Wickerhamomyces patagonicus sp. nov., an ascomycetous yeast species from Patagonia, Argentina

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Eight strains of a novel yeast species were isolated from tree saps of 'Coihue' (*Nothofagus dombeyi*, Nothofagaceae) and glacial meltwater (Castaño Overo River) in the Nahuel Huapi National Park, Patagonia, Argentina. The sequences of the D1/D2 domains of the large subunit of the rRNA gene showed that this novel yeast species belongs to the *Wickerhamomyces* genus (Order Saccharomycetales, Family Wickerhamomycetaceae). The closest related species were *Candida ponderosae* and *Wickerhamomyces chambardii*. *Wickerhamomyces patagonicus* sp. nov. is proposed to accommodate these novel strains, with the type strain CRUB 1724^T (=CBS 11398^T =JCM 16381^T).

The diversity of ascomycetous yeasts in pristine environments, such as those found in the Nahuel Huapi National Park (NHNP) in north-western Patagonia (Argentina), has not yet been fully explored. This park has a cold to temperate climate, and includes vast areas with little or no human influence. So far, studies on the occurrence of yeasts in aquatic and terrestrial environments in the NHNP have characterized native yeast communities and described several novel species (Brizzio & van Broock, 1998; de García *et al.*, 2007; Libkind *et al.*, 2003, 2005, 2007, 2008, 2009). However, these studies have focused mainly on basidiomycetous yeasts of aquatic environments.

Analysis of the sequences of the D1/D2 domains of the large-subunit rRNA gene of eight yeast isolates, from natural environments, showed that these strains represent a novel yeast species of the genus *Wickerhamomyces*. This novel species was related to *Candida ponderosae* and *Wickerhamomyces chambardii*. In this paper, we describe this novel species as *Wickerhamomyces patagonicus* sp. nov.

Seven strains of *W. patagonicus* sp. nov. were recovered from two different sap exudates on two cut branches of one tree of *Nothofagus dombeyi* in the pathway of the Frias Glacier in the NHNP, February 2007. The sap found on *N.*

dombeyi was bright beige and translucent with some turbidity due to the presence of microbial growth. Exudates were collected in the forest that surrounds Mount Tronador in sterile plastic plates; the samples were serially diluted, stirred with a sterile loop and streaked onto YM agar (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3 % yeast extract, 2 % agar) containing 100 mg chloramphenicol l⁻¹. The YM plates were incubated at room temperature (20 ± 2 °C) for 3 to 8 days. Yeast strains were purified and maintained on potato dextrose agar slants (Yarrow, 1998). An additional yeast isolate of the novel species was recovered from a meltwater river, originating from the Castaño Overo glacier of Mount Tronador, as described by de García *et al.* (2007). This glacial meltwater river runs through the *Nothofagus* forest where the other seven samples were obtained. The strains considered in this study are listed in Table 1.

The isolates were characterized morphologically and physiologically according to Yarrow (1998). Ascospore production was observed after growth on glucose-yeast extract (GY; 0.2 % glucose, 0.1 % yeast extract, 2 % agar) at 18 °C for 3 weeks. For PCR fingerprinting, the microsatellite-primed PCR (MSP-PCR) technique was applied (Libkind *et al.*, 2003). Protocols for DNA extraction, primers, PCR and electrophoresis conditions were as described by Libkind *et al.* (2003). For sequencing of 26S rRNA gene D1/D2 domains, forward primer NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and reverse primer NL4 (5-GGTCCGTGTTTCAAGACGG-3) were employed. The internal transcribed spacer (ITS) region

Abbreviations: ITS, internal transcribed spacer; MSP-PCR, microsatellite-primed PCR; NHNP, Nahuel Huapi National Park.

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA gene D1/D2 domain sequences of strains CRUB 1724^T and CRUB 1219 are FJ666399 and FJ952145, respectively, and for the ITS sequence of strain CRUB 1724^T FJ793131.

Table 1. List of isolates of *Wickerhamomyces patagonicus* sp. nov.

CRUB, Regional University Center of Bariloche (Centro Regional Universitario Bariloche). CBS, Centraalbureau voor Schimmelcultures the Netherlands; JCM, Japan Collection of Micro-organisms.

Source	Location	Strains
Exudates of <i>Nothofagus dombeyi</i> (Coihue, Nothofagaceae)	Pathway to Frias Glacier, Mount Tronador, NHNP, Patagonia, Argentina	CRUB 1724 ^T (=CBS 11398 ^T =JCM 16381 ^T), CRUB 1723, CRUB 1725, CRUB 1726, CRUB 1727, CRUB 1728, CRUB 1729
Meltwater of Castaño Overo River	Castaño Overo Glacier	CRUB 1219

was sequenced using the forward primer ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and the reverse primer ITS4 (5-TCCTCCGCTTATTGATATGC-3). Sequencing was carried out using an ET Dynamic Terminator kit in a MegaBACE 1000 automated 96 capillary DNA sequencer (GE Healthcare). The nucleotide sequences and other related sequences were multiply aligned using the CLUSTAL w software package (EMBL-EBI; <http://www.ebi.ac.uk/clustalw/>). Phylogenetic relationships were estimated by using MEGA version 4.0.2 (Tamura *et al.* 2007); a phylogenetic tree was reconstructed using the neighbour-

joining algorithm, and bootstrap values were calculated from 1000 replicate runs. The Kimura two-parameter model was used to estimate evolutionary distance.

Classification, ecology and species delineation

Results of MSP-PCR fingerprinting characterization for all eight strains showed no differences in DNA banding patterns (data not shown). Sequence analysis of the D1/D2 region of the large-subunit rRNA gene of strains CRUB 1724^T and CRUB 1219 placed these strains in the recently

Fig. 1. Phylogenetic placement of *Wickerhamomyces patagonicus* sp. nov. obtained by neighbour-joining (distance K2P method) of the D1/D2 domains of the large subunit of the rRNA gene. Bar, number of substitutions accumulated every 100 nucleotides. Bootstrap values higher than 50% are shown (1000 replicates).

proposed genus *Wickerhamomyces* (Kurtzman *et al.*, 2008). The novel strains differed by nine nucleotide substitutions (1.5%) from *C. ponderosae*, and by 13 nucleotide substitutions (2.2%) from *W. chambardii* (Fig. 1). ITS sequence analysis showed that strain CRUB 1724^T differed by 14 nucleotide substitutions (2.4%) from *C. ponderosae* and by 37 nucleotide substitutions (6.4%) from *W. chambardii*. The phenotypic profile of this novel species fitted the genus description proposed by Kurtzman *et al.* (2008). Hat-shaped ascospores were produced, and methanol and hexadecane were not assimilated. This evidence allowed the designation of these strains as a novel species in the *Wickerhamomyces* clade, according to criteria proposed by Kurtzman *et al.* (2008). We named this novel species *Wickerhamomyces patagonicus*, with the type strain CRUB 1724^T.

The physiological profiles of *W. patagonicus* sp. nov., *C. ponderosae* and *W. chambardii* were markedly different. *W. patagonicus* sp. nov. showed positive growth on vitamin-free medium while *C. ponderosae* and *W. chambardii* could not grow in this substrate. Separation of *W. patagonicus* sp. nov. from *C. ponderosae* could be based on growth on D-mannitol and citrate, which are positive for *C. ponderosae* and negative for the novel species. *W. chambardii* cannot grow, on D-xylose or lysine while *W. patagonicus* sp. nov. gives positive responses for these physiological tests (Table 2). Single colonies of *W. patagonicus* sp. nov. were

able to produce two to four hat-shaped ascospores when growing on GY agar.

Some studies reporting yeasts associated with trees of the species *Nothofagus* have been reported (Ramirez & González, 1984; Serjeant *et al.*, 2008), but none of the isolates were obtained from exudates. In this paper, we suggest that *W. patagonicus* sp. nov. is associated with the *Nothofagus dombeyi* exudates. Its presence in the glacial meltwater river may be attributed to surface run-off from the surrounding *Nothofagus* forest. Species related to *W. patagonicus* sp. nov. have been isolated from tree-related systems. *C. ponderosae* was obtained from the frass of an unidentified beetle in Ponderosa pine in the USA, and *W. chambardii* from a banana cultivar in Indonesia (Kurtzman, 2001; Nakase *et al.*, 2006), which could suggest that these yeasts are associated with trees, and that probably the insects that visit these substrates are vectors for these species.

Latin diagnosis of *Wickerhamomyces patagonicus* de García, Brizzio, Rosa, Libkind et van Broock sp. nov.

Cultura in agar malti post dies 2 (20 °C) parva, convexa, glabra et candida. In agar farinae Zea mays post dies 14 mycelium nec pseudomycelium non formantur. Species homothallica. Asci inconjugati et stabiles, et habentes 2–4 ascospores petasiformes. Glucosum non fermentantur. Glucosum, D-galactosum (exiguum), D-xylosum, L-rhamnosum, cellobiosum (exiguum), salicinum (exiguum), raffinose (exiguum), amyllum solubile (exiguum), glucitolum (exiguum), glycerolum, xylitolum, acidum lacticum (exiguum), N-acetylglucosaminum (exiguum), acidum succinicum, ethanolum et ethyl acetat assimulantur, at non L-sorbosum, D-glucosaminum, D-ribosum, L-arabinosum, D-arabinosum, maltosum, trehalosum, melibiosum, lactosum, melezitolum, inulinum erythritolum, ribitolum, mannitolum, galactitolum, meso-inositolum, gluconatum, acidum citricum, methanolum, hexadecanum, acetolum et 2-propanolum. Lysinum et natrium nitrosum assimulantur. Ad crescentiam vitaminae externae non necessariae. 25 °C crescit neque 37 °C. In media cum 15 % NaCl, cum 1 % acido acetico aut cum 50 % glucose non crescit. Materia amyloidea iodophila non formantur. Typus CRUB 1724^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 11398^T typus stirps deposita est.

Description of *Wickerhamomyces patagonicus* de García, Brizzio, Rosa, Libkind and van Broock sp. nov.

Wickerhamomyces patagonicus (pa.ta.go'ni.cus. N.L. masc. adj. *patagonicus* referring to Patagonia, the region from where the species was isolated).

Growth on 5 % malt extract agar. After 3 days on 5 % malt extract agar at 20 °C, cells are ovoid, single or in pairs. Budding is multilateral. After 2 days on YM agar at 20 °C,

Table 2. Salient physiological differences between *Wickerhamomyces patagonicus* sp. nov., *Candida ponderosae* and *Wickerhamomyces chambardii*

Species: 1, *Wickerhamomyces patagonicus* sp. nov.; 2, *Candida ponderosae*; 3, *W. chambardii*. –, Negative; +, positive; w, weak; v, variable.

Physiological test	1	2	3
Galactose	w	+	+
Raffinose	w	–	–
Cellobiose	w	+	+
Soluble starch	w	–	–
D-Xylose	+	+	–
L-Rhamnose	+	–	–
N-Acetyl-D-glucosamine	w	–	–
D-Mannitol	–	+	–
D-Glucitol	w	–	–
Salicin	w	+	+
DL-Lactate	w	+	+
Citrate	–	+	v
Propane-1,2-diol	–	–	w, v
Nitrite	w	–	–
Lysine	+	+	–
Vitamin-free medium	+	–	–

colonies are white, smooth and glistening. In Dalmau plates after 2 weeks on cornmeal agar, pseudomycelium or true mycelium is not formed. The species is homothallic. After 5 days on glucose-yeast extract agar, cells give rise to deliquescent asci containing two to four hat-shaped ascospores (Fig. 2). Glucose is not fermented. Assimilates the following carbon compounds: glucose, D-galactose (weakly), D-xylose, L-rhamnose, cellobiose (w), salicin (w), raffinose (w), soluble starch (w), D-glucitol (w), glycerol, xylitol, DL-lactate (w), N-acetyl-D-glucosamine (w), succinate, ethanol and ethylacetate. No growth occurs on L-sorbose, D-glucosamine, D-ribose, L-arabinose, D-arabinose, maltose, α , α -trehalose, melibiose, lactose, melezitose, inulin, meso-erythritol, ribitol, D-mannitol, galactitol, myo-inositol, D-gluconate, citrate, methanol, hexadecane, acetone or 2-propanol. Assimilates the following nitrogen compounds: lysine and nitrite (w). Growth at 37 °C is negative. Growth in vitamin-free medium is positive. Growth on YM agar with 10 % sodium chloride is negative. Growth on 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μ g cycloheximide ml⁻¹ growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative.

W. patagonicus was isolated from exudates of 'Coihue' (*Nothofagus dombeyi*, Nothofagaceae), and a meltwater river of the Castaño Overo glacier in Nahuel Huapi National Park, Patagonia, Argentina. The type strain is CRUB 1724^T (=CBS 11398^T =JCM 16381^T).

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Fig. 2. Phase-contrast micrograph of strain CRUB 1724^T on GY agar after 5 days at 22 °C. (a) Cells and an ascus with hat-shaped ascospores; (b) budding cells and three released hat-shaped ascospores. Bar, 5 μ m.

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