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 (CLOCA 10-161T) 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 Clyo, 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 CLOCA 10-161T (=CBS 12941T=NCYC 3743T) 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 Issatchenka, 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 Issatchenka. Pichia dryadoideae 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/DBJ accession numbers for the LSU D1/D2 and ITS sequences of CLOCA 10-161T 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. CLOCA 10-161T 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 Wicker-
hamomyces (Kurtzman et al., 2008). Since then, additional
species have been described and assigned to this genus,
including Wickerhamomyces chaumieriensis, Wickerhamomyces
edaphicus, Wickerhamomyces ochangensis, Wickerhamomyces
pataconicus, Wickerhamomyces querolae and Wickerhamo-
myces 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
odontosovae, 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 pro-
erties that can reliably delineate the genus Wicker-
hamomyces, which as Kurtzman (2011b) noted is typical of
many of the more recently created genera that have been
deined 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 Wicker-
hamomyces are often recovered from arboreal habitats and
have been isolated from a variety of different tree species
including acacia (Wickerhamomyces ciferrii), alder (Wicker-
hamomyces alni), cherry (Wickerhamomyces silvicola), oak
(Wickerhamomyces hampshirensis), pine and spruce (Wicker-
hamomyces canadensis). In addition, another frequent source
for several of these species, notably Wickerhamomyces
bisporus, Wickerhamomyces bovis, Wickerhamomyces hamp-
shirensis 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 10-161T, 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 10-161T
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
nuttall 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
Clyo, Georgia, USA (Harrington & Fraedrich, 2010). Strains
A543 and A546 were both isolated from fruit-tree pinhole
borer beetles (Xyleborinus saxe sensi), 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). Spo-
ulation 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 sus-
pensions 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 SEQUENcher, 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 recon-
structed 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 10-161T 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 Wickerhamo-
myces lynferdii and 1.4 % divergence (6 nt substitutions
and 2 indels in 571 nt) with Wickerhamomyces subpellicu-
losa. 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 10-161T 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 assexual 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 189 nt). Furthermore, the Ecuadorian isolate (CLQCA 10-161T) 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-7130T), 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-7130T), 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 Xyleborus 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 surface-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 habitat 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 10-161T 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 10-161T 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**

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 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 10-161T grown in YM broth for 2 days at 25 °C with agitation. Bar, 2 μm.](image-url)
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 10-161T (=CBS 12941T=NCYC 3743T), 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.

Acknowledgements
S. A. J., C. J. B. and I. N. R. were supported at the IFR by a BBSRC Competitive Strategic Grant. The NCYC is a BBSRC-supported National Capability. E. J. C. B. and P. P. B. were supported by the Pontificia Universidad Católica del Ecuador and SENESCYT (awarded to E. J. C. B.). C.-F. L. was supported by a Grant-in-Aid from the National Science Council, Executive Yuan, Taiwan (NSC 97-2621-B-134-001-MY3).

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