Yeasts in the *Sugiyamaella* clade associated with wood-ingesting beetles and the proposal of *Candida bullrunensis* sp. nov.

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During a survey of yeasts associated with wood-ingesting insects, six strains of the *Sugiyamaella* clade were isolated from the gut of passalid and tenebrionid beetles and the decayed wood inhabited by them. Phylogeny based on rRNA gene sequences placed these yeasts as members of *Sugiyamaella smithiae*, *Sugiyamaella americana*, *Candida lignohabitans* and a novel species closely related to *Su. americana*. The only strain of the novel species, EH008, could be unquestionably distinguished from its relatives by DNA sequences and other taxonomic characteristics. Ascospore production was not observed under the laboratory conditions tested. Therefore, this novel species is proposed as *Candida bullrunensis* sp. nov. (type strain EH008 = ATCC MYA-4660 = CBS 11840).

INTRODUCTION

The digestive tract of insects is a good source of highly diverse yeasts (Suh *et al.*, 2005a). While some of these yeasts are consistently isolated from the insect gut, the role of gut yeasts is not fully understood. Evidence suggests that the enzymes produced by these yeasts aid in the digestion and detoxification of the diet ingested by the hosts and/or the yeasts directly provide essential nutrients to the insects, such as amino acids, vitamins, and lipids (Dowd, 1992; Vega & Dowd, 2005). Gut-inhabiting yeasts are often found in insects that feed on nutritionally poor substrates, such as termites and beetles feeding on wood (e.g. Prillinger *et al.*, 1996; Schäfer *et al.*, 1996; Suh *et al.*, 2003).

Passalid beetles (Coleoptera: Passalidae) are one of the most common types of wood-ingesting beetles that live in colonies on rotten logs. Several previous studies have confirmed the presence of yeasts in the gut of the beetle by microscopic techniques or by using molecular methods (Lichtwardt *et al.*, 1999; Nardi *et al.*, 2006; Zhang *et al.*, 2003). Phylogenetically distinct yeasts, including several novel species, have been reported in association with passalid beetles (e.g. Nguyen *et al.*, 2006; Suh *et al.*, 2003, 2004b, 2005b). Interestingly, a few yeasts isolated from the gut of passalid and other wood-feeding beetles, such as *Scheffersomyces* (Pichia) *stipitis*, *Spathaspora passalidarum* and *Candida jeffriesii*, are able to produce ethanol from xylose, a pentose that forms the backbone of hemicellulose, which is a major component of plant cell walls (Nguyen *et al.*, 2006; Suh *et al.*, 2003).

During a survey of the microbial flora in the gut of passalid beetles and other wood-inhabiting insects, we found several yeasts belonging to the *Sugiyamaella* clade (Kurtzman, 2007), subphylum Saccharomycotina, which is composed of yeasts mainly derived from woody substrates. Here we describe a novel species of the genus *Candida* and discuss the phylogeny and ecology of the insect-associated yeasts in the *Sugiyamaella* clade.

METHODS

Yeast isolation and identification. The wood-feeding insects and woody substrates near insect tunnels that were tested in this study were collected from rotten logs on Bull Run Mountain in Broad Run, Virginia, USA. The methods used for isolating yeasts from insects have been described in detail in previously published papers (Suh & Blackwell, 2004; Suh *et al.*, 2004a). The morphological observations and metabolic tests that constitute the yeast standard description were performed according to established methods (Yarrow, 1998; Barnett *et al.*, 2000). The assimilation tests for carbon and nitrogen sources were conducted in liquid media. Starved inocula were used in the nitrogen and vitamin assimilation tests. The yeasts were observed individually; similar isolates were crossed in all combinations for possible ascospore formation on YM agar, 2 % malt extract agar, cornmeal agar, V8 juice agar and modified KNO₃ agar (Pincus *et al.*, 1988) at 20 °C and 25 °C for up to 6 weeks.

DNA sequencing and molecular phylogenetic analyses. The methods used for nucleic acid extraction, PCR amplification and sequencing of rRNA genes were those discussed in Suh & Zhou (2010). The complete sequences of the small subunit (SSU) rRNA gene, the internal transcribed spacers (ITS) including the 5.8S rRNA...
gene and the D1/D2 region of the large subunit (LSU) rRNA gene were compared to those of other yeasts in the GenBank database using the BLAST search program (Altschul et al., 1990). Selected DNA sequences were aligned with the CLUSTAL_X multialignment program (Thompson et al., 1997) and adjusted visually.

The GenBank accession numbers for the DNA sequences generated in this study are: HM208601/HM208602/HM208603 (D1/D2 region of LSU rRNA gene/ITS including 5.8S rRNA gene/SSU rRNA gene) for strain EH008T; HM208604/HM208605/HM208606 for strain EH053; HM208607/HM208608/HM208609 for strain EH184; HM208610/HM208611/* for strain EH188; HM208612/HM208613/* for strain EH075 and HM208614/HM208615/* for strain EH076.

Maximum-parsimony analyses were performed using PAUP 4.0b10 (Swofford, 2002). Heuristic tree searches were executed using the tree bisection–reconnection branch–swapping algorithm with random sequence analysis. Bootstrap values of the most parsimonious tree were obtained from 1000 replications. Base pair differences were counted using BLAST2 sequences (Tatusova & Madden, 1999) or from a manually aligned sequence database.

**RESULTS AND DISCUSSION**

**Yeast isolates and novel species**

A total of 91 yeast strains were isolated from wood-feeding insects and woody substrates found near the insects. Among those yeasts, six isolates were identified as species in the Sugiyamaella clade (Kurtzman, 2007) on the basis of DNA sequence comparisons. The strains and their sources are listed as below: EH008T (=ATCC MYA-4660T =CBS 11840T) and EH053 (=ATCC MYA-4662), gut of Odontotaenius disjunctus (Coleoptera: Passalidae) from a rotten log; EH184 (=ATCC MYA-4663) and EH188 (=ATCC MYA-4664), gut of an unidentified tenebrionid beetle (Coleoptera: Tenebrionidae) from a rotten log; EH075 (=ATCC MYA-4665) and EH076, insect tunnel on wood.

From DNA sequence comparisons and other taxonomic characterization methods, five of the strains were identified as three recognized species within the Sugiyamaella clade: strain EH053 as Sugiyamaella americana, strains EH184 and EH188 as Candida lignohabitans and strains EH075 and EH076 as Sugiyamaella smithiae. The D1/D2 sequences of the LSU rRNA gene from these strains were identical to those of the type strains of each recognized species. The sequences of the ITS and D1/D2 regions of strains EH184 and EH188 were identical to each other, as were those of strains EH075 and EH076. No ascospores were observed from individual or mixed cultures of C. lignohabitans strains EH184 and EH188 under the conditions tested. Further studies crossing the strains with the type strain of C. lignohabitans may provide an opportunity to determine their ascosporic state. The results from physiological tests and morphological observation of these five strains were well matched with the descriptions of each species, except for some variations between strains EH184 and EH188 and C. lignohabitans NRRL YB-1473T in their assimilation of D-ribose and D-arabinose.

Strain EH008T could be clearly distinguished from any recognized species in the Sugiyamaella clade. Based on rRNA gene sequence comparisons, the closest species to this strain was Su. americana. The nucleotide difference in the D1/D2 region was 27–30 bp including five gaps between strain EH008T and the strains of Su. americana. The differences in the complete sequences of the SSU rRNA gene and ITS including 5.8S rRNA gene were 39 bp (20 gaps) and 25 bp (five gaps), respectively. Such degrees of variation are considered sufficient to distinguish strain EH008T as a separate species (Kurtzman & Robnett, 1998; Kurtzman, 2006). In addition to the molecular differences, strain EH008T could also be distinguished from Su. americana based on physiological characteristics: e.g. fermentation of glucose, assimilation of D-ribose, glyceral, erythritol, ribitol, L-arabininitol and growth at 37 °C. No ascospores were observed for strain EH008T on five media tested after 6 weeks at 20 °C or 25 °C. Based on the molecular, physiological and morphological data, we propose that strain EH008T represents a novel species in the genus Candida.

**Latin diagnosis of Candida bullrunensis Suh, Houseknecht and Zhou sp. nov.**

*Cultura in agaro dextrosum et peptonum et extractum levidinis continente post 7 dies ad 25 °C, candida aut creacea, teres et butyrosa. Cellulae vegetatiae globosae et subglobosae (2.5–6.3 × 2.5–7.5 μm), singulæ vel binae, pseudohyphae non fiunt. Cultura in agaro farinae Zeae maydis confection post 11 dies ad 25 °C candida aut creacea. Hyphae verae et pseudohyphae fiunt. Ascoporae non fiunt. Saccharas non fermentantur. Assimilantur glucosum, galactosum, L-sorosum (lente), D-glucosaminum (lente), D-xilosum, L-arabinosum, D-arabinosum (infrime), trehalosum, cellobiosum, salicinum, arbutinum, xylitolum, D-glucitolum, D-mannitolum, glucalonactonum, 2-keto-D-gluconatum, D-gluconatum (infrime), D-gluconuratum (infrime), D-galacturonatum, Dl-acidum lacticum (lente), acidum succinicum (lente), ethanolum et propane-1, 2-diolum (lente). Non assimilantur D-ribosem, L-rhamnosum, sucrosum, malossum, methyl x-D-glucosidum, melibiosum, lactosum, raffinosum, melezitosum, inulinum, amyllum solubile, glycerolum, erythritolum, ribitolum, L-arabinitolum, galactitolum, inositolum, acidum citricum, methanolum, butano-2, 3-diolum et acidum quinicum. Assimilantur ethylaminum, L-lysinum, cadaverinum, glucosaminum et D-trytophanum. Non assimilantur kali nitratum, sodii nitritum, creatium, creatinimum et imidazolum. Amyllum non formatum. Thiiumanum externum ad crescentiam necessarium est. 30 °C crescit necque 37 °C. In medio 0.01 % cycloheximido addito crescit. Typus: EH008T (=ATCC MYA-4660T =CBS 11840T), designat stirpem typicum. Isolata a ile coleopterorum (Odontotaenius disjunctus: Passalidae), Broad Run, Virginia, USA, depositata in Collectione Culturarum American Type Culture Collection (ATCC), Manassas, Virginia, USA, et Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

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Description of Candida bullrunensis Suh, Houseknecht & Zhou sp. nov.

Candida bullrunensis (bull.ru.nen’sis. N.L. fem. adj. bullrunensis referring to Bull Run Mountain, Virginia, USA, the collection locality of the type strain).

After 7 days of growth on YM agar at 25 °C, cells are globose to subglobose (2.5–6.3 × 2.5–7.5 μm), and occur singly, in pairs, or in small clusters (Fig. 1a). Pseudohyphae and true hyphae are not present. Colonies are white to cream coloured, butyrous, smooth and have a clear margin. After 11 days of growth on Dalmau plate culture on cornmeal agar at 25 °C, pseudohyphae and true hyphae with blastoconidia are present (Fig. 1b and 1c). Aerobic growth is white to cream coloured with a slightly fuzzy margin. After 6 weeks at 25 °C on YM agar, 2 % malt extract agar, cornmeal agar, V8 juice agar or modified KNO₃ agar, no ascospores are observed. Glucose, galactose, maltose, methyl β-D-glucoside, sucrose, trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, inulin, starch and D-xylene are not fermented. Glucose, galactose, L-sorbose (delayed), D-glucosamine (delayed), D-xylene, L-arabinose, D-arabinose (weak), trehalose, cellobiose, salicin, arbutin, xyliotol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-glucuronate (weak), D-galacturonic acid, DL-lactate (delayed), succinate (delayed), ethanol and propane 1, 2-diol (delayed) are assimilated. D-Ribose, L-rhamnose, sucrose, maltose, methyl β-D-glucoside, melibiose, lactose, raffinose, melezitose, inulin, soluble starch, glycerol, erythritol, ribitol, L-arabinitol, galactitol, myo-inositol, citrate, methanol, butane 2,3 diol and quinic acid are not assimilated. Ethylamine, L-lysine, cadaverine, D-glucosamine (as nitrogen source) and D-tryptophan are assimilated; potassium nitrate, sodium nitrite, creatine, creatinine and imidazole are not assimilated. Thiamine is required for growth. Growth in 0.01 % cycloheximide is positive. Growth on 1 % acetic acid, 50 % D-glucose, 60 % D-glucose, 10 % NaCl and 16 % NaCl is negative. Growth at 30 °C is positive, while growth at 37 °C is negative. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative.

The type strain, EH008ᵀ (= ATCC MYA-4660ᵀ = CBS 11840ᵀ), was isolated from the gut of Odontotaenius disjunctus (Coleoptera: Passalidae) found on a rotten log, Bull Run Mountain, Broad Run, Virginia, USA.

Molecular phylogeny and ecology of yeasts in the Sugiyamaella clade

In a recent multigene phylogenetic analysis of yeasts, the ascosporic genus Stephanoascus was determined to be polyphyletic (Kurtzman & Robnett, 2007). The type species Stephanoascus ciferrii along with Stephanoascus farinosus was grouped with members of the Trichomonascus clade and these species were reassigned to the genus Trichomonascus. The remaining species of the genus Stephanoascus, Stephanoascus smithiae, formed a phylogenetically distinct clade with a group of anamorphic species in the genus Candida and was assigned as the type species of the new genus Sugiyamaella (Kurtzman & Robnett, 2007). Since its proposal, three other species of the genus Sugiyamaella and several related species of the genus Candida form the Sugiyamaella clade (Kurtzman, 2007; Wang et al., 2010). The genera Sugiyamaella, Trichomonascus, Wickerhamiella and Zygaonas along with related anamorphs were assigned to the newly established family Trichomonascaceae by Kurtzman & Robnett (2007), and a novel genus Spencermartinsiella was recently added in the family based on a multigene analysis (Péter et al., 2011).

A phylogenetic tree was constructed from the D1/D2 sequences of the LSU rRNA gene (about 600 bp) of 30 yeasts in the Sugiyamaella clade, including the strains isolated from this study (Fig. 2). Due to the unavailability of data, sequences of the ITS and the SSU rRNA gene were not included in the phylogenetic analysis. Although there were a few unresolved relationships among some of the yeasts, our results showed similar topologies within the Sugiyamaella clade compared with previous studies on this yeast group (Fig. 2; Kurtzman, 2007; Wang et al., 2010; F. Y. Bai, personal communication). Yeasts in the clade were divided into two subclades with high statistical support: 1) Su. americana and C. bullrunensis sp. nov., along with several undescribed anamorphic yeasts, and 2) Su. smithiae, Su. chiloensis, Su. japonica and related species of the genus Candida (Fig. 2). C. bullrunensis sp. nov. was closely related to the strains of Su. americana, including strain EH053 from this study, and formed a clade with these strains with a comparatively high bootstrap value. Several undescribed species of the genus Candida, i.e. strains BG02-3-7-5-1-2, CBS 10851, CBS 10852, CBS 10853, SD4S01 and GA3M12, showed close relationships with C. bullrunensis sp. nov.

**Fig. 1.** Candida bullrunensis sp. nov. EH008ᵀ. Budding yeast cells (a) after 7 days on YM agar at 25 °C, and hyphae (b) and blastoconidia (c) on cornmeal agar after 11 days at 25 °C. Bar, 5 μm.
Su. americana (Fig. 2). The remaining strains from this study, C. lignohabitans strains EH184 and EH188 and Su. smithiae strains EH075 and EH076, grouped well with their type strains NRRL YB-1336T and NRRL Y-17850T respectively (Fig. 2).

Interestingly, most of the members of the Sugiyamaella clade have a common ecology that is closely related to wood. They were isolated either directly from wood-ingesting insects and insect frass or from common insect habitats such as decayed wood, forest soil, mushrooms and peat (e.g. Kurtzman & Robnett, 2007; Kurtzman, 2007; Suh et al., 2005a; Wang et al., 2010). Su. americana NRRL YB-17336T and NRRL Y-17850T were isolated from insect frass, while C. bullrunensis sp. nov. EH008 T, Su. americana EH053 and Candida strains BG02-3-7-5-1-1 were isolated from the gut of the wood-ingesting beetle Odontotaenius disjunctus. Some other undescribed Candida strains near C. bullrunensis sp. nov. also have similar isolation sources. Strains CBS 10851, CBS 10852 and CBS 10853 were isolated from rotten wood in China (F.-Y. Bai, personal communication), while strains GY43S04 and SD4S01 and strain GA3M12 were isolated from forest soil and a mushroom in Taiwan, respectively (C.–F. Lee, personal communication). Similarly, the Candida lignohabitans strains EH184 and EH188 were isolated from tenebrionid beetles inhabiting a rotten log and the type strain NRRL YB-1473T was isolated from decayed wood. Su. smithiae strains EH075 and EH076 were derived from insect tunnels in wood, as were a few other strains in this species (Smith & de Hoog, 1998). All of the other species in the Sugiyamaella clade were also isolated either from decayed wood or insect tunnels (Kurtzman, 2007; Wang et al., 2010).

The connection between rotten wood and wood-ingesting insects raises interesting questions concerning the function of these yeasts in the gut of insects: are the yeasts merely acquired by eating yeast-infected wood or are they performing some important or essential functions in the gut? Significantly, all the yeasts in the Sugiyamaella clade, including the isolates from this study, are able to assimilate cellobiose, salicin and D-xylose (Kurtzman, 2007; Wang et al., 2010; F.-Y. Bai, personal communication; this study), which are indicator substrates of enzymes involved in metabolic routes associated with the digestive and detoxification processes of woody substrates (Vega & Dowd, 2005; Rivera et al., 2007). Therefore, based on the circumstantial evidence mentioned above, we assume that the yeasts in the Sugiyamaella clade may play an important role in the digestion process for wood-ingesting insects. However, compared with the consistent isolation of Scheffersomyces (Pichia) stipitis from the gut of O. disjunctus (Nardi et al., 2006; Suh et al., 2003; Zhang et al., 2003; unpublished data from this study), the isolation of Sugiyamaella yeasts directly from the beetle is somewhat
limited. For example, *C. bullrunensis* sp. nov. was isolated only once from more than 30 individual beetle samples in this study. Therefore, it is possible that the relationship between the *Sugiyamaella*-related yeasts and the wood-ingesting beetles could be more of a dispersal mechanism rather than performing any physiological function in the body of the host. However, more physiological studies and more extensive sampling will be necessary to clarify the ecological relationship between the gut yeasts of the *Sugiyamaella* clade and their host insects.

**ACKNOWLEDGEMENTS**

We are grateful to Dr Brian Beck for his advice throughout this study and to Mr Eric Emerick for assisting with physiological tests. Dr Albert P. Torzilli allowed us to use his laboratory space in George Mason University for insect dissection. We also thank Drs Feng-Tan Bai and Ching-Fu Lee for kindly providing their unpublished data. This work was supported in part by National Science Foundation Grant DBI-0548684 and the ATCC Internship Program.

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