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Published in:
G3: Genes, Genomes, Genetics

DOI:
10.1093/g3journal/jkab342

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

Document license:
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Citation for published version (APA):
Arrey, G., Li, G., Murphy, R., Guimaraes, L., Alizadeh, S., Poulsen, M., & Regenberg, B. (2021). Isolation, characterization, and genome assembly of Barnettozyma botsteinii sp. nov. and novel strains of Kurtzmaniella quercitrusa isolated from the intestinal tract of the termite Macrotermes bellicosus. G3: Genes, Genomes, Genetics, 11(12), [jkab342]. https://doi.org/10.1093/g3journal/jkab342
Isolation, characterization, and genome assembly of *Barnettozyma botsteinii* sp. nov. and novel strains of *Kurtzmaniella quercitrusa* isolated from the intestinal tract of the termite *Macrotermes bellicosus*

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Abstract

Bioconversion of hemicelluloses into simpler sugars leads to the production of a significant amount of pentose sugars, such as D-xylose. However, efficient utilization of pentoses by conventional yeast production strains remains challenging. Wild yeast strains can provide new industrially relevant characteristics and efficiently utilize pentose sugars. To explore this strategy, we isolated gut-residing yeasts from the termite *Macrotermes bellicosus* collected in Comoé National Park, Côte d’Ivoire. The yeasts were classified through their Internal Transcribed Spacer/Large Subunit sequence, and their genomes were sequenced and annotated. We identified a novel yeast species, which we name *Barnettozyma botsteinii* sp. nov. 1118 (MycoBank: 833563, CBS 16679 and IBT 710) and two new strains of *Kurtzmaniella quercitrusa* var. *comoensis* (CBS 16678, IBT 709) and var. *filamentosus* (CBS 16680, IBT 711). The two *K. quercitrusa* strains grow 15% faster on synthetic glucose medium than *Saccharomyces cerevisiae* and grow on D-xylose as the sole carbon source at a rate of 0.35 h⁻¹. At neutral pH, the yeast form of *K. quercitrusa* var. *filamentosus*, but not var. *comoensis*, switched to filamentous growth in a carbon source-dependent manner. Their genomes are 11.0–13.2 Mb in size and contain between 4888 and 5475 predicted genes. Together with closely related species, we did not find any relationship between gene content and ability to grow on xylose. Besides its metabolism, *K. quercitrusa* var. *filamentosus* has a large potential as a production organism, because of its capacity to grow at low pH and to undergo a dimorphic shift.

Keywords: *Barnettozyma botsteinii*; D-xylose; insect-associated yeasts; *Kurtzmaniella quercitrusa*; new yeast species

Introduction

The biotechnology industry has historically relied on yeast species to carry out fermentation processes (Demain 2000). A major challenge today is the conversion of raw plant biomass directly into organic compounds of added value, such as organic acids or biogas (Qin et al. 2017; Martínez-Gutiérrez 2018; Lee et al. 2019). Hemicellulose material is mainly composed of cellulose (30–50%), hemicellulose (20–30%), and lignin (15–25%) (Kricka et al. 2014). To break down these complex polysaccharides, filamentous fungi, such as *Trichoderma reesi*, *Aspergillus niger* and *Aspergillus oryzae*, and their secreted enzymatic cocktails have been used with success (Mäkelä et al. 2014; Lange 2017). Because these fungi do not efficiently ferment pentose sugars such as D-xylose and D-arabinose produced during depolymerization of hemicelluloses, yeast species or genetically engineered *Saccharomyces cerevisiae* strains have been suggested for downstream processes (Bettiga et al. 2009). However, co-fermentation of hexoses and pentoses is still a major challenge during the efficient conversion of lignocellulosic plant biomass, mainly due to difficulties in integrating a catabolic pathway within an existing metabolic network (Young et al. 2010). There is therefore a need to identify and optimize yeasts that can efficiently ferment both pentoses and hexoses. One strategy is to isolate novel wild yeast strains that can metabolize these sugars, which can either serve as a production organism on their own or as a genetic resource for metabolic pathways that can be engineered into industrial strains (Steenseels et al. 2014).

The unicellular yeasts are commonly found in soil, plants, and flowers, and within the microbiome of insects (bees, beetles, termites, and ants), where they establish a variety of relationships, ranging from obligate to facultative mutualisms (Ganter 2006; Greig and Leu 2009; Kurtzman et al. 2011c; Buzzini et al. 2017). Described yeast species are found both in the Basidiomycota and Ascomycota phyla and are thought to represent only 1% of the expected yeast diversity worldwide (Robert 2006). In addition, yeast species from tropical rainforest, desert, or tundra ecosystems are underrepresented among characterized species (Kurtzman et al. 2015).
and supplemented with 0.1 g/l of chloramphenicol, 0.05 g/l of luting 39 g/l of PDA (Sigma Aldrich, 70139) in water, autoclaved, buffered saline (PBS) supplemented with Tween 80 (5 mg/ml). Together to increase the starting material and manual tissue dissection for biotechnological production.

The termite and gut (Figure 1) (Bos et al. 2020). In this study, we present and characterize two novel strains belonging to the species Kurtzia quercitrusa, phylum Ascomycota, and they were thus named K. quercitrusa var. comoeensis (strain number in this work 1112) and K. quercitrusa var. filamentosus (strain number in this work 1120). We also identified a previously unknown species from the Ascomycota, which we name Barnettzyma botsteinii sp. nov. 1118T (strain number in this work 1118). All three strains assimilate pentose sugars, which make them potentially suitable for biotechnological applications and interesting for further genomic and metabolic studies. Their degradation of plant-derived polymers suggests a potential role in assisting termites to assist in the degradation of up to 90% of all dead wood in Kenya (Buxton 1981). Altogether, their foraging and digestive capabilities substantially contribute to carbon cycling in these ecosystems (Sugimoto et al. 2000) converting wood, leaves, and roots (Buxton 1981; Collins 1983; Smith et al. 2019).

In this study, we present and characterize two novel strains and one new species of yeast isolated from the gut of M. bellicosus. The two novel strains belong to the species Kurtzia quercitrusa, phylum Ascomycota, and they were thus named K. quercitrusa var. comoeensis (strain number in this work 1112) and K. quercitrusa var. filamentosus (strain number in this work 1120). We also identified a previously unknown species from the Ascomycota, which we name Barnettzyma botsteinii sp. nov. 1118T (strain number in this work 1118). All three strains assimilate pentose sugars, which make them potentially suitable for biotechnological applications and interesting for further genomic and metabolic studies. Their degradation of plant-derived polymers suggests a potential role in assisting termites and Termitomyces metabolism and supports their potential for biotechnological production.

**Methods**

**Sample collection**

We collected M. bellicosus minor workers from the inside of wild nests in Comoe National Park, Côte d’Ivoire during the month of November 2018. Specifically, the termite nests were located in the proximity of the Comoe National Park Research Station (8°46’11”N, 3°47’21”W), in the southwest end of the park next to the Comoe River. The gut of small workers (25 termites per nest) was dissected in situ and divided into midgut, hindgut, and foregut (Figure 1) (Bos et al. 2020). Five to ten replicates were pooled together to increase the starting material and manual tissue dissection. The gut-residing microbes were then subjected to genomic DNA extraction and rDNA sequencing.

**Internal Transcribed Spacer and D1/D2 sequencing and phylogeny**

For genomic DNA (gDNA) extraction, a standardized protocol for yeasts was applied to all the isolates. Briefly, yeast cell wall was broken using glass beads and vortexing. Buffers P1, P2, and N3 (Qiagen, Cat. No.: 19051, 19052, and 19064) were used to isolate the DNA fraction, which was further precipitated by the addition of isopropanol. Finally, after an ethanol wash, pellets were resuspended in TE buffer (10 mM Tris-HCl, pH = 8, 1 mM EDTA) and DNA concentration was assessed by Nanodrop. Primers ITS4 (5‘-TCCTCCGCTATGATATGC) and ITS5 (5‘-CTTGGTCATTTAGAGGAAGTAA) (Kuiper-Goodman and Scott 1989) were used to amplify Internal Transcribed Spacer (ITS) regions while primers NL1 (5‘-GCATATCAATAACCGAGGAAAAAG) and NL4 (5‘-GGTCCGTTTCTAAGACGGG) were used to amplify the Large Subunit of the nuclear ribosomal RNA (LSU rRNA) gene region (Kurtzman and Robnett 1997). Both PCR products were purified, Sanger sequenced and results aligned to NCBI database using BLASTn (Altschul et al. 1990). Nucleotide sequences for a set of the most closely yeast species were retrieved from GenBank (Supplementary Table S1). Alignment was performed with Mafft v.7.471 (http://mafft.cbrc.jp/alignment/soft ware/) and a maximum-likelihood (ML) phylogeny based on ITS and LSU was constructed in MEGA v. X (Kumar et al. 2018). Branch support was obtained from bootstrap analysis with 1000 repetitions.

**Genome sequencing and analyses**

We sequenced and assembled the genomes of isolates 1118, 1112, and 1120 in the following steps. Code used is provided in Supplementary File S1.

**DNA extraction, sequencing, and assembly**

Whole-genome sequencing of isolate 1118, 1112, and 1120 was done by first extracting DNA using a scaled-up CTAB extraction (Poulsen et al. 2014). Whole-genome sequencing was performed using a combination of 100/150 bp paired-end shotgun (BGIseq/ DNBseq) and long-read (PacificBio sequencer) sequencing by BGI. The resulting sequences were checked for quality using FastQC v0.11.9 (Andrews et al. 2012). Genomes were assembled using three different approaches and the best result was chosen for subsequent analyses. First, short and long reads were assembled together and used to generate scaffolds using SPAdes 3.14.1 (Nurk et al. 2013). Second, long reads were assembled into scaffolds with Canu 2.0 (Koren et al. 2017) and then polished by the short reads using NextPolish 1.3.1 (Hu et al. 2020). Finally, short reads were assembled into scaffolds alone, also using SPAdes 3.14.1 (Nurk et al. 2013). Contigs that were shorter than 500 bp were discarded in the final assemblies, and assembly quality was assessed by quantifying genome completeness based on the expected gene content of the Benchmarking Universal Single-Copy Orthologs (BUSCO), version 4.4.1, against the database for fungal genomes (Seppey et al. 2019).

**Genome annotation**

Genomes were annotated using MAKER v3.01.03 (Holt and Yandell 2011). Two ab initio gene predictors were used with the MAKER pipeline: SNAP v2013-11-29 (Leskovec and Sosic 2016) and AUGUSTUS v3.3.3 (Stanke et al. 2004), each of which was trained for individual genomes. The training of both SNAP and AUGUSTUS requires pre-existing gene models as training data.
Therefore, an initial MAKER analysis was carried out where gene annotations were generated directly from homology evidence without using the \textit{ab initio} gene predictors. The resulting gene annotations supported by homology evidence were then used to train SNAP and AUGUSTUS. Once both \textit{ab initio} gene predictors were trained, they were used together with homology evidence in a full MAKER analysis. Homology evidence was only used to inform gene predictions. Resulting gene models supported by homology evidence were used to re-train SNAP and AUGUSTUS. A second-round analysis was conducted using the newly trained SNAP and AUGUSTUS parameters, and once again the resulting gene models with homology supports were used to re-train SNAP and AUGUSTUS. Finally, a third round of MAKER analysis was performed using the new SNAP and AUGUSTUS parameters. All resulting gene models are reported, and these comprise the final set of annotated genes.

Functional gene annotation was performed using InterProScan version 5.48-83.0 (Jones et al. 2014) with annotation of gene ontology (GO) terms (Ashburner et al. 2000), KEGG pathways (Kanehisa et al. 2021), and Pfam 33 (Mistry et al. 2021). The predicted proteins sequences were blasted against NCBI-NR (O’Leary et al. 2016) and Swiss-Prot databases (Bateman 2019) with an E-value cutoff of 1e-5. Noncoding RNA (ncRNA) genes were identified by B\textit{a}\textit{s}ic Rapid Ribosomal RNA Predictor v0.9, Barrnap (Seemann 2013) and \textit{t}RNA genes were predicted using the \textit{t}RN\textit{A}sc\textit{a}n-\textit{SE} v2.0.5 (Lowe and Chan 2016) algorithm with default parameters. Repeat sequences were identified and classified using RepeatMasker v4.1.0 (Smit et al. 2019). The criterion used for LTR family classification was that the 5\textsuperscript{0} LTR sequences should share at least 80\% identity over at least 80\% of their length for the same family.

\textbf{Multi-locus-sequence-typing analysis}

We performed multi-locus-sequence-typing (MLST) using a set of Universal Single-Copy Orthologous genes for all genomes that were identified with BUSCO v4 (Seppey et al. 2019) with default settings and using the fungi\_odb10.2019-11-20 dataset. We collated 344 (of 758 potential) genes present in all 48 genomes and generated multi-fasta files of the orthologous genes (Supplementary Table S2). These multi-fasta files were aligned with Clustal Omega v1.2.4 (Sievers et al. 2011). Phylogenies were generated using RAxML-NG v0.9.9 (Kozlov et al. 2019), employing the -all mode, GTR+G model, and a seed of 2. Branch support based on bootstrapping and transfer distance were obtained, before generating a
randomly rooted consensus tree using ASTRAL-Pro v1.12 (Zhang et al. 2020).

**Yeast media and growth conditions**

Strains were grown and kept on YPD medium (10 g/l of yeast extract, 20 g/l of bacto-peptone, and 20 g/l of glucose) at 30°C. To study growth dynamics and carbon source assimilation, strains were grown and kept on minimal medium (1.6 g/l of yeast nitrogen base without amino acids, 5 g/l of ammonium sulfate with a pH of 6, supplemented with 1% glucose or 1% α-xylene). To check α-xylene assimilation in different concentrations and aeration conditions, single colonies from minimal glucose agar plates were used to inoculate glass-test tubes (max. vol. 35 ml) at an OD₆₀₀ = 0.15, containing minimal medium with 2% or 10% α-xylene at different volumes: 3 ml (high aeration), 7.5 ml (low aeration) or 20 ml (microaeration). The tubes were then kept at 30°C with shaking (150 rpm) for 14 days. To calculate the growth rate in α-xylene and α-glucose medium, starter cultures were prepared by growing single colonies from glucose or xylene minimal medium agar plates into corresponding liquid medium buffered to a pH of 3.2 or pH = 6 overnight at 30°C. The next day, the overnight cultures were used to inoculate 50 ml medium to an OD₆₀₀ ~ 0.1 in 250 ml baffled Erlenmeyer flasks containing the same buffered medium. For both carbon sources, the medium was buffered to a pH = 3.2 with 0.1 M final concentration of phosphoric buffer (1M KH₂PO₄ adjusted to pH = 3.2 with phosphoric acid) or to a pH = 6 with 0.1 M final concentration of potassium phosphate buffer (pH = 6). Cultures were incubated at 30°C and 150 rpm. To calculate each μ, OD₆₀₀ measurements from two different single colonies were used. Each individual OD₆₀₀ measurement was obtained by the average of triplicates. As previously described, colonization patterns were made on semi-solid YPD 0.3% agar plates and cultures were grown for 14 days at room temperature (Kurtzman et al. 1991).

**Carbon assimilation**

Carbon-source assimilation was assessed using microplates (Kurtzman et al. 2011c) using Phenotypic Microarray plates (Biolog, PM1 # 12111 and PM2 # 12112). Briefly, the plates were inoculated with strains 1112, 1118, and 1120 in buffer FM containing Dye mix E to facilitate and improve detection, incubated at 30°C for 144 h and the absorbance measured at 550 nm, as described by the manufacturer. Values at +144 h were used to assess growth on each of the carbon sources. For each time point, background absorbance was subtracted and the fold change (FC) over negative control (no carbon source) was calculated. Signals were assigned to a strong growth (FC >3 at 144 h, +++), intermediate growth (FC >2 and <3 at 144 h, ++), weak growth (FC >1 and <2), and no growth (FC ≤1). Strains were seeded in duplicate plates in two independent experiments. Mean of the FC was calculated.

**Microscopy**

Yeast cells for microscopy were grown overnight in YPD medium at 30°C at 150 rpm. Cells were washed once with distilled sterile water and pellets were re-suspended in water. One to five microliters were mounted in a glass slide with a 0.17 mm thin cover slip. Samples were visualized using a Nikon Eclipse E600 and pictures taken with an Optronics MagnaFire CCD Microscope Camera.

**Xylose metabolism gene analysis**

We performed detailed analyses of the genes putatively involved in xylose metabolism by comparing the newly assembled genomes to 27 genomes available from GenBank. We inferred the potential for α-xylene utilization if the following orthologues were present: Xylose reductase (XYL1), xyitol dehydrogenase (XYL2), and α-xylulokinase (XYL3), which translate α-xylene to xylulose-5P, where the xylulose-5P enters the pentose phosphate pathway (PPP). Furthermore, we included genes from the PPP oxidative phase involving the enzymes glucose 6-phosphate dehydrogenase (ZWF1), 6-phosphogluconate dehydrogenase (GND1), and a nonoxidative phase carried out by α-ribulose-5-phosphate 3-epimerase (RPE1), ribose-5-phosphate ketol-isomerase (RKI1), transketolase (TKL1), and transaldolase (TAL1), while phosphoglycerate isomerase (PGI1) completes the cycle. We counted the number of copies of the genes encoding these enzymes in the genome and visualized differences across yeasts in a heatmap generated in Excel. We obtained and aligned genes coding for these enzymes from all 27 genomes using Mafft v.7.471 (http://mafft.cbrc.jp/alignment/software) and subsequently predicted the secondary protein structure using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

**Results**

**Phylogenetic analysis of yeast isolates**

We collected worker termites of the species M. bellicosus in Comôed National Park (Côte d’Ivoire) and dissected their intestines in foregut, midgut, and hindgut for yeast isolation (Bos et al. 2020). We identified a total of 20 individual yeast colonies spanning all three different gut compartments and four independent termite colonies (Figure 1). To identify novel species and subspecies, we next characterized the isolates taxonomically by sequencing the ITS and D1/D2 rDNA regions of LSU and aligning the sequences to GenBank repository using BLASTN (Altschul et al. 1990) (Figure 1).

From the set of 20 isolates, 11/20 were assigned to Meyerozyma caribrica. Next, 3/20 isolates were assigned to Candida sp. TMS-2011 and the rest (6/20 isolates) did not have a clear match with any NCBI sequence, showing similarities between 84.4% and 99% to their respective best matches (Figure 1). From the six uncategorized isolates, four (IDs: 1112, 1113, 1105 and 1106) showed three gaps with K. quercitrusa KMC-Y23, isolate 1120 showed one mismatch with K. quercitrusa WHFS-5 and isolate 1118 showed 26 gaps and 37 mismatches with Barnettzyma sp. EN30502, leading to a similarity of 91%. Because 1112, 1113, 1105, and 1106 had identical rDNA D1/D2 sequences, we grouped them and selected 1112 as a representative of this group (Figure 1).

The best ML model based on ITS and LSU was TN93+G. The ML tree allowed us to include all closely related yeast species within the genera (Figure 2). B. kosteri sp. nov. (1118) clusters as an independent branch within Barnettzyma salicaria. The next characterized the isolates taxonomically by sequencing the ITS and D1/D2 rDNA regions of LSU and aligning the sequences to GenBank repository using BLASTN (Altschul et al. 1990) (Figure 1).

To gain more confidence on its phylogenetic placement, we sequenced the genomes of isolates 1118, 1112, and 1120. We generated a Multiple-Locus-Sequence-Typing (MLST) analysis based on a set of 176 BUSCO genes inferred to be Ascomycota-specific and single-copy in at least 85% of the available yeast genomes with high (<80%) genome completeness (for general genome features and completeness, see Table 1). The genome sizes and gene content of 1112 and 1120 are similar to Kurtzmanniella cederata, which has a 12.1 Mb large genome and contains 5478 genes, while 1118 is 11.1 Mb with 4888 genes; comparable to the closest relative with a genome available (Barnettzyma salicaria; 11.01 Mb, 5586 genes). The MLST phylogeny thus confirmed the placement of 1112 and 1120 with high (<80%) genome completeness (for general genome features and completeness, see Table 1).
of these new isolates in their respective genera and clades (Figure 2).

**Metabolic profile characterization**

To characterize their carbon metabolism, we grew strains 1112, 1120, and 1118 in an array of carbon sources (Supplementary Table S4). Metabolism was detected as a function of respiratory activity (production of NADH) by monitoring the color change in the medium (reduction of tetrazolium dye). From the 190 carbon sources tested, *K. quercitrusa* var. *comoensis* (1112) metabolized among others pentose sugars (2-reoxy-D-ribose, D-arabinose, L-arabinose, D-mannose, D-ribose, L-lyxose, and D-xylose), hexose sugars (α-D-glucose, D-fructose, D-galactose, and D-tagatose), disaccharides (isomaltulose, maltose, sucrose, gentiobiose, palatinose, and turanose), trisaccharides (D-melezitose and maltotriose), organic acids (5-keto-D-gluconic acid, citramalic acid, D,L-malic acid, D-amino-aleric acid, γ-aminobutyric acid, L-malic acid, N-acetyl-neuraminic acid succinic acid), Tween (Tween 20, Tween 40, and Tween 80), etc. (see the complete list in Supplementary Table S4).
K. quercitrusa var. filamentosus (1120) metabolized among others pentose sugars (2-reoxy-D-ribose, D-arabinose, L-arabinose, D-ribose and D-mannose, and L-lyxose), (hexose sugars (α-D-glucose, D-fructose, D-galactose), disaccharides (D-trehalose and gentiobiose), amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-isoleucine, L-lysine, L-proline, and L-pyroglutamic acid), etc. (see the complete list in Supplementary Table S4).

B. botsteinii sp. nov. 1118T metabolized among others pentose sugars (2-reoxy-D-ribose, L-arabinose, D-ribose, L-xylose, and L-lyxose), methyl pentose (L-rhamnose), hexose sugars (α-D-glucose, D-glucose-1-Phosphate, D-fructose, D-mannose, and D-psicose), disaccharides (D-cellobiose, gentiobiose, and maltose), trisaccharides (maltotriose), sugar alcohols (D-mannitol, D-sorbitol, and glycerol), organic acids (5-keto-D-glutaric acid, acetic acid, citric acid, fumaric acid, pyruvic acid, L-malic acid, and succinic acid), Tween (Tween 20, Tween 40, and Tween 80), etc. (see Methods section) (Figure 3A). Colonies on rich glucose-based medium (YPD) and minimal glucose medium (see Methods section). Cells divide mitotically by a small bud that fully separates from the mother cell (Figure 3A). Colonies on YPD agar are white, bright, and divide mitotically by a small bud that fully separates from the mother cell (Figure 3A). Colonies on rich glucose-based medium (YPD) and minimal glucose medium (see Methods section). Cells divide mitotically by a small bud that fully separates from the mother cell (Figure 3A). Colonies on YPD agar are white, bright, and divide mitotically by a small bud that fully separates from the mother cell (Figure 3A).

We then compared the metabolic profile characterization of the 1118 with other known and closely related Barnettozyma and Candida species such as B. salicaria, B. siamensis and C. montana. The result revealed that it has different metabolic characteristics, as it can assimilate D-ribose, L-arabinose, gelatine, and maltose, but not salicin and sucrose (Supplementary Table S5). Similarly, the comparison of the physiology of 1112 and K. quercitrusa CBS 4412 showed that 1112 differed by being able to assimilate D-arabinose, D-xylose, inulin, L-arabinose, N-acetyl-D-glucosamine and salicin, but not α-glucosamine, D-mannitol, D-trehalose, glycerol, and xylitol. In contrast to K. quercitrusa CBS 4412, 1120 grows on D-arabinose, gelatine, L-arabinose and gelatine but not D-mannitol, maltose, sucrose, and D-sorbose (Supplementary Table S5).

Morphology description

**Kurtzmaniella quercitrusa var. comoensis**

Strain ID in this work: 1112. Microscopic examination of this isolate confirmed that it grew as yeast. Cells appear as black, oval shaped and were 3–4 μm long and 2–3 μm wide during exponential growth in rich (YPD) and minimal glucose medium (see Methods section). Cells divide mitotically by a small bud that fully separates from the mother cell (Figure 3A). Colonies on YPD agar are white, bright, and round, while they appear as a flat and round with an even rim on medium with a low concentration of agar, 0.3% YPD agar (Figure 3D).

**Kurtzmaniella quercitrusa var. filamentosus**

Strain ID in this work: 1120. Yeast cells of this strain are clearly distinguishable from K. quercitrusa var. comoensis. Being 5–6 μm in length and 1–2 μm wide, cells appear as longer and thinner than 1112 (Figure 3B). Moreover, during exponential growth in glucose-based medium, YPD, and minimal glucose medium (see Methods section), some cells do not fully separate after division leading to formation of 5–6-cell-long pseudohyphal structures. After 5 days of growth in liquid 1% xylose medium, formation of pseudohyphae is strongly enhanced, leading to truly septated hyphae. Hyphae first appear as germ tubes (Figure 4A), which are 2–7 cell long chains growing from a single round cell. They later elongate and mature to true hyphal structures showing bifurcations (Figure 4, C and C'). Colonies on rich glucose-based medium (YPD) and minimal glucose medium show a rough, butyrous, and ivory-colored phenotype. In addition, their colonization pattern on 0.3% agar plates is non-even with visible edges and with some growth elevated from the agar (see Methods section) (Figure 3E).

Figure 3 Morphology description. Bright field microscopy pictures (left images) of cells grown in YPD at exponential phase and colonization pattern on YPD 0.3% agar (right images) of K. quercitrusa var. comoensis (A and D), K. quercitrusa var. filamentosus (B and E), and B. botsteinii sp. nov. 1118T (C and F). Colonies were grown at room temperature for 14 days. Bars show respective scales.

Figure 4 Filamentous growth of K. quercitrusa var. filamentosus (1120). Cells grown in the synthetic liquid medium 1% xylose (pH = 6) grow as filaments. Single yeast colonies from xylose agar plates were grown in 1% xylose liquid medium buffered at pH = 6 (see Methods). After 48–72h at 30°C at 150 rpm, small cell chains are visible (A and B) elongating either from single cells (*) or a cluster of cells (**). Eventually, after 96–120 h, these structures develop to longer septate filaments (C and C') with bifurcations (arrowhead in C'). Bright-field microscopy images at indicated scale.

**Barnettozyma botsteinii** sp. nov.

B. botsteinii sp. nov. 1118T yeast cells are mostly spherical and small (3–4 μm × 2–3 μm) during exponential growth in liquid rich...
Table 2 Growth rates in defined liquid media

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<td></td>
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<td>DT</td>
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Growth rate ($\mu_{\text{max}}, \text{h}^{-1}$) and doubling times (DT, h) of CEN.PK, B. botsteinii sp. nov. 1118, K. quercitrusa var. nov. 1112, and K. quercitrusa var. nov. 1120 in liquid SC-1% glucose and SC-1% xylose at pH 3.2 or 6. All growth rates are averages of two independent experiments from two independent clones.

**D-Xylose metabolism**

After observing that the novel isolates grow on pentose sugars, we next characterized their $\alpha$-xylose utilization, using different aeration and concentrations of sugar in the growth medium. Strains K. quercitrusa var. comoensis (strain 1112) and K. quercitrusa var. filamentosus (strain 1120) show an aeration-dependent growth preference on $\alpha$-xylose (Figure 5). From the conditions tested, the highest biomass is reached with high aeration in combination with 2% $\alpha$-xylose, where the OD$_{600}$ after 14 days of growth reaches 16.6 ± 0.6 and 14.9 ± 1.0, respectively. For both K. quercitrusa strains, we observed a correlation between aeration levels and OD$_{600}$. A decrease in aeration level correlates with a decrease in OD$_{600}$ both with 2% $\alpha$-xylose and 10% $\alpha$-xylose. For example, in low aeration conditions 1112 grows to OD$_{600}$ of 5.8 ± 0.2 with 2% $\alpha$-xylose and 5.8 ± 0.2 with 10% $\alpha$-xylose. Similarly, 1120 grows to an OD$_{600}$ of 5.4 ± 0.8 with 2% $\alpha$-xylose and 2.3 ± 0.6 with 10% $\alpha$-xylose. We observed a further reduction in the OD$_{600}$ in microaeration conditions, especially in 1112 strain, where it reaches only 3.2 ± 0.29 in 2% $\alpha$-xylose and 2.4 ± 0.1 in 10% $\alpha$-xylose.

B. botsteinii sp. nov. 1118$^T$, on the other hand, did not efficiently grow on $\alpha$-xylose. Only marginal to very limited growth was observed with microaeration and high $\alpha$-xylose concentration (Figure 5). Interestingly, K. quercitrusa var. filamentosus (strain 1120) undergoes a dimorphic shift and forms hyphae in 2% $\alpha$-xylose medium combined with high or low aeration (Figure 5). The dimorphic shift consisted of formation of pseudohyphae and hyphae of various lengths. No changes in morphology were observed with the other strains.

We next compared the growth rates of K. quercitrusa when propagate in $\alpha$-xylose and $\alpha$-glucose at low and neutral pH (Table 2). K. quercitrusa var. comoensis (strain 1112) cells grew in 1% synthetic glucose medium with a doubling time of 2.0 h at pH = 3.2. At pH = 6, the doubling time was similar at 1.9 h. In 1% xylose medium, cells grew at a doubling time of 19.3 h, while they exhibited no growth at pH = 3.2. Cells grew as single yeast cells and divided by budding through exponential phase at both carbon source medium (Table 2). For comparison, a S. cerevisiae CEN.PK type strain grew only in synthetic glucose medium at a doubling time of 2.3 h at pH = 3.2 while at pH = 6 it grew with a doubling time of 2.1 h.

K. quercitrusa var. filamentosus (strain 1120) grew on 1% glucose with a doubling time of 2.0 h at both tested pH (3.2 and 6.0). In xylose, the strain showed pH-dependent morphologic differences (Table 2). At pH = 3.2, the strain grew as single yeast cells with a doubling time of 19.6 h, while at pH = 6.0 medium, pseudohyphae accumulated and matured into true hyphae (Table 2 and Figure 5). Both strains of K. quercitrusa (strains 1112 and 1120) showed very similar growth rates in 1% glucose medium and slightly lower than S. cerevisiae CEN.PK$^T$ strain (Table 2).

B. botsteinii sp. nov. 1118$^T$ grew in 1% glucose medium at pH = 6 with doubling time of 2.8 h (Table 2). Interestingly, the strain was sensitive to acidic pH that drastically increased its doubling time of 25.7 h at pH = 3.2. Compared to the K. quercitrusa strains and S. cerevisiae CEN.PK$^T$, this species showed the slowest growth rate at both pH (Table 2). Under this experimental growth condition, the strain was not able to grow on 1% xylose medium at either of the tested pH (Table 2).

Copy number changes of genes coding for enzymes in metabolic pathways are known to affect metabolic pathway efflux (Steenwyk and Rokas 2018). Therefore, we investigated the copy number of genes involved in xylose and pentose metabolism. Our comparative genomics analysis for XL and PPP genes revealed that the vast majority of yeasts contained all the orthologues necessary for xylose metabolism (Figure 6). The lack of growth of some of the species on xylose thus does not appear to be as a result of the absence of xylose-metabolizing genes (Figure 6).

**Taxonomy**

Description of B. botsteinii sp. nov. 1118

Mycobank: MB 833563

Barnetozyma botsteinii (bot’stani, N.L. gen. n. botsteinii, named after scientist David Botstein) isolated from the hindgut of termite M. bellicosus in Côte d’Ivoire. Holotype is preserved at the Westerdijk Institute as CBS 16679$^T$ and an isotype at the IBT Culture Collection of Fungi as IBT 710. Cells appear as spherical and small (3–4 μm × 2–3 μm), typically forming clusters of 3–5 cells in pseudohyphal structures. No sign of true hyphae formation was observed in the conditions tested. The colonies appear ivory colored, flat and circular in YPD or minimal 1% glucose media (Figure 5). Accumulation and matured into true hyphae (Table 2 and Figure 5). Both strains of K. quercitrusa (strains 1112 and 1120) showed very similar growth rates in 1% glucose medium and slightly lower than S. cerevisiae CEN.PK$^T$ strain (Table 2).

B. botsteinii sp. nov. 1118$^T$ grew in 1% glucose medium at pH = 6 with doubling time of 2.8 h (Table 2). Interestingly, the strain was sensitive to acidic pH that drastically increased its doubling time of 25.7 h at pH = 3.2. Compared to the K. quercitrusa strains and S. cerevisiae CEN.PK$^T$, this species showed the slowest growth rate at both pH (Table 2). Under this experimental growth condition, the strain was not able to grow on 1% xylose medium at either of the tested pH (Table 2).

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important figure for the yeast research community (Botstein et al. 1980; Botstein and Fink 1988).

Discussion

In this study, we isolated novel and biotechnologically relevant yeast strains from termite guts. We describe two novel strains of K. quercitrusa and a new species of yeast, which we name B. botsteinii based on genomic information. The criteria to classify these three novel taxa follows Kurtzman et al. (2011c), in which conspecific strains differ by no more than three nucleotides, whereas distinct species show six or more nucleotide differences (Kurtzman et al. 2011b).

Here, the novel yeast species B. botsteinii sp. nov. 1118T is described based on a single isolate. Novel single-strain fungal taxa descriptions are debated as complete intraspecific variability (including genetic and phenotypic) and its ecological role cannot be fully captured using one strain. However, nearly one-third of described yeast species is based on single isolates (Kurtzman et al. 2011a). The documentation and publishing of single strains novel taxa serve as a nucleation point for further studies and the possibility of combining information from strains independently isolated in time. While it was not possible for us to collect new material or find any other available strain with a complete match in GenBank, it is possible that more isolates of B. botsteinii sp. nov. are found in the future. This debate was recently reviewed in Brysch-Herzberg et al. (2020).

We find that both novel strains of K. quercitrusa are able to grow on d-xylose, and their overall metabolic profile makes them interesting for further applied studies. The complex phenotypic change that K. quercitrusa var. filamentosus (strain 1120) undergoes (filamentation when growing on certain carbon sources) reveals the existence of a metabolic shift that can control alterations in protein secretion and enzyme production similar to that of other yeasts with a dimorphic shift (Gauthier 2017; Celinańska and Nicaud 2019).

We identified two novel strains and a new species of yeast by studying a relatively small number of individuals (around 100 termites from only one termite species, M. bellicosus). Other yeast strains, without a clear match to described species, have previously been isolated from Macrotermes subhyalinus (Yoro et al. 2014) and other fungus-growing termites (Odontotermes formosanus) (Mathew et al. 2012). Therefore, the Macrotermiteinae termite subfamily, comprising 330 described species, appears as a potentially extensive biological reservoir for new yeast species and strains of biotechnological relevance.

K. quercitrusa was first isolated from the insect frass on an oak tree (Quercus sp.) (Meyer and Yarrow 1998), type strain CBS 4412T. Other isolates of the same species have been collected from other insects (beetles) and from flowers around the world (e.g., Oceania, South America, and South-east Asia) (Crous et al. 2004; Molnár et al. 2008). Although generally considered nonpathogenic, adult and pediatric human infections have been reported in immunocompromised patients, widening the spectrum of non-albicans candidiasis (Xiao et al. 2014; Westblade et al. 2015).

Yeasts are widely used in the biotechnological production of metabolites and protein. However, most do not metabolize pentose sugars well. The two new K. quercitrusa strains (var. comoensis 1112 and var. filamentosus 1120) grow well on hexose sugars, such as glucose, fructose, mannose, and galactose, and are also able to assimilate pentose sugars such as ribose, xylose, arabinose, and lyxose. They show differences in growth on short oligosaccharides in which var. comoensis grows on maltose and sucrose while var. filamentosus grows on trehalose and adonitol. Furthermore, they both can grow in the presence of complex plant-derived polysaccharides such as mannann, dextrin, and laminarin.
The metabolic profile resembles that of K. quercitrusa-type strain CBS 4412T, including xylose assimilation (Kurtzman et al. 2011c). Strain var. filamentosus is different to var. comoensis in its ability to differentially metabolize both monosaccharides (adonitol) and disaccharides (D-trehalose). The filamentous growth of var. filamentosus has been reported in another member of K. quercitrusa, strain NRRL Y-5392, although described as pseudohyphae (Kurtzman et al. 2011c). In addition, other members of the Kurtzmaniella clade, such as Candida raienensis, also show hyphae formation under certain conditions (Ramírez and González 1984; Kurtzman et al. 2011c). We observed that this phenotype is highly variable and dependent on factors that are common, for example, among Candida species, namely: carbon source and concentration, pH, and aeration level (Caplice and Moran 2015).
We also describe a new yeast species, named B. botsteinii sp. nov. 1118\textsuperscript{T}, closely related to \textit{Barnettzyma salicaria} NNRL Y-6780\textsuperscript{T}. Metabolically, \textit{B. botsteinii} grows on an array of hexose and pentose sugars. Notably, the strain showed positive growth on short organic acids: acetic and glyoxylic acids (2 carbons), pyruvic acid (3C), fumaric and malic acids (4C), \(\alpha\)-ketoglutaric acid (5C), and citric acid (6C). Because many of these are part of the Krebs cycle, this metabolic profile suggests that this species could be classified as Krebs positive (Barnett and Kornberg 1960; Casal et al. 2008). The isolated strain grew in culture conditions although at relatively low rates, suggesting that we did not find the best growth conditions for this species. \textit{B. botsteinii} could be dependent on the environment provided by the termite for optimal growth like some other known gut-associated yeasts, such as \textit{Coccidioscocus legeri}, which have an obligate relationship with their host insect and are unable to grow on any tested laboratory conditions (Kurtzman et al. 2011c; Stefanini 2018).

The annotation and analyses of genes involved in xylose metabolism did not spur differences in the novel strains and other known yeasts, suggesting that the phenotypic variation in growth in this group of yeasts is unlikely to be driven by differences in the gene content. Although no effect of gene copy number was observed, other genetic factors could influence gene expression and explain the observed phenotypic diversity such as single-nucleotide polymorphisms (SNPs), small inversions–deletions, or translocations. In addition, differences in the balance of key metabolic components and co-factors can influence enzyme activities, for example, NADH/NAD\textsuperscript{+}, which are important for xylose metabolism (Riley et al. 2016). This suggests that the capacity to assimilate and metabolize xylose may be ancient and conserved in yeasts.

A question that remains open is whether these yeast species stably colonize the termite gut or are ingested during foraging. \textit{K. quercitrusa} strain CBS 4412\textsuperscript{T} was isolated from insect frass and we isolated \textit{K. quercitrusa} var. \textit{filamentosus} from the termite hindgut (i.e., last compartment before faces deposition), suggesting that at least this particular yeast species can survive gut passage, a feature not common to all yeasts nor insect guts (Madden et al. 2016). Gut passage is exploited by other yeast species for their dispersal and breeding (Reuter et al. 2007; Stefanini et al. 2012; Madden et al. 2018) and it is possible that \textit{K. quercitrusa} and \textit{B. botsteinii} are also dependent on gut passage for growth, mating, and dispersal.

Biotechnologically, the strains and species characterized here show interesting features that warrant further exploration. \textit{K. quercitrusa} growth on xylose makes them interesting for industrial applications, especially if they are able to co-ferment xylose in the presence of hexoses (Hahn-Hägerdal et al. 2007; Ceccato-Antonini et al. 2017). Especially, the characteristics of \textit{K. quercitrusa} var. \textit{filamentosus} make it suited for biotechnologically production, since growth at low pH, metabolism of pentose sugars, and a controllable filamentous state are particularly desired traits. In addition, all three strains appear to be able to metabolize polysorbate, also known as Tween. This is a complex molecule formed by polyoxyethyleneated sorbitol molecules with lauric acid and used as a detergent and surfactant. Other yeasts such as \textit{Candida albicans} or \textit{Candida parapsilosis} sustain growth on Tween as a sole carbon source, a trait considered as a virulence factor as its lipid content triggers the expression of extracellular phospholipases, including LIP1 (Fu et al. 1997; Slifkin 2000).

In addition, other metabolic processes other than pentose consumption are also biotechnologically attractive. The cellubiose respiration by \textit{B. botsteinii} sp. nov. 1118\textsuperscript{T} suggests the presence of \(\beta\)-glucosidases enzymes (EC 3.2.1.21), which are able to break down the cellubiose molecule into single glucose molecules. During the depolymerization of cellulose, cellubiose is produced which inhibits cellulose-degrading enzymes (cellulose 1,4-\(\beta\)-celllobiosidase and endoglucanases), reducing the overall efficiency (Murphy et al. 2013; Sørensen et al. 2013). Thus, the addition of \(\beta\)-glucosidases to the process can greatly improve its efficiency (Woodward and Wiseman 1982).

In conclusion, the intestine of fungus-growing termites appears to be a promising niche for the identification of new yeast strains and species. The metabolic profiles of the yeasts isolated from termite gut reflect the adaptations to a plant-based diet found either within the wild or within the gut of the termite, and these gut-residing yeasts of \textit{Macrotermes} termites have interesting traits of relevance to the biotechnology industry.

### Data availability

Sanger sequences of D1/D2 rDNA regions of LSU and ITS for isolate 1112, 1120, and 1118 are deposited in the GenBank under the accesses MN497060.1, MN497056.1, MN509221.1, MN497061.1, MN497057.1, and MN509222.1. Genomes of three strains are deposited in the GenBank under the BioProject number PRJNA695125, and the accession numbers are JAF1D00000000, JAFII100000000, and JAFIDH00000000. The strains are available as follows: \textit{B. botsteinii} sp. nov. 1118\textsuperscript{T} (MycoBank: 833563, CBS 16679\textsuperscript{T} and IBT 710), \textit{K. quercitrusa} var. \textit{comensis} 1112 (CBS 16678, IBT 709) and \textit{K. quercitrusa} var. \textit{filamentosus} 1120 (CBS 16680, IBT 711).

Supplementary material is available at figshare: https://doi.org/10.25387/g3.14899278.

### Acknowledgments

The authors would like to thank N’golo A. Kone, Kolotchêlêma Simon Silue, and the staff at the Comôé National Park Research Station in Côte d’Ivoire for assistance with collections. They also thank Josefín Stiller, Thomas Laessøe, and Bolette Rud. Fallesen for their advice on phylogenetics, morphological description, and phonetics.

### Funding

This work was supported by an ERC Consolidator grant (771349) to M.P. G.A. received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 801199.

### Conflicts of interest

The authors declare that there is no conflict of interest.

### Author contributions

Termite collection and dissection were performed by Leandro Guimaraes. Yeast isolation, identification, and characterization were performed by Gerard Arrey and Sefa Alizadeh. Guangshuo Li performed genome analyses and Robert Murphy performed MLST. Michael Poulsen and Birgitte Regenberg raised funding for the project and designed the study, with input from all authors. The first draft of the manuscript was written by Gerard Arrey and Birgitte Regenberg. All authors commented on previous versions of the manuscript and read and approved the final manuscript.
Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Termite colonies were collected under permit 189/UNA/CRE/SREC issued by Université Nangui Abrogoua and Research Station Comoe on December 12, 2018. *M. bellissus* is not an endangered species of insect. This article does not contain any studies with human participants performed by any of the authors.

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Communicating editor: C. Boone