Wickerhamiella verensis f.a. sp. nov., a novel yeast species isolated from subsoil groundwater contaminated with hydrocarbons and from a human infection

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Running title: *Wickerhamiella verensis* isolated from hydrocarbon contaminated samples and clinical environment.

Repositories: The GenBank accession numbers for the D1/D2 LSU rRNA gene and ITS-5.8S rDNA region sequences of strain YEALI 107^T (=CECT 12028^T) are EF141077 and EU818715, respectively.

Abstract

Yeast strains belonging to a novel anamorphic yeast species were isolated from subsoil groundwater contaminated with hydrocarbons in a metal working factory located in Northern Spain, and from a human infection in the USA. Comparison of ITS sequences between the isolates revealed 0.2% divergence between the Spanish isolates and 0.46% divergence between those and the USA isolate. Phylogenetic analysis based on the D1/D2 domains of the LSU rRNA gene showed that these isolates belong to the *Wickerhamiella* clade and *W. sorbophila* and *W. infanticola* are their closest relatives. Sequence divergence between the new isolates and *W. sorbophila* and *W. infanticola* is 1.97 and 1.79 %, respectively. The isolates in the new species are not fermentative and pseudohyphae were not produced. Sexual reproduction was not observed for individual isolates or in mixtures of isolates. Conjugation between the isolates in the new species and close relatives *W. sorbophila* and *W. infanticola* was not observed. These data support the proposal of *Wickerhamiella verensis* as a novel species, with CECT 12028^{T} as the holotype.

Main text

The genus *Wickerhamiella* was erected in 1973 (1) to accommodate yeast strains forming very small vegetative cells and showing conjugation and ascospore formation. Based on these characteristics four new species *W. australiensis*, *W. occidentalis*, *W. lipophila* and *W. cacticola* were later described (2, 3). Phylogenetic analysis of D1/D2 LSU rRNA gene sequences placed *W. domercqiae*, the type species in the genus *Wickerhamiella*, in a clade together with several *Candida* species but separated from the other four species of the genus (4). Later, based on D1/D2 LSU rRNA gene sequences, 18 new species transferred from the genus *Candida* were described (5). The genus Wickerhamiella comprises now more than 40 species (www.Mycobank.org). The ecology of the genus is not well stablished, most species have been isolated from flowers and associated insects, fruit, wineries or distilleries, but some have been recovered from human sources. Most species appear to be endemic to tropical regions and Asia, but other species have a cosmopolitan distribution.

During a survey of the microorganisms present in a hydrocarbon-contaminated factory in Northern Spain, 78 yeasts were isolated and identified. After the identification process, two strains YEALI 107 (=CECT 12028) and YEALI 108 (CECT 12036) could not be assigned to a known yeast species. In addition, a strain (CDC 11278) isolated in Atlanta, Georgia USA from an infant with a blood infection also proved to be a member of the new species. In this study, we present the phenotypic characterization and phylogenetic analyses of the strains representing the novel asexual species, which we propose as *Wickerhamiella verensis*, a new species in the *Wickerhamiella* clade.

Collection site and isolation of yeasts

Norther Spain samples

Samples collection site were subsoil groundwater of an old metal working factory in Vera de Bidasoa (Spain) (Longitude -1.6830042, Latitude 43.2808504) polluted by different mixtures of hydrocarbons released over several decades of industrial activity. Vera de Bidasoa is located in Northern Spain, and the weather is oceanic temperate, strongly influenced by the Cantabrian Sea (Bay of Biscay) with abundant rains, mist and drizzle but

relatively warm. The annual average temperature lies between 8.5°C and 14.5°C and annual average precipitation ranges from 1,100 to 2,500 mm. In the metal working factory, four zones of contamination were identified: Z1, fuel oil spilled from heating systems; Z2, lubricant-oil leaked from metal working units; Z3, diesel fuel spilled from underground tanks; and Z4, perimeter with mixtures of the three contaminants. The hydrocarbon-polluted areas were initially treated using physico-chemical techniques followed by a bioremediation approach based on the injection of an oleophilic fertilizer (S200, IEP www.iepeurope.com), diluted hydrogen peroxide, and a surfactant (Bioversal HC, IEP). Yeasts were isolated before, during and after the bioremediation process using a synthetic medium for hydrocarbon degradation (DM medium 0.3% NH_4NO_3; 0.05% $MgSO_4.7H_2O$; 2% $CaCl_2.2H_2O$; 0.007% K_2HPO_4; 0.05% KH_2PO_4; 2% agar) supplemented with 2% fuel oil, 2% lubricant oil or 2% diesel (Repsol, S.A., Spain) as sole carbon source. Information about bacterial counts and hydrocarbon degradation along the bioremediation process are found in Menendez-Vega *et al.* (6). Seventy-eight yeast strains were isolated from three contaminated areas Z1, Z2 and Z4 before, during and after the bioremediation process (Table 1).

USA samples

An unidentified strain (CDC 11278) was received from the U.S. Center for Disease Control and Prevention, Atlanta, Georgia USA. The strain had been isolated from an infant suffering from a blood infection.

Ecology of yeast species found in subsoil groundwater samples from Vera de Bidasoa, Spain

During this study, 78 yeast strains pertaining to species different from *W. verensis* were isolated and identified from a hydrocarbon contaminated factory in Vera de Bidasoa, Spain. Most of the strains pertained to the species *Candida viswanathii*, and 6 other yeast species (Table 1) were represented with less strains. Most of these yeast species contain strains isolated from clinical or human environment. The most represented species *C. viswanathii,* isolated from a fatal case of meningitis in India and subsequently isolated in 1962 from sputum, is considered an opportunistic pathogen (7). Moreover, strains in species *C. parapsilosis*, *Rhodotorula mucilaginosa*, *Meyerozyma guilliermondii*

and *Lodderomyces elongisporus* are commonly associated with fungemia (8). The new species *W. verensis* also includes environmental and clinical strains.

Sequencing and phylogenetic analysis

D1 and D2 domains of the nuclear LSU rRNA gene were amplified by PCR using the primers NL-1 and NL-4 (9). PCR products were cleaned and sequenced. Sequences were edited and assembled using MEGA X (10) and then subjected to a sequence similarity search using the BLASTN tool in NCBI to identify them by sequence homology of described taxa (Table 1). The D1/D2 LSU rRNA gene sequence of CECT 12028^T as the holotype was included in a multiple alignment generated using MEGA X, which included described species of the *Wickerhamiella* clade and selected reference species (11). A phylogenetic tree was inferred using the Maximum Likelihood method in MEGA X. Sequence HQ111062 representing the D1/D2 LSU rRNA gene of *Starmerella bombicola* was used as outgroup (11). Additionally, the ITS1-5.8S-ITS2 rRNA region of the new isolates and closely related species was amplified and sequenced using primers ITS1 and ITS4 (12). Sequences were aligned using MEGA X and nucleotide substitutions were determined.

Species delimitation

Wickerhamiella verensis strains CECT 12028^T and CECT 12036 had the same D1/D2 nucleotide sequence. The clinical isolate, CDC 11278, differed from the type of *W*. *verensis* by 3 substitutions and 1 deletion for D1/D2. BLAST alignment of the D1/D2 LSU rRNA sequences showed 11 substitutions between neighbouring species *W. verensis* and *W. sorbophila,* and 10 substitutions between *W. verensis* and *W. infanticola* in the 577 nucleotide positions examined.

The ITS1-5.8S-ITS2 rRNA sequences alignment showed 1 substitution between the Spanish strains (433 bp, including PCR primers) and by 2 substitutions in case of the USA clinical isolate. The ITS1-5.8S-ITS2 rRNA sequences alignment of *W. verensis* CECT 12028^T with closely related *Wickerhamiella* species (446 positions) showed 12 substitutions between *W. verensis* and *W. sorbophila* and 9 substitutions between *W. verensis* and *W. infanticola*. The nucleotide substitutions found between *W. verensis* and *W.*

infanticola or *W. sorbophila* indicate that *W. verensis* is a new species different from its closest relatives.

Figure 1 shows the phylogenetic position of CECT 12028^T and related species based on LSU D1/D2 sequences. *W. verensis* is closely related to isolate SJ-1. D1/D2 LSU alignment (598 positions) shows that they differ in one nucleotide substitution and 2 gaps, indicating that they probably pertain to the same species. The subclade constituted by *W. verensis*, *W*. *sorbophila* and *W. infanticola* is related to isolate *Candida* sp. SN-102 and *W. siamensis*. Strains SJ-1 and SN-102 have been isolated from sea surface microlayer in Taiwan, two isolates from *W. verensis* and *W. sorbophila* were isolated from industrial waste water, and one isolate from *W. verensis* and *W. infanticola* were from human source.

Phenotypic characterization of the yeasts

Yeast strains were maintained for short periods on GPYA (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar). For long-term storage, yeasts were frozen at -80ºC in 15% glycerol or maintained as lyophilized preparations. Morphological and physiological characterization of *W. verensis* CECT 12028^T and closely related strains *W. sorbophila* NRRL Y-7921 and *W. infanticola* NRRL Y-17858, was performed according to standard methods (13). Hydrolysis of Tween 80 was tested on media devised by Sierra *et al.* (14). Cell morphology was examined in cultures grown on malt extract agar (Difco) using a light microscope (Nikon Eclipse E800). Media tested to detect sporulation in the isolates included glucose-yeast extract-malt extract-peptone (YM) agar, potato dextrose agar (PDA), restricted growth (RG) agar and yeast carbon base-ammonium sulphate (YCBAS) agar (13). Strains were examined microscopically for ascospore formation individually and as a group on the four media at 15 °C and 25 °C.

The ability of the strains to mate was tested by mixing equal amounts of young actively growing cells (2 d, 25 ºC) from *W. verensis* CECT 12028^T, CECT 12036, CDC 11278, *W. sorbophila* NRRL Y-9721, *W. infanticola* NRRL Y-17858 onto slants of YM, 5% malt extract, RG and YCBAS agar media. Incubation was at 15 ºC and 25 ºC for 6 weeks.

Table 2 shows the ability of Spanish yeast isolates to grow on different hydrocarbons. All yeasts isolated from Spain were able to grow on lubricant oil (isolation medium). The new species *W. verensis* was not able to grow on any hydrocarbon tested. Table 3 shows the physiological differences between the Spanish isolates of W. verensis and closest relatives *W. sorbophila* NRRL Y-7921 and *W. infanticola* NRRL Y-17858. Sporulation media were observed for two months, but neither asci nor ascospores were found. Mating experiments revealed that neither conjugation nor ascospores were observed.

Description of *Wickerhamiella verensis f.a*., sp. nov. Belloch, Pelaez, Menendez-Vega, Sanchez & Kurtzman

Wickerhamiella verensis (ve.ren'sis. N.L. fem. adj. *verensis* of Vera de Bidasoa, a town in Navarra (Northern Spain).

In malt extract medium after 3 days at 25ºC, cells are globose to ellipsoidal (1-2 x 1.5-3.5 µm), occur singly or in parent-bud pairs (Figure 1). After one month at 25 º C, sediment is present. After one month on GPY agar at 25 º C, colonies are cream coloured, smooth and the margin is entire. No true hyphae or pseudohyphae develop in Dalmau plate cultures. Conjugation and ascospore formation is absent. Fermentation is absent. Assimilates D-glucose, D-galactose, L-sorbose, sucrose, inulin, D-glucitol, D-mannitol, ethanol, succinate and hexadecane. Does not assimilate cellobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, soluble starch, D-arabinose, L-arabinose, D-ribose, L-rhamnose, D-xylose, methanol, glycerol, meso-erythritol, inositol, ribitol, xylitol, galactitol, citrate, DL-lactate, D-gluconate, methyl-α-D-glucoside, salicin, D-glucosamine, N-acetyl-D-glucosamine, saccharate, D-glucuronate and L-arabinitol. Assimilates ethylamine and L-lysine but not nitrate, nitrite, imidazole and glucosamine. No starch-like substance is produced. Urease reaction is negative. Hydrolysis of Tween 80 is positive. Growth on vitamin free medium is negative. Optimal growth temperature is 25ºC, but growth at 37ºC is weak. Growth on 0.01% cycloheximide is positive, whereas growth on 0.1% cycloheximide is negative. Acid formation on chalk agar is negative. Growth on 50% D-glucose and 10% NaCl / 5% glucose media is positive and growth on 1% acetic acid is negative.

The designated holotype of *Wickerhamiella verensis* sp. nov. is strain CECT 12028^T and the ex-type is CBS 10548^T. The holotype is preserved in a metabolically inactive state (frozen in 15% glycerol at -80ºC and lyophilised) in the CECT collection of the University of Valencia, Spain. It was isolated from subsoil water polluted with heavy lubricating oil in Vera de Bidasoa (Spain). The MycoBank number is MB 833012.

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Conflict of interest

The authors declare that there are no conflicts of interest

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317 **Figure legends**

318 **Figure 1.** Phylogenetic placement of *Wickerhamiella verensis* among species of
319 the *Wickerhamiella* clade based on LSU rRNA gene D1/D2 sequences. The
320 tree was inferred using Maximum Likelihood analysis of 598 aligned positions
321 using the general Time Reversible model and a discrete Gamma distribution to
322 model evolutionary rate differences among sites. Bootstrap values are based on
323 1000 replicates and the outgroup species is *Starmerella bombicola*.
324
325 **Figure 2.** Budding cells of *Wickerhamiella verensis* CECT 12028 grown on malt
326 extract agar for 3 days at 25ºC. Bar represents 5μm.
327
328

Table 1.- Yeasts isolated form collection sites Z1, Z2, and Z4, before (A), during (D) and after (F) the bioremediation process. SN: number of samples taken from each area and YN: number of yeasts isolated in the different areas.

Species	**collection site**	**bioremediation**	**SN**	**YN**	**BLAST**
Candida viswanathii	Z1, Z2, Z4	A, D	6, 2, -2	27, 4, 3	100% U45752
C. parapsilosis	Z1, Z2, Z4	A, D	1, 2, 3	6, 7, 3	100% U45754
Wickerhamiella verensis sp. nov.	Z2	D	1	2	new *W. verensis*
Rhodotorula mucilaginosa	Z1, Z2, Z4	A, D, F	4, 1, 2	5, 1, 1	99% AF070432
Yarrowia lipolytica	Z2, Z4	A, D	1, 2	5, 2	100% AJ508570
Rhodosporidium toruloides	Z1	D	1	2	99% AF444746
Meyerozyma guilliermondii	Z2	D	2	7	100% U45709
Lodderomyces elongisporus	Z1	D	1	3	99% U45763

Z1, fuel oil; Z2, lubricant-oil; Z3, diesel; Z4, mixture of Z1, Z2 and Z3

Table 2.- Hydrocarbon utilization spectrum for different yeast species isolated from the same collection sites.

Yeast species	**Utilization of hydrocarbons [a]**					
	naphthalene	**fluorene**	**tetradecane**	**kerosene**	**diesel**	**octane**
Candida viswanathii	v	+	+	-	v	v
Candida parapsilosis	v	+	+	-	v	v
Lodderomyces elongisporus	-	+	+	-	+	-
Wickerhamiella verensis sp. nov.	-	-	-	-	-	-
Meyerozyma guilliermondii	+	+	+	-	v	-
Rhodosporidium toruloides	+	+	+	-	+	-
Rhodotorula mucilaginosa	+	+	-	-	-	+
Yarrowia lipolytica	v	-	+	-	+	-

[a] Results are presented as follows: +, positive; -, negative; v, variable: some isolates are positive, others negative.

Table 3.- Physiological properties differentiating *W. verensis* sp. nov. CECT 12028^{T} from phylogenetically related species *W. sorbophila* NRRL Y-7921^{T} and *W. infanticola* NRRL Y-17858^{T}.

Assimilation of	**Strains**		
	CECT 12028	**NRRL Y-7921**	**NRRL Y-17858**
inulin	+	-	-
sucrose	+	-	-
ethanol	+	-	-
glycerol	-	+	+
xylitol	-	+	+

Figure 1

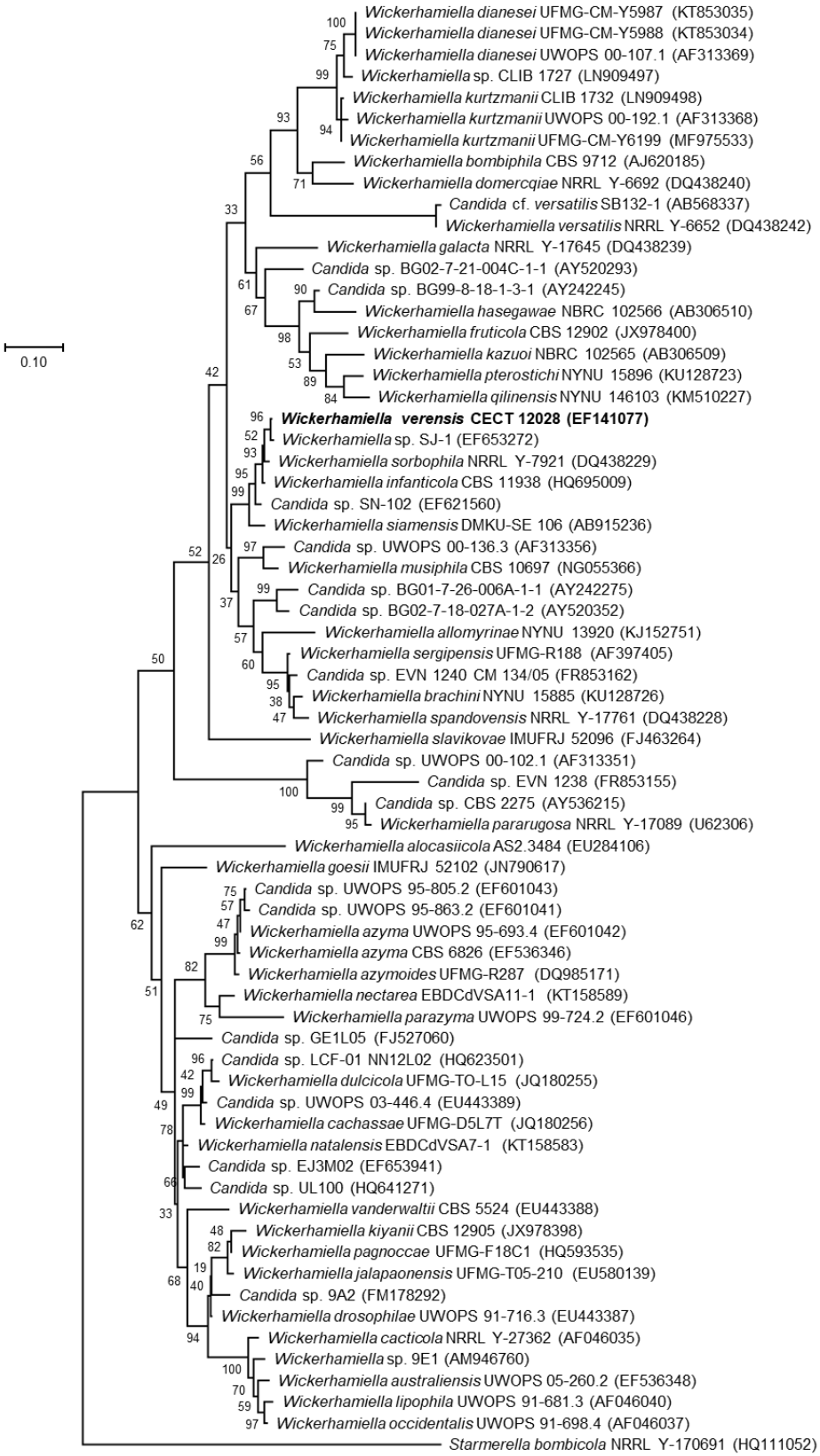
0.10
Wickerhamiella dianesei UFMG-CM-Y5987 (KT853035)
Wickerhamiella dianesei UFMG-CM-Y5988 (KT853034)
Wickerhamiella dianesei UWOPS 00-107.1 (AF313369)
Wickerhamiella sp. CLIB 1727 (LN909497)
Wickerhamiella kurtzmanii CLIB 1732 (LN909498)
Wickerhamiella kurtzmanii UWOPS 00-192.1 (AF313368)
Wickerhamiella kurtzmanii UFMG-CM-Y6199 (MF975533)
Wickerhamiella bombiphila CBS 9712 (AJ620185)
Wickerhamiella domercqiae NRRL Y-6692 (DQ438240)
Candida cf. versatilis SB132-1 (AB568337)
Wickerhamiella versatilis NRRL Y-6652 (DQ438242)
Wickerhamiella galacta NRRL Y-17645 (DQ438239)
Candida sp. BG02-7-21-004C-1-1 (AY520293)
Candida sp. BG99-8-18-1-3-1 (AY242245)
Wickerhamiella hasegawae NBRC 102566 (AB306510)
Wickerhamiella fruticola CBS 12902 (JX978400)
Wickerhamiella kazuoi NBRC 102565 (AB306509)
Wickerhamiella pterostichi NYNU 15896 (KU128723)
Wickerhamiella qilinensis NYNU 146103 (KM510227)
Wickerhamiella verensis CECT 12028 (EF141077)
Wickerhamiella sp. SJ-1 (EF653272)
Wickerhamiella sorbophila NRRL Y-7921 (DQ438229)
Wickerhamiella infanticola CBS 11938 (HQ695009)
Candida sp. SN-102 (EF621560)
Wickerhamiella siamensis DMKU-SE 106 (AB915236)
Candida sp. UWOPS 00-136.3 (AF313356)
Wickerhamiella musiphila CBS 10697 (NG055366)
Candida sp. BG01-7-26-006A-1-1 (AY242275)
Candida sp. BG02-7-18-027A-1-2 (AY520352)
Wickerhamiella allomyrinae NYNU 13920 (KJ152751)
Wickerhamiella sergipensis UFMG-R188 (AF397405)
Candida sp. EVN 1240 CM 134/05 (FR853162)
Wickerhamiella brachini NYNU 15885 (KU128726)
Wickerhamiella spandovensis NRRL Y-17761 (DQ438228)
Wickerhamiella slavikovae IMUFRJ 52096 (FJ463264)
Candida sp. UWOPS 00-102.1 (AF313351)
Candida sp. EVN 1238 (FR853155)
Candida sp. CBS 2275 (AY536215)
Wickerhamiella pararugosa NRRL Y-17089 (U62306)
Wickerhamiella alocasiicola AS2.3484 (EU284106)
Wickerhamiella goesii IMUFRJ 52102 (JN790617)
Candida sp. UWOPS 95-805.2 (EF601043)
Candida sp. UWOPS 95-863.2 (EF601041)
Wickerhamiella azyma UWOPS 95-693.4 (EF601042)
Wickerhamiella azyma CBS 6826 (EF536346)
Wickerhamiella azymoides UFMG-R287 (DQ985171)
Wickerhamiella nectarea EBDCdVSA11-1 (KT158589)
Wickerhamiella parazyma UWOPS 99-724.2 (EF601046)
Candida sp. GE1L05 (FJ527060)
Candida sp. LCF-01 NN12L02 (HQ623501)
Wickerhamiella dulcicola UFMG-TO-L15 (JQ180255)
Candida sp. UWOPS 03-446.4 (EU443389)
Wickerhamiella cachassae UFMG-D5L7T (JQ180256)
Wickerhamiella natalensis EBDCdVSA7-1 (KT158583)
Candida sp. EJ3M02 (EF653941)
Candida sp. UL100 (HQ641271)
Wickerhamiella vanderwaltii CBS 5524 (EU443388)
Wickerhamiella kiyanii CBS 12905 (JX978398)
Wickerhamiella pagnoccae UFMG-F18C1 (HQ593535)
Wickerhamiella jalapaonensis UFMG-T05-210 (EU580139)
Candida sp. 9A2 (FM178292)
Wickerhamiella drosophilae UWOPS 91-716.3 (EU443387)
Wickerhamiella cacticola NRRL Y-27362 (AF046035)
Wickerhamiella sp. 9E1 (AM946760)
Wickerhamiella australiensis UWOPS 05-260.2 (EF536348)
Wickerhamiella lipophila UWOPS 91-681.3 (AF046040)
Wickerhamiella occidentalis UWOPS 91-698.4 (AF046037)
Starmerella bombicola NRRL Y-170691 (HQ111052)

Figure 2