

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

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Running Title: *Wickerhamomyces arborarius* sp. nov.

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ABSTRACT

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* var. *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 large-subunit (LSU) ribosomal RNA 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 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 as the type strain.

Keywords: Ascomycota, *Wickerhamomyces*, arboreal habitat; wood-boring beetles, novel species

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* (*P. 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 of a varied number of *Pichia* and *Williopsis* species, were recognised 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 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 Garcia *et al.* 2011; Groenwald *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 which can reliably delineate the genus *Wickerhamomyces*, which as Kurtzman (2011b) noted is typical of many of the more newly created genera which have been defined from phylogenetic analysis. Most *Wickerhamomyces* produce hat-shaped ascospores, although *W. 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; Kurtzman, 2011b). Collectively, *Wickerhamomyces* species are often recovered from arboreal habitats and have been isolated from a variety of different tree species including acacia (*W. ciferrii*), alder (*W. alni*), cherry (*W. silvicola*), oak (*W. hampshirensis*), pine and spruce (*W. canadensis*). In addition, another frequent source for several of these species, notably *W. bisporus*, *W. bovis*, *W. hampshirensis* and *W. 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 new *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* var. *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 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 corneal 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 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), 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 constructed using the neighbour-joining method (Saitou & Nei, 1987), with the Jukes-Cantor distance measure, and *Candida 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 and 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 *W. anomalus* subclade of the genus *Wickerhamomyces*, and forms a distinct (bootstrap value, 98%) species pair with *W. sydowiorum* (Fig. 1). Interestingly, and somewhat surprisingly, *Candida nitratorans* the designated anamorph of *W. sydowiorum* (Kurtzman, 2011b) was found to belong to a separate species group, comprising of *W. lynferdii*, *W. subpelliculosa* and *W. 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. nitratorans* has been incorrectly identified as the asexual state of *W. sydowiorum*. In fact this level of sequence variation would suggest, although not necessarily prove, that *C. nitratorans* represents a distinct species. Clearly additional work will be required to resolve the taxonomic status of *C. nitratorans*, as well as that of *W. 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 *Wickerhamomyces* species and *W. 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 *W. sydowiorum* were also analysed. With the exception of NRRL Y-10997, whose current taxonomic status is somewhat unclear, the other three *W. 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 *Wickerhamomyces* species, and once again illustrate the value of ITS sequencing for differentiating between closely related (sibling) species. In the online version of this paper, two supplementary figures are included showing separate ITS alignments: i) between representative strains of the novel *Wickerhamomyces* species and the *W. sydowiorum* type strain (NRRL Y-7130^T) (Fig. S1), and ii) between four different strains of *W. sydowiorum* (Fig. S2).

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. *W. alni*, *W. canadensis*, *W. hampshirensis* and *W. 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, 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 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 *X. glabratus* itself has not yet been found on mainland Ecuador, three other *Xyleborus* species have, while *Xyleborinus saxeseni* has been found recently in the Galápagos (first detected in 1996), suggesting it also might 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 *Wickerhamomyces* species and *W. 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 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. In the online version of this paper, a supplementary table (Table S1) is included which lists the key growth characteristics that can be used to distinguish between the five strains.

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 *W. 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 as 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* James, Carvajal, Barahona, Harrington, Lee, Bond & Roberts, sp. nov.

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

In YM broth, after 2 days incubation at 25°C, cells are ovoid (2-7 x 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 and 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, melizitose, 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, 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 in 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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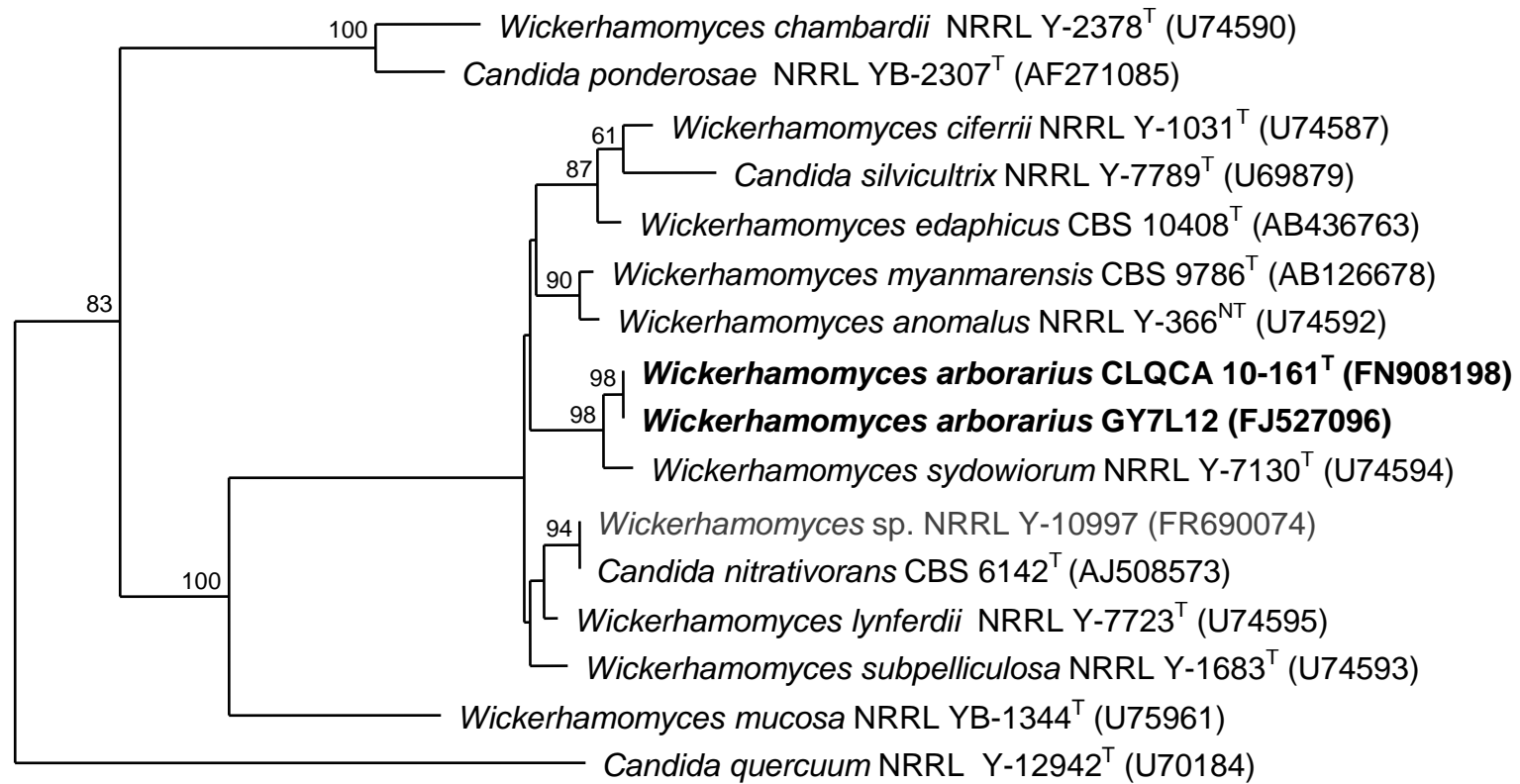
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Figure Legends

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. *Candida quercuum* was used as the outgroup species for the analysis. Bootstrap values $\geq 50\%$, determined from 1000 replicates, are shown at branch nodes. Bar, 2 base substitutions per 100 nucleotides.

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 .



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