

Wickerhamomyces arborarius f.a., sp. nov., an ascomycetous yeast species found in arboreal habitats on three different continents

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Five strains representing a novel yeast species belonging to the genus *Wickerhamomyces* were independently isolated from Ecuador, Taiwan and the USA. One strain (CLQCA 10-161^T) was isolated from the white flower of an unidentified plant species collected in the Maquipucuna cloud forest reserve, near Quito, in Ecuador. A second strain (GY7L12) was isolated from the leaf of a Chinese sumac or nutgall tree (*Rhus chinensis* 'roxburghiana') collected in the Taoyuan mountain area, Kachsiung, in Taiwan. Three additional strains (A543, A546 and A563) were isolated from two species of wood-boring beetle (*Xyleborus glabratus* and *Xyleborinus saxeseni*) collected near Clio, Georgia, USA. Analysis of the D1/D2 domains of the LSU rRNA gene indicated that the novel species belongs to the genus *Wickerhamomyces*, and is most closely related to *Wickerhamomyces sydowiorum*, an insect-associated species predominantly found in South Africa. The North American and Taiwanese strains have identical internal transcribed spacer (ITS) sequences and can be distinguished from the Ecuadorian strain based on a single nucleotide substitution in the ITS1 region. The species name of *Wickerhamomyces arborarius* f.a., sp. nov. is proposed to accommodate these strains, with CLQCA 10-161^T (=CBS 12941^T=NCYC 3743^T) designated the type strain.

The ascomycetous genus *Wickerhamomyces* was created, along with two other genera (*Barnettozyma* and *Lindnera*), following a recent detailed phylogenetic study carried out by Kurtzman *et al.* (2008) of 140 coenzyme Q-7 producing yeast taxa. The aim of the study was to determine the phylogenetic placement of species (and species varieties) assigned to the genera *Issatchenkia*, *Pichia*, *Starmera*

and *Williopsis*. Using a multigene sequencing approach, Kurtzman and colleagues were able to resolve these species into five distinct and well-supported clades. As a result, the genus *Pichia* underwent a significant reduction in overall size (to just 20 species) and was redefined and restricted to only those species closely related to *Pichia membranifaciens* (*Pichia membranifaciens* clade), including all those previously assigned to the genus *Issatchenkia*. *Pichia dryadoides* and *Pichia quercuum* were both transferred, as basal members, to the genus *Starmera* (*Starmera* clade). The three remaining clades, each comprising a varied number of *Pichia* and *Williopsis* species, were recognized as representing new genera, and were named *Barnettozyma*, *Lindnera* and *Wickerhamomyces*.

When first created, sixteen *Pichia* species, including the former *Pichia anomala* (renamed as *Wickerhamomyces anomalus*) and *Pichia sydowiorum* (renamed as *Wickerhamomyces*

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the LSU D1/D2 and ITS sequences of CLQCA 10-161^T are FN908198 and FR670785, respectively. The accession number for the LSU D1/D2 sequence of GY7L12 is FJ527096.

The MycoBank number for *Wickerhamomyces arborarius* f.a., sp. nov. CLQCA 10-161^T is MB805568.

Two supplementary figures and one supplementary table are available with the online version of this paper.

sydowiorum) were transferred along with *Williopsis mucosa* (renamed *Wickerhamomyces mucosus*) to the genus *Wickerhamomyces* (Kurtzman *et al.*, 2008). Since then, additional species have been described and assigned to this genus, including *Wickerhamomyces chaumierensis*, *Wickerhamomyces edaphicus*, *Wickerhamomyces ochangensis*, *Wickerhamomyces patagonicus*, *Wickerhamomyces queroliae* and *Wickerhamomyces xylosica* (Limtong *et al.*, 2009; Rosa *et al.*, 2009; de García *et al.*, 2010; Groenewald *et al.*, 2011; Shin *et al.*, 2011; Limtong *et al.*, 2012). Six anamorphic species, *Candida odintsovae*, *Candida peoriensis*, *Candida ponderosae*, *Candida quercuum*, *Candida silvicultrix* and *Candida ulmi*, are also accommodated within the genus along with a number of novel and as yet un-described taxa (Kurtzman, 2011b).

From a phenotypic perspective, there are no obvious properties that can reliably delineate the genus *Wickerhamomyces*, which as Kurtzman (2011b) noted is typical of many of the more recently created genera that have been defined from phylogenetic analysis. Most members of the genus *Wickerhamomyces* produce hat-shaped ascospores, although *Wickerhamomyces mucosus* (previously assigned to the genus *Williopsis*) is somewhat of an exception as it forms saturn-shaped ascospores, a characteristic more commonly associated with *Saturnispora* yeasts (Kurtzman, 2011a, b). Collectively, species of the genus *Wickerhamomyces* are often recovered from arboreal habitats and have been isolated from a variety of different tree species including acacia (*Wickerhamomyces ciferrii*), alder (*Wickerhamomyces alni*), cherry (*Wickerhamomyces silvicola*), oak (*Wickerhamomyces hampshirensis*), pine and spruce (*Wickerhamomyces canadensis*). In addition, another frequent source for several of these species, notably *Wickerhamomyces bisporus*, *Wickerhamomyces bovis*, *Wickerhamomyces hampshirensis* and *Wickerhamomyces sydowiorum*, is insects and insect frass (Kurtzman, 2011b).

In the present study, we describe the discovery of five strains independently isolated in North and South America as well as in East Asia, and the formal taxonomic description of a novel *Wickerhamomyces* species, *Wickerhamomyces arborarius* sp. nov., to accommodate them. The first strain, CLQCA 10-161^T, was isolated along with over 70 other yeast strains during a pilot study to survey the yeast diversity found in the Maquipucuna Cloud Forest Reserve, located 50 miles to the north-west of Quito, Ecuador. Strain CLQCA 10-161^T was isolated from the white flower of an unidentified plant species collected at 1668 m above sea level. The second strain, GY7L12, was isolated from the leaf of a Chinese sumac or nutgall tree (*Rhus chinensis* 'roxburghiana') collected in the Yushan mountain area, Taoyuan, Kaohsiung, Taiwan. Three additional strains were isolated by grinding and dilution-plating of whole wood-boring beetles excavated from stems of redbay (*Persea borbonia*) with laurel wilt collected near Clio, Georgia, USA (Harrington & Fraedrich, 2010). Strains A543 and A546 were both isolated from fruit-tree pinhole borer beetles (*Xyleborinus saxeseni*), while strain A563 was isolated from a redbay ambrosia beetle (*Xyleborus glabratus*). The latter beetle species, native to Asia, is most notable for

the fact it carries (as a fungal symbiont) the laurel wilt pathogen *Raffaelea lauricola*, and is believed to be the insect vector that introduced this fungus to the south-eastern United States (Harrington *et al.*, 2008, 2011). The yeast strains were characterized biochemically, morphologically and physiologically according to the standard methods described by Kurtzman *et al.* (2011). Growth temperature testing was determined by cultivation on yeast extract-malt extract (YM) agar (Becton, Dickinson & Company). Sporulation tests were performed on cornmeal agar, 5 % malt extract 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 internal transcribed spacer (ITS) region were amplified by PCR directly from whole yeast cell suspensions as described 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), and sequenced using a Life Technologies 3730XL sequencer at the Genome Analysis Centre (TGAC), Norwich, UK. 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 EMBL, 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 reconstructed using the neighbour-joining method (Saitou & Nei, 1987), with the Jukes–Cantor distance measure and *C. quercuum* used as the outgroup species. Confidence limit values were estimated from bootstrap analyses of 1000 replicates (Felsenstein, 1985).

The LSU D1/D2 sequences of strains A543, A546, A563, CLQCA 10-161^T and GY7L12 were 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 five strains displayed 0.9 % divergence (3 nt substitutions and 1 indel in 571 nt) with *Wickerhamomyces sydowiorum*, 1.2 % divergence (5 nt substitutions and 2 indels in 571 nt) with *Wickerhamomyces lynferdii* and 1.4 % divergence (6 nt substitutions and 2 indels in 571 nt) with *Wickerhamomyces subpelliculosa*. Although somewhat limited, these levels of sequence divergence are nevertheless still greater than those observed between *Wickerhamomyces lynferdii* and *Wickerhamomyces subpelliculosa* (0.5 %; 3 nt substitutions in 571 nt), and between *Wickerhamomyces anomalus* and *Wickerhamomyces myanmarensis* (0.3 %; 2 nt substitutions in 573 nt).

A phylogenetic analysis based on LSU D1/D2 sequences showed that the novel taxon (as represented by strains CLQCA 10-161^T and GY7L12) belongs to the *Wickerhamomyces anomalus* subclade of the genus *Wickerhamomyces*, and forms a distinct (bootstrap value, 98 %) species pair with *Wickerhamomyces sydowiorum* (Fig. 1). Interestingly, and somewhat surprisingly, *Candida nitrativorans* the designated anamorph of *Wickerhamomyces sydowiorum* (Kurtzman, 2011b) was found to belong to a separate species group, comprising *Wickerhamomyces lynferdii*, *Wickerhamomyces subpelliculosa* and *Wickerhamomyces sydowiorum* NRRL Y-10997 (Fig. 1). Statistically, this species grouping is poorly supported (bootstrap value, <50 %). However, the fact the LSU D1/D2 sequences for the respective type strains display 1.6 % sequence divergence (8 nt substitutions, 1 indel in 571 nt) would suggest *C. nitrativorans* has been incorrectly identified as the asexual state of *Wickerhamomyces sydowiorum*. In fact this level of sequence variation would suggest, although not necessarily prove, that *C. nitrativorans* represents a distinct species. Clearly, additional work will be required to resolve the taxonomic status of *C. nitrativorans*, as well as that of *Wickerhamomyces sydowiorum* NRRL Y-10997, which has an identical LSU D1/D2 sequence (Fig. 1).

Despite the close phylogenetic relationship based on LSU D1/D2 sequences, the novel species of the genus *Wickerhamomyces* and *Wickerhamomyces sydowiorum* can be readily distinguished from one another by ITS sequencing. In the ITS1 region, the two taxa differ by 11 nt substitutions and 5 indels (in 182 nt), and in the ITS2 region by 10 nt substitutions and 1 indel (in 188 nt). Furthermore, the Ecuadorian isolate (CLQCA 10-161^T) can be distinguished from the American (A543, A546 and A563) and Taiwanese (GY7L12) isolates based on a single

(T/C) base substitution in the ITS1 region. To assess the potential usefulness of ITS sequencing for differentiating between isolates of these two taxa, four strains of *Wickerhamomyces sydowiorum* were also analysed. With the exception of NRRL Y-10997, whose current taxonomic status is somewhat unclear, the other three *Wickerhamomyces sydowiorum* strains, including the type strain (NRRL Y-7130^T), were found to have identical ITS1 sequences, and ITS2 sequences which differed by no more than 1 nt substitution and 2 indels (in 189 nt). These results provide further evidence that the five strains belong to a separate and distinct species of the genus *Wickerhamomyces*, and once again illustrate the value of ITS sequencing for differentiating between closely related (sibling) species. Separate ITS alignments: (i) between representative strains of the novel *Wickerhamomyces* species and the *Wickerhamomyces sydowiorum* type strain (NRRL Y-7130^T), and (ii) between four different strains of *Wickerhamomyces sydowiorum* are shown in Figs S1 and S2 (available in the online Supplementary Material), respectively.

Based upon the origins of the five strains reported here, it would seem the ecological niche of the novel species, like that of other members of the genus (e.g. *Wickerhamomyces alni*, *Wickerhamomyces canadensis*, *Wickerhamomyces hampshirensis* and *Wickerhamomyces silvicola*), is an arboreal habitat. The species appears to be widely distributed, and to date has been found on three separate continents (i.e. Asia, and North and South America). Three of the strains were isolated from wood-boring beetles: A543 and A546 from *Xyleborinus saxeseni* (also known as the Asian ambrosia beetle) and A563 from *Xyleborus glabratus* (also known as the redbay ambrosia beetle). One of the beetles was surfaced-sterilized before grinding and isolation by dilution plating, and it was speculated that the yeast was a gut-inhabitant of

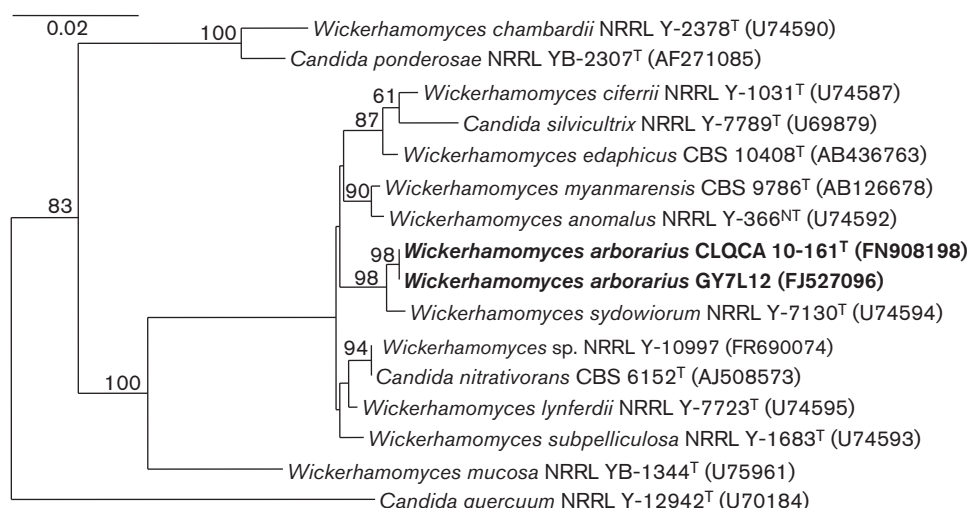


Fig. 1. Neighbour-joining dendrogram based on sequences of the D1/D2 domain of the LSU rRNA gene of *Wickerhamomyces arborarius* sp. nov. and its closest relatives. *C. quercuum* was used as the outgroup species for the analysis. Bootstrap values ≥ 50 %, determined from 1000 replicates, are shown at branch nodes. Bar, two base substitutions per 100 nt.

the beetle (Harrington & Fraedrich, 2010). This raises the possibility, albeit rather speculative at this stage, that ambrosia beetles could represent the primary habitat of this yeast. The Asian ambrosia beetle, also called the fruit-tree pinhole borer, is commonly found in forests and is native to Eurasia. Similarly, the redbay ambrosia beetle is native to Asia (including Taiwan), and was recently identified as the insect vector which first introduced the laurel wilt pathogen *Raffaelea lauricola* into the south-eastern United States (Harrington *et al.*, 2011). It is interesting to note the ITS sequences of all three beetle strains as well as the Taiwanese strain (GY7L12) are identical, possibly suggesting the North American isolates may have originated from Asia, where both ambrosia beetles are native. Although *Xyleborus glabratus* itself has not yet been found on mainland Ecuador, three other species of the genus *Xyleborus* have, while *Xyleborinus saxeseni* has been found recently in the Galápagos (first detected in 1996), suggesting it might also be present on the mainland. However, further sampling will need to be done in order to gain a better understanding of both the origin and primary habit of this novel *Wickerhamomyces* species.

Physiologically, the novel species of the genus *Wickerhamomyces* and *Wickerhamomyces sydowiorum* appear to be indistinguishable from one another. Although some strain variation exists within each species, there does not appear to be a single standard phenotypic test which can be used reliably to separate them. With regard to the novel species, the fermentation of maltose and raffinose appear to be variable growth characteristics, as do the assimilation of lactose, cellobiose, L-sorbose, L-arabinose and D-arabinose. Indeed, the five strains can be subdivided according to their geographical origins. The Ecuadorian strain CLQCA 10-161^T can be distinguished from the other four strains based on its ability to assimilate L-sorbose. The Taiwanese strain GY7L12 can be distinguished from the others as it is the only one unable to assimilate lactose. The three North American strains (A543, A546 and A563) can be distinguished as they assimilate L-arabinose but not D-arabinose, whereas the Ecuadorian and Taiwanese strains both assimilate D-arabinose but not L-arabinose. Key growth characteristics that can be used to distinguish between the five strains are presented in Table S1.

Based on the molecular and phenotypic data presented here, we conclude that the five strains, found in different arboreal habitats on three separate continents, represent a novel species. Although neither sporulation nor conjugation was observed in either mixed or pure cultures grown on a variety of different sporulation media, phylogenetic analysis based on LSU D1/D2 sequences clearly demonstrated that the species belongs to the genus *Wickerhamomyces*, and specifically to the *Wickerhamomyces anomalus* clade. In accordance with the recent amendment made to Article 59 of the International Code of Botanical Nomenclature (Norvell, 2011), the novel species was assigned to the genus *Wickerhamomyces*, and the name *Wickerhamomyces arborarius* f.a., sp. nov. was proposed,

with CLQCA 10-161^T designated the type strain. In view of the fact no sexual cycle was observed, the suffix 'f.a.' (for 'forma asexualis') was added to indicate the species description is based solely upon the asexual form (anamorph). The use of this abbreviation was first (informally) proposed by Lachance (2012), and has since been adopted, firstly by Groenewald & Smith (2013) in their reassignment of *Candida yakushimensis* to *Yarrowia yakushimensis* f.a., comb. nov., and subsequently by Lachance & Kurtzman (2013) in their description of the genus *Tortispora* gen. nov.

Description of *Wickerhamomyces arborarius*
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Wickerhamomyces arborarius (ar.bo.ra'ri.us. L. masc. adj. *arborarius* of or pertaining to trees).

In YM broth, after 2 days of incubation at 25 °C, cells are ovoid (2–7 × 4–7 µm) and occur singly, in pairs, in short chains or in groups (Fig. 2). Budding is multilateral. Sediment is formed after 1 month, but no pellicle is observed. Pseudomycelia are well formed with irregular branching. True mycelia are not formed. No sexual state is observed from either mixed or pure cultures grown for 1 month at 25 °C on cornmeal agar, 5 % malt extract agar, potassium acetate agar or YM agar. Glucose (positive or latent), galactose (latent or latent but weak), sucrose (positive or latent), maltose (latent or negative) and raffinose (latent or negative) are fermented, but not cellobiose, trehalose, lactose, melibiose, melezitose, inulin, starch, xylose or methyl α-D-glucoside. Glucose, sucrose, raffinose, melibiose, galactose, lactose (variable), trehalose, maltose, melezitose, methyl α-D-glucoside, starch, cellobiose (variable), salicin (positive or latent), sorbose (latent or negative), L-rhamnose (positive or latent), D-xylose, L-arabinose (variable), D-arabinose (variable), D-ribose (variable), ethanol, glycerol, erythritol, ribitol, D-mannitol,

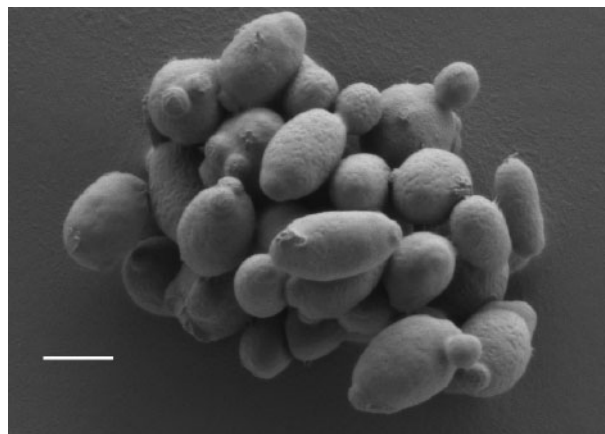


Fig. 2. Scanning electron microscopic image of vegetative cells of *Wickerhamomyces arborarius* sp. nov. CLQCA 10-161^T grown in YM broth for 2 days at 25 °C with agitation. Bar, 2 µm.

D-glucitol, DL-lactate, succinate, citrate and xylitol (positive or latent) are assimilated. No growth occurs on inulin, methanol, galactitol, inositol or D-glucosamine. Ethylamine hydrochloride, lysine, cadaverine and nitrate are assimilated. Growth occurs at 30 °C, but not at 37 °C. Growth occurs on YM agar with 10 % (w/v) NaCl and on 50 % glucose/yeast extract, but is variable on 60 % glucose/yeast extract. Starch-like compounds are not produced. No growth occurs with 10 µg cycloheximide ml⁻¹.

The type strain, CLQCA 10-161^T (=CBS 12941^T=NCYC 3743^T), was isolated in April 2007 from the white flower of an unidentified plant species collected in the Maquipucuna Cloud Forest Reserve, Pichincha Province, Ecuador. The Mycobank deposit number is MB805568.

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