogenetic relationships and genus boundaries of the ca. 80 species belonging to the '*Saccharomyces* complex'. In the most recent (fifth) edition of 'The Yeasts, A Taxonomic Study', 32 species were listed as belonging to the genus (Vaughan-Martini *et al*. 2011). Since then, the genus has continued to increase in size as additional species have been discovered and described including *K. bromeliacearum*, *K. ichnusensis*, *K. intestinalis*, *K. pyschrophila*, *K. rupicola*, *K. taianensis*, *K. wufongensis* (Lee *et al*. 2009; Chen *et al*. 2010; Suh & Zhou, 2011; Araújo *et al*. 2012; Cardinali *et al*. 2012; Kabisch *et al*. 2013; Safar *et al*. 2013).

On the basis of multigene sequencing, using both nuclear- and mitochondrial-encoded genes, Kurtzman and Robnett (2003) resolved the genus into three main lineages (see Fig. 1; Kurtzman & Robnett, 2003). However, with only moderate statistical support (bootstrap value, 76%), Kurtzman (2003) concluded that the newly redefined genus was likely to be provisional, as the species assigned to it were clearly polyphyletic. A similar result was obtained more recently by Vaughan-Martini *et al*. (2011) using LSU D1/D2 sequences, where at least five separate subgroups/subclades, with varying statistical support, were identified (see Fig. 34.1; Vaughan-Martini *et al*. 2011). The overall consensus is that as additional species are discovered and further multigene sequencing is carried out, stronger and more reliable species partitioning can be achieved (Kurtzman, 2003; Lu *et al*., 2004; Vaughan-Martini *et al*., 2011). If so, then this in turn will inevitably lead to a reclassification of

the genus as presently defined, and result in the creation of a number of new sister genera (Kurtzman, 2003; Vaughan-Martini *et al*., 2011).

From a phenotypic perspective, there are no distinctive morphological or physiological traits that can reliably delineate the genus *Kazachstania* (Kurtzman, 2003; Kurtzman & Robnett, 2003). This lack of phenotypic identity is a characteristic common to many of the recently created genera that have been defined from phylogenetic analysis (e.g. *Wickerhamomyces*; Kurtzman, 2011). Species of the genus *Kazachstania* have been isolated from a wide variety of habitats, such as animals, fermented foods, fruit, leaves, mushrooms, silage, soil and wastewater (Wu & Bai, 2005; Limtong et al., 2007; Nisiotou & Nychas, 2008; Lee et al. 2009; Chen et al. 2010; Vaughan-Martini et al. 2011). Some species are heterothallic (e.g. *K. gamospora* and *K. zonata*; Imanishi *et al*., 2007), whereas others are homothallic (e.g. *K. hellenica*; Nisiotou & Nychas, 2008).

Since 2007, the Colección de Levaduras Quito Católica (CLQCA) has been conducting a yeast bio-prospecting programme to catalogue and characterise the indigenous yeast species present in the many differing ecological habitats found in Ecuador, both on the mainland and on the Galápagos Islands. To date, more than 3000 yeast strains have been collected and a number of new species have been discovered and formally described, including *Candida carvajalis* (James *et al*. 2009), *Saturnispora quitensis* (James *et al*. 2011) and most recently *Wickerhamomyces arborarius* (James *et al*. 2014). In October 2013, the CLQCA carried out a preliminary study to catalogue the yeast diversity at several sites in the Yasuní National Park (Yasuní), a 9,800 km^2 region of prime Amazonian rainforest habitat situated in eastern Ecuador, approx. 250 km from Quito. Yasuní is widely recognised as representing one of the most biologically diverse regions on Earth, and harbours the greatest variety of tree species found anywhere on the planet. Furthermore, many of the plants and animals found there are endemic to the region (e.g. the bat species *Lophostoma yasuni*). Yasuní is also incorporated within the territory of two indigenous tribes of people, the Tagaeri and Taromenan, who live in voluntary isolation from the outside world. One key objective of

the Yasuní yeast collecting project was to investigate whether or not it was possible to isolate ethanol tolerant species (e.g. *Saccharomyces*) in this arboreal habitat. In order to do this, a selective sampling and enrichment method, as developed by Sniegowski *et al*. (2002), was used.

Here we describe the discovery of seven novel *Kazachstania* strains isolated at separate sites on mainland Ecuador and in the Galápagos archipelago, and the formal taxonomic description of a new *Kazachstania* species, *Kazachstania yasuniensis* sp. nov., to accommodate them. Six ethanol-tolerant *Kazachstania* strains were isolated from substrates collected in the Yasuní National Park. All were isolated by enrichment culturing using a medium containing 7.6% (v/v) ethanol (Sniegowski *et al*. 2002). One strain (CLQCA 20-132^{T}) was isolated from rotten wood, two strains (CLQCA 20-280 and CLQCA 20-348) were isolated from soil samples, and a further three strains (CLQCA 20-198, CLQCA 20-374 and CLQCA 20-431) were isolated from decaying fruits. A seventh strain, CLQCA 24SC-045, was subsequently identified following a re-examination of *Kazachstania* strains previously collected during a 2009 trip to four of the human-inhabited islands of the Galápagos (i.e. Floreana, Isabela, San Cristobal and Santa Cruz). Strain CLQCA 24SC-045 was found at Los Gemelos (approx. 600 m above sea level) on Santa Cruz Island, and was isolated from a sample of rotten wood collected from a daisy tree *(Scalesia pedunculata*), a tree species endemic to the Galápagos.

The seven yeast strains were characterised biochemically, morphologically, and physiologically according to the standard methods described by Kurtzman *et al*. (2011). Growth temperature testing was determined by cultivation on YM (yeast extract-malt extract) agar. Sporulation tests were performed on cormeal agar, Gorodkowa agar, potassium acetate agar and YM agar, and plates were incubated at 25˚C for 1 month in individual and mixed cultures.

The variable D1/D2 domains of the LSU rRNA gene and ribosomal ITS region were amplified by PCR directly from whole yeast cell suspensions as described previously by James *et al*. (1996). The LSU D1/D2 domain was amplified and sequenced using primers NL1 and NL4 (O'Donnell, 1993). The ITS region was amplified using primers ITS5 and ITS4, and sequenced using these primers as well as internal primers ITS2 and ITS3 (White *et al*. 1990). The amplified DNA was checked by 1.0% agarose gel electrophoresis, purified and concentrated using QIAquick PCR purification spin columns (Qiagen). A NanoDrop 1000 spectrophotometer (Thermo Scientific) was used for measuring DNA concentration and samples were sequenced by a commercial sequencing facility (Eurofins MWG Operon, Germany). Sequence traces were edited manually and consensus sequences generated using the program SEQMAN, version 7 (DNASTAR). The LSU D1/D2 sequences were compared pairwise using a FASTA similarity search (Pearson & Lipman, 1988), and were aligned with the sequences of closely related taxa, retrieved from the EMBL sequence database, using the multiple alignment program CLUSTAL W (Thompson *et al*., 1994), included in the DNAMAN software package, version 5.1.5 (Lynnon BioSoft). A phylogenetic tree was constructed from the combined sequences of the LSU D1/D2 and ITS regions (including 5.8S rDNA) using the neighbour-joining method (Saitou & Nei, 1987), with the Jukes-Cantor distance measure, and *Kazachstania aquatica* used as the outgroup species. Confidence limit values were estimated from bootstrap analyses of 1000 replicates (Felsenstein, 1985).

The LSU D1/D2 sequences of the six Yasuní strains CLQCA 20-132^{T}, CLQCA 20-198, CLQCA 20-280, CLQCA 20-348, CLQCA 20-374 and CLQCA 20-431 as well as the Galápagos strain CLQCA 24SC-045 were all found to be identical. A FASTA sequence similarity search of the EMBL fungal sequence database revealed no other yeast taxon with a LSU D1/D2 sequence identical to these strains. In terms of pairwise sequence similarity, the seven strains displayed 0.7% divergence (4 nt substitutions in 581 nt) with *Kazachstania servazzii and Kazachstania unispora*, and 1.0% divergence (6 nt substitutions in 572 nt) with

Kazachstania aerobia and *Kazachstania solicola*. Although somewhat limited, these levels of sequence divergence are comparable to those observed between *Kazachstania aerobia* and *Kazachstania unispora* (4 nt substitutions in 572 nt), and between *Kazachstania servazzii* and *Kazachstania unispora* (6 nt substitutions in 572 nt). In fact, they are notably greater than those observed between *Kazachstania aerobia* and *Kazachstania servazzii*, whose LSU D1/D2 sequences differ by only two nucleotide substitutions (in 572 nt), and between *Kazachstania aerobia* and *Kazachstania solicola*, which have identical LSU D1/D2 sequences.

As reported previously by Vaughan-Martini *et al*. (2011), the four member species of the *K. unispora* subclade (viz. *K. aerobia*, *K. servazzii*, *K. solicola* and *K. unispora*) are closely related to one another, and form a distinct species group within the genus. Reliable taxonomic resolution of these *Kazachstania* species based on LSU D1/D2 sequences is at best limited and in the case of *K.aerobia* and *K. solicola* impossible. Levels of sequence divergence in this rDNA region range from 0 (between *K.aerobia* and *K. solicola*) to 6 nt substitutions (between *K. servazzii* and *K. unispora*). However, despite their close phylogenetic relationships based on LSU D1/D2 sequences, the four current members of the *K. unispora* subclade as well as the Ecuadorian novel species can be readily distinguished from one another by ITS sequencing. Levels of ITS sequence divergence are significantly greater, ranging from 22 nt subsitutions and 3 indels (in 659 nts) between *K. aerobia* and *K. servazzii*, to 36 nt substitutions and 15 indels (in 671 nts) between *K. solicola* and *K. unispora*. In the case of the novel species, this taxon differs from its two closest relatives *K. servazzii* and *K. unispora* by 26 nt substitutions and 7 indels (in 659 nts), and by 28 nt substitutions and 7 indels (655 nts), respectively.

Furthermore, the levels of ITS sequence divergence are such that the seven Ecuadorian strains can be differentiated into three separate sub-groups based upon the type of substrate from which each was isolated. The two strains isolated from rotten wood (CLQCA 20-132^{T} and CLQCA 24SC-045) can be distinguished from the two soil strains (CLQCA 20-280 and

CLQCA 20-348) based on two nt substitutions in the ITS1 region, and from the three decaying/rotten fruit strains based on three nt substitutions in the ITS2 region. Interestingly, and perhaps rather unexpectedly, CLQCA 20-132^T and CLQCA 24SC-045 were found to have identical ITS sequences, despite the fact that one was isolated on the mainland in eastern Ecuador (CLQCA 20-132^T) while the other was isolated approx. 1700 km to the west in the Galápagos archipelago. A comparative alignment of the ITS sequences for all seven novel *Kazachstania* strains is shown in Supplementary Fig. S1 (available in IJSEM Online).

At present, there are relatively few ITS sequences available for strains belongings to the *K. unispora* subclade. In their study of yeast biota involved in silage deterioration, Lu *et al*. (2004) characterised both *K. aerobia* strains (NS14^T and NS26) upon which the species description is based and found them to have identical ITS sequences. Likewise, in their phylogenetic study of the '*Saccharomyces* complex', Kurtzman and Robnett (2003) examined two strains of *K. unispora*, including the type strain (NRRL Y-1556^T), and found them to have identical ITS sequences. The ITS sequences for two additional *K. unispora* strains, one from fermented orange juice and the other from nasal mucus (GenBank accession nos AF321542 and AF455430, respectively) differ from that of the type strain by a single indel in the ITS2 region. Collectively, this data would suggest that the ITS sequences of these *Kazachstania* species are well conserved, and exhibit very limited intra-specific variation. This supports our proposal that the seven Ecuadorian strains clearly belong to a distinct species, rather than simply represent South American variants of *K. unispora*.

The levels of LSU D1/D2 sequence divergence exhibited by members of the *K. unispora* subclade are extremely low (ranging from 0 to 6 nt substitutions), which makes accurate species delineation difficult. However, as Figure 1 demonstrates, far better and more statistically significant resolution can be achieved by combining LSU D1/D2 and ITS sequences. Using this approach, the four known species along with the novel Ecuadorian species can be readily distinguished from one another. This includes *K. aerobia* and *K. solicola* which, as reported previously by Wu and Bai (2005), have identical D1/D2

sequences. Indeed, as this and previous studies have shown, ITS sequencing represents a far more reliable method of species discrimination for *K. unispora* and its close relatives (Lu *et al*., 2004; Wu & Bai, 2005).

Based upon the origins of the seven strains reported here, it would seem plausible to speculate that the ecological niche of *K. yasuniensis* sp. nov. is possibly an arboreal habitat. Although the strains were isolated from three different substrates, namely decaying fruits, rotten wood and soil, all were found in densely wooded environments. The six Yasuní strains were collected at separate sites within the Amazonian rainforest region of eastern Ecuador, while the Galápagos strain was found in a *Scalesia* forest in the highlands of Santa Cruz Island.

An ecological analysis of the Ecuadorian yeast strains registered in the CLQCA database was recently performed in order to develop a simple mathematical model for calculating how well individual yeast species have adapted to the differing habitats found in Ecuador (Carvajal *et al*. 2014). In this study, a set of 881 yeast strains, representing 104 species, were analysed using a mathematical approach which focused on the number of different natural regions of Ecuador each species was found to colonize as well as the number of different types of substrate from which they had been isolated. From these analyses it was possible to calculate the Relative Specialization Index (*Si*) for each species. The *Si* value measures the degree of specialization related to the habitats and substrates studied. Thus, the higher the *Si value,* the more specialized the yeast species. In Ecuador it was possible to find yeast species exhibiting *Si* values ranging from 0.02 (generalist) to 0.92 (specialist) (Carvajal *et al*. 2014).

With regard to *K. yasuniensis* sp. nov., the *Si* value was calculated to be 0.62. This meant it grouped with the majority of yeast species (67%) analysed which were found to be highly specialized and restricted to a small number of habitats and substrates, both on mainland Ecuador and in the Galápagos archipelago (Carvajal *et al*. 2014). Other species sharing the

same *Si* value as *K. yasuniensis* sp. nov. included *Candida ecuadorensis*, *C. natalensis*, *C. oleophila*, *Geotrichum silvicola*, *Hanseniaspora meyeri*, *Rhodotorula glutinis*, and *Wickerhamiella occidentalis*. Although the actual distribution of each of these species differed from that of *K. yasuniensis* sp. nov.

From the same study it was also possible to establish a correlation between the percentage of plant species that were originally from the mainland and which had subsequently migrated, via different means of dispersal (e.g. birds), to the oceanic archipelago. In 1976, Porter (1976) determined that ~30% of all vascular plant species found in the Galápagos have a Neotropical origin. Remarkably in their more recent study, Carvajal *et al*. (2014) identified that 31% of the yeast species isolated on the Galápagos Islands were also found on mainland Ecuador. This would indicate that plant dispersal may have played an important role in the dispersal of yeast species from the mainland to the archipelago. However, it is as yet unclear as to how a species such as *K. yasuniensis* sp. nov., which to date has only been found in the Ecuadorian Amazon, could have been introduced into the Galápagos Islands. Further sampling will be need to be carried out in order to gain a better insight into the origins and distribution of this novel *Kazachstania* species, and to establish how it may have been dispersed from the mainland to the Galápagos archipelago.

Physiologically, the species group of *K. aerobia*, *K. servazzii*, *K. solicola*, *K. unispora* and *K. yasuniensis* sp. nov. are very similar to one another. Supplementary Table S1 lists the key characteristics that can be used to differentiate between the five *Kazachstania* species. With regard to the novel species, the assimilation of trehalose and ethanol as well as growth on ethylamine hydrogen chloride and sodium chloride (10%) appear to be variable growth characteristics. *Kazachstania yasuniensis* sp. nov. differs from its closest genealogical relatives *K. unispora* (Fig. 1) on its ability to assimilate sucrose (positive or delayed) and inability to grow at 37˚C, and from *K. servazzii* (Fig. 1) on its ability to grow in the presence of 0.01% cycloheximide and inability to assimilate glycerol. In view of the fact that these five species have such similar overall phenotypic profiles, making accurate discrimination

255 difficult, we strongly recommend that ITS sequencing should be adopted as a more reliable
256 and robust method for determining species identity.

257 The *K. unispora* subclade, which with the discovery of *K. yasuniensis* sp. nov. now
258 comprises of five closely related species, represents a distinct and statistically well-
259 supported species group within the genus *Kazachstania* (Fig. 1). The five species have
260 similar overall phenotypes, and whilst not a distinct characteristic of the subclade each
261 typically forms persistent asci which are transformed directly from vegetative cells and
262 contain one spheroidal ascospore each (Lu *et al*., 2004; Wu & Bai, 2005; Vaughan-Martini *et*
263 *al*., 2011; this study). Results from the present study as well as from previous studies would
264 strongly suggest that these five species represent a separate genus (Lu *et al*., 2004; Wu &
265 Bai, 2005). However, it is also evident that while the genus as currently defined appears to
266 be polyphyletic (Kurtzman, 2003; Kurtzman & Robnett, 2003; Wu & Bai, 2005; Vaughan-
267 Martini *et al*., 2011), further multigene sequencing is still required to establish clear, and well-
268 defined genus boundaries prior to any future reclassification of these yeasts.

269

270 **Description of *Kazachstania yasuniensis* James, Carvajal, Portero, Nueno-Palop,**
271 **Bond & Roberts, sp. nov.**

272 *Kazachstania yasuniensis* (ya.su.ni'en.sis. N.L. fem. adj. yasuniensis of or belonging to
273 Yasuní, where the majority of these yeasts were found).

274 In YM broth, after 2 days of incubation at 25˚C, cells are ovoid (4-6 x 5-10 μm) and occur
275 singly, in pairs, in short chains or in groups (Fig. 2a). Budding is multilateral. Sediment is
276 formed after 1 month, but no pellicle is observed. In Dalmau plate culture on corn meal agar,
277 pseudohyphae are not formed. Sporulation observed on cornmeal agar, Gorodkowa agar,
278 potassium acetate agar and YM agar after 3-7 days at 25˚C; vegetative cells transform
279 directly into persistent asci each containing one spheroidal ascospore (Fig. 2b).

Glucose and galactose are fermented, but not sucrose, maltose, lactose, melibiose, melezitose, raffinose, trehalose, starch, cellobiose, inulin, D-xylose or methyl α-D-glucoside. Glucose, sucrose (positive or latent), raffinose (latent but weak), galactose, trehalose (seldom positive) and ethanol (latent but weak or negative) are assimilated. No growth occurs on inulin, melibiose, lactose, maltose, melezitose, methyl α-D-glucoside, starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose, D-arabinose, D-ribose, methanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, inositol, DL-lactate, succinate, citrate, D-glucosamine, glucono-D-lactone or xylitol. Cadaverine (latent) and ethylamine hydrochloride (latent or negative) are assimilated. No growth occurs on lysine or nitrate. Growth occurs at 30˚C, but not at 37˚C. Growth occurs on YM agar with 10% (w/v) NaCl (variable) and on 100 ug cycloheximide ml^{-1}. No growth occurs on 50% glucose/yeast extract. Starch-like compounds are not produced.

The type strain, CLQCA 20-132^{T} (=CBS 13946^{T} = NCYC 4008^{T}), was isolated in October 2013 from a rotten wood sample collected in the Yasuní National Park, Ecuador. The Mycobank deposit number is MB810753.

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392 **Figure Legends**

393 **Fig. 1.** Neighbour-joining dendrogram based on the combined sequences of the LSU D1/D2
394 and ITS regions (including 5.8S rDNA) of *Kazachstania yasuniensis* sp. nov. and its closest
395 relatives. Species names are followed by CBS, CLQCA or NRRL strain accession numbers
396 and, respectively, the EMBL/GenBank accession numbers for the LSU D1/D2 and ITS
397 regions. *Kazachstania aquatica* was used as the outgroup species for the analysis.
398 Bootstrap values of ≥50%, determined from 1000 replicates, are shown at branch nodes.
399 Bar, 1 base substitutions per 100 nt.

400

401 **Fig. 2.** *Kazachstania yasuniensis* sp. nov. CLQCA 20-132^{T}. (a) Scanning electron
402 microscopic image of vegetative cells grown in YM broth for 2 days at 25˚C with agitation.
403 Bar, 10 μm. (b) Photomicrograph of asci formed on YM agar after 3 days at 25˚C. Bar, 10
404 μm.

405

Figure 1

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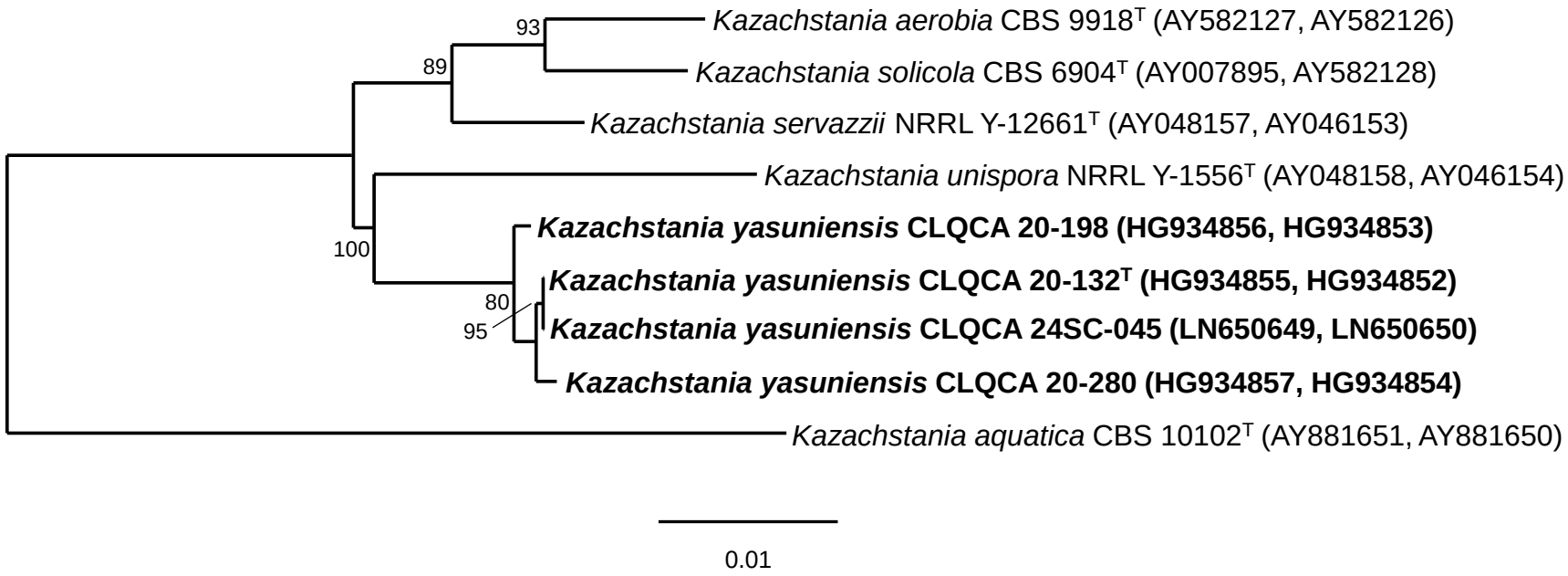

Figure 2a
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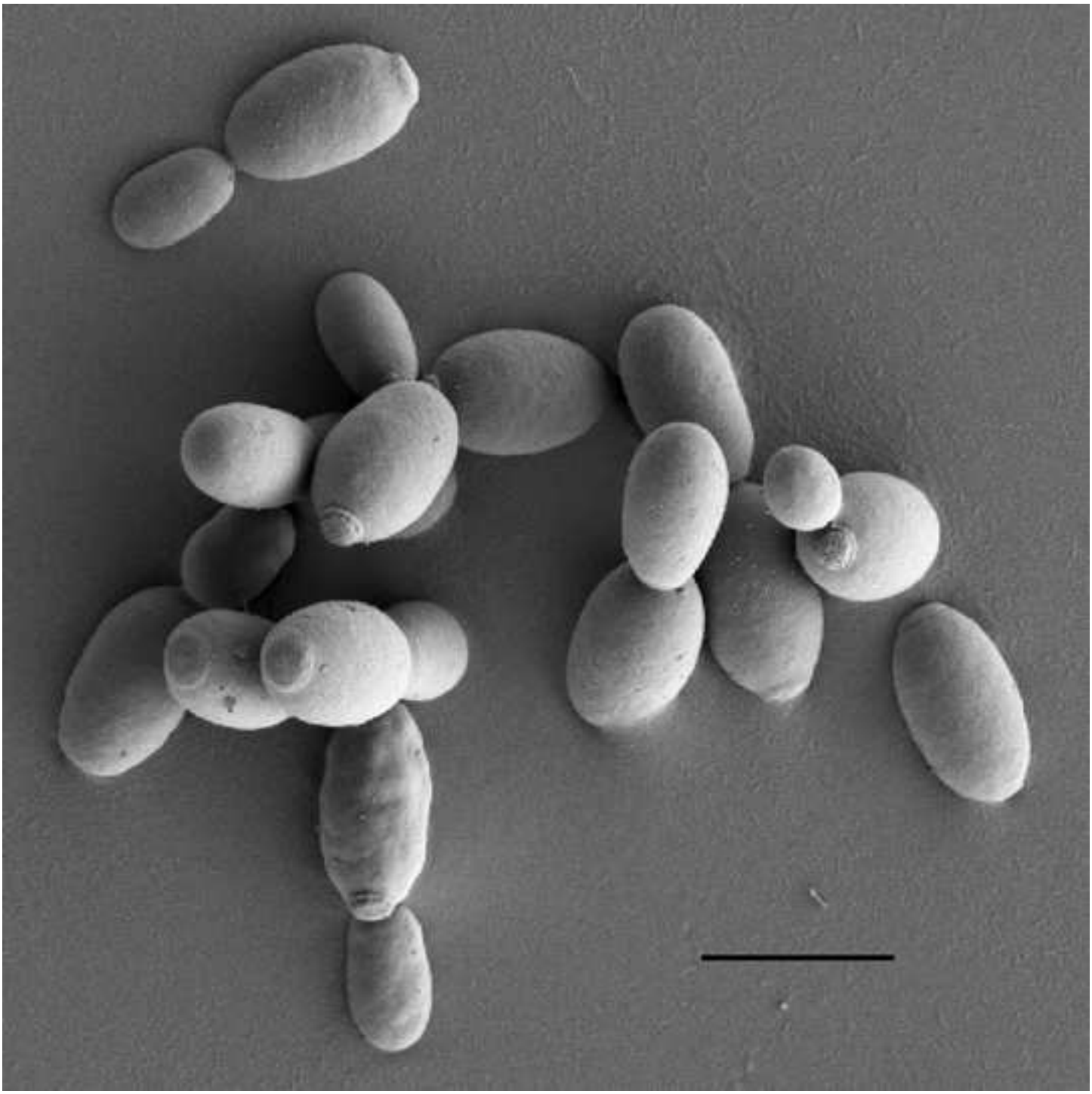

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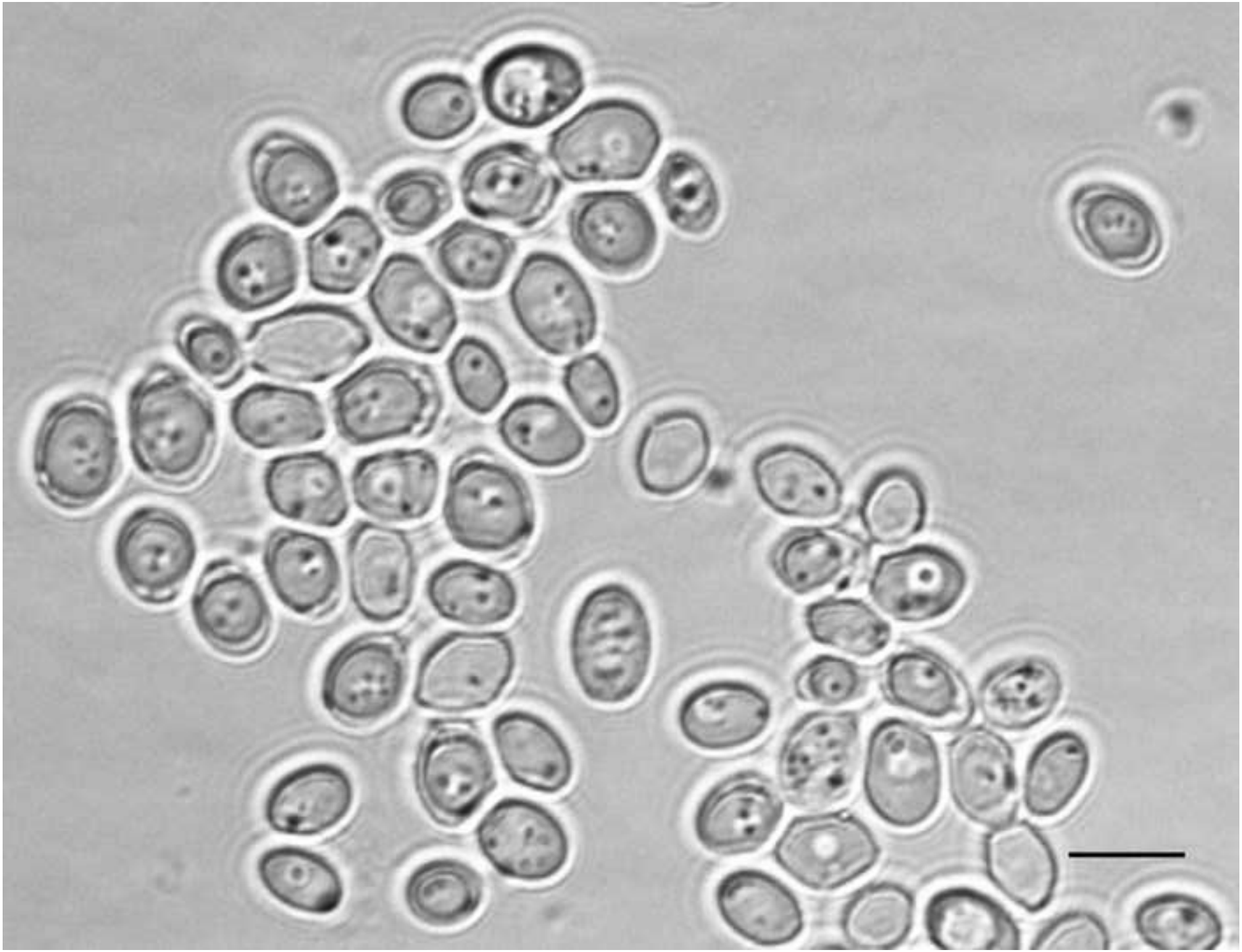

Supplementary Data

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