Description of *Holtermanniella* gen. nov., including *Holtermanniella takashimae* sp. nov. and four new combinations, and proposal of the order Holtermanniales to accommodate tremellomycetous yeasts of the *Holtermannia* clade

Michael Wuczkowski,¹ Volkmar Passoth,² Benedetta Turchetti,³ Ann-Christin Andersson,² Matilda Olstorpe,² Arja Laitila,⁴ Bart Theelen,⁵ María van Broock,⁶ Pietro Buzzini,³ Hansjörg Prillinger,¹ Katja Sterflinger,¹ Johan Schnürer,² Teun Boekhout⁵ and Diego Libkind⁶

Correspondence

Diego Libkind

libkind@crub.uncoma.edu.ar

¹University of Natural Resources and Applied Life Sciences, Institute of Applied Microbiology (IAM), Austrian Center of Biological Resources and Applied Mycology (ACBR), Muthgasse 18, 1190 Wien, Austria

²Swedish University of Agricultural Sciences (SLU), Department for Microbiology, Uppsala, Sweden

³Department of Applied Biology & Industrial Yeasts Collection DBVPG, Università di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy

⁴VTT Technical Research Centre of Finland, PO Box 1000, Tietotie 2, Espoo, 02044 VTT, Finland

⁵CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

⁶Lab. Microbiología Aplicada y Biotecnología, INIBIOMA, CONICET-UNComahue, Quintral 1250, (8400), Bariloche, Río Negro, Argentina

The novel genus *Holtermanniella* is proposed here to accommodate four *Cryptococcus* species closely related to *Holtermannia corniformis* that are included in the *Holtermannia* clade (Basidiomycota, Agaricomycotina). Thus, four novel combinations are proposed: *Holtermanniella nyarrowii* comb. nov., *Holtermanniella festucosa* comb. nov., *Holtermanniella mycelialis* comb. nov. and *Holtermanniella wattica* comb. nov. In addition, a novel anamorphic yeast species was studied with 15 isolates obtained from different habitats around the world. Analysis of the sequences of the D1/D2 region of their large subunit rDNA showed that the novel species is placed phylogenetically within the *Holtermannia* clade of the Tremellomycetes (Agaricomycotina, Basidiomycota). PCR fingerprinting and sequencing of ITS1–5.8S–ITS2 showed genetic intraspecific variability among the strains: three groups were formed, which did not correlate with geographical origin or substrate. This novel species, designated the type species of *Holtermanniella* gen. nov., is described as *Holtermanniella takashimae* sp. nov.; the type strain is CBS 11174^T (=HB 982^T =DBVPG 8012^T). The order Holtermanniales ord. nov. is proposed here to include *Holtermannia* (the type genus) and *Holtermanniella*.

The GenBank/EMBL/DDBJ accession numbers for the ITS/large subunit rDNA sequences obtained in the present study are FM246501/FM242574 (strain HB 982^T), FM246503/DQ377667 (VTT C-04546), FM246504/DQ377668 (VTT C-04547), FM882240/DQ000213 (DBVPG 4685), FM246502/EF595760 (CRUB 1154), FM246505 (HB 981; ITS only), FJ473372/FJ473377 (CBS 10506), GU945549/GU945548 (CRUB 1845), GU937752/GU937759 (CBS 7088), GU937753/GU937760 (CBS 7676), GU937754/GU937757 (CBS 7677), GU937755/GU937758 (CBS 7678) and GU937756/GU937761 (CBS 7675).

On the basis of molecular phylogenetic studies, the polyphyletic genus *Cryptococcus* is distributed across the phylum Basidiomycota, subphylum Agaricomycotina (Hibbett *et al.*, 2007), in at least four orders: Filobasidiales, Tremellales, Trichosporonales and Cystofilobasidiales (Scorzetti *et al.*, 2002). Four *Cryptococcus* species, namely *Cryptococcus nyarrowii*, *C. festuosus*, *C. mycelialis* and *C. watticus*, occupy an isolated phylogenetic position within the Tremellomycetes and are related to *Holtermannia* (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). The genus *Holtermannia* comprises

dimorphic fungi with a sexual state characterized by the production of minute basidiocarps, typically erect, coralloid gelatinous, and occurring in dense aggregates (Kobayashi, 1937; Bandoni & Boekhout, 1998). Such characteristics make it impossible to assign the aforementioned *Cryptococcus* species to a teleomorphic genus such as *Holtermannia*. Six *Holtermannia* species are presently known (Kobayashi, 1937), although *Holtermannia corniformis* is the only species for which living cultures (anamorphic yeast phase) and sequence data are available (only for two strains).

Holtermannia corniformis and related *Cryptococcus* species, normally referred as the *Holtermannia* clade, constitute a monophyletic cluster basal to the Filobasidiales (Sampaio, 2004). In this study, we propose the new genus *Holtermanniella* to include the anamorphic *Cryptococcus* species of this clade. In addition, the new order Holtermanniales is proposed to include tremellomycetous yeasts of the *Holtermannia* clade. Finally, we studied 11 isolates that represent a novel species, and the name *Holtermanniella takashimae* sp. nov. is proposed to accommodate these strains. *Holtermanniella takashimae* is the type species of the genus *Holtermanniella* gen. nov.

Strains

Strains HB 981, HB 982^T, HB 1002 and HB 1003 from the Austrian Center of Biological Resources Culture Collection were isolated from litter of an agricultural field near Gr. Enzersdorf, Austria (Wuczkowski *et al.*, 2003a, b), and strains VTT C-04546 and VTT C-04547 (Technical Research Centre of Finland Culture Collection) were

obtained from processed barley samples (Finnish malting barley cultivar 'Saana' from the 2001 crop) after steeping and germination (Laitila *et al.*, 2006). Strains CRUB 1154 and CRUB 1845 from the Centro Regional Universitario Bariloche Culture Collection were isolated from subsurface water from Negra lake (Libkind *et al.*, 2009) and from the phylloplane of *Nothofagus pumilio* at Cerro Otto, respectively; both sites are within the Nahuel Huapi National Park, Patagonia, Argentina. Strain DBVPG 4685 from the Industrial Yeast Collection DBVPG (University of Perugia, Italy) was obtained from ascocarps of *Tuber melanosporum* Vitt., collected in a typical Mediterranean forest in Italy (Buzzini *et al.*, 2005). Strain SLU J596 (=CBS 10506) from the collection of the Department of Microbiology, Swedish University of Agricultural Sciences, was isolated from a 19-day-fermented mixture of cereal grains, collected east of Uppsala (Lyberg *et al.*, 2008; Olstorpe *et al.*, 2008). Four of the isolates included in this work were not obtained by the authors of this paper and data on these strains were kindly provided by the respective investigators. Two of these isolates (SEG-9-3 and SEG-5-6) were obtained by A. Yurkov from grassland soil at Brandenburg in northern Germany, in the Biosphere reserve Schorfheide-Chorin. A third isolate (NFMay08DNXL-Y5 =CBS 11507) was obtained by H. Vishniac from prairie soil at Oklahoma State University, Stillwater, OK, USA. The remaining strain (ZIM 629) was isolated in Slovenia by N. Cadez from the surface of a grape berry of the Rebula cultivar. Finally, a 26S rDNA sequence from strain GY38L01 was included in some analyses. Details of the strains are given in Table 1.

Table 1. Known isolates of *Holtermanniella takashimae* sp. nov.

Culture collections: CRUB, Centro Regional Universitario Bariloche yeast culture collection; CBS, Centraalbureau voor Schimmelcultures; HB, Austrian Center of Biological Resources culture collection (ACBR); VTT, Finland Culture Collection; DBVPG, Industrial Yeast Collection, University of Perugia, Italy; ZIM, Collection of Industrial Microorganisms, Slovenia. Grouping was based on molecular data (PCR fingerprinting) and/or ITS sequencing unless indicated. ND, Not determined.

Strain	Other code(s)	Origin	Group
HB 982 ^T	CBS 11174 ^T , DBVPG 8012 ^T	Plant litter, agricultural field, Austria	A
HB 1002		Plant litter, agricultural field, Austria	A
HB 1003		Plant litter, agricultural field, Austria	A
VTT C-04546	HB 1265	Processed barley, cultivar 'Saana', Finland	A
CBS 11581	SEG-5-6	Grassland soil, Brandenburg, Germany	A
CBS 11507	NFMay08DNXL-Y5	Prairie soil, Stillwater, OK, USA	A*
ZIM 629		Grape berries, Slovenia	A*
VTT C-04547	CBS 11175	Processed barley, cultivar 'Saana', Finland	B
CRUB 1154	HB 1263	Negra lake, Patagonia, Argentina	B
CBS 10506	SLU J596	Fermented cereal feed, Sweden	B
CRUB 1845		<i>Nothofagus pumilio</i> phylloplane, Patagonia, Argentina	B
CBS 11582	SEG-9-3	Grassland soil, Brandenburg, Germany	B
DBVPG 4685	HB 1264	Ascocarps of <i>Tuber melanosporum</i> , Italy	C
HB 981		Plant litter, agricultural field, Austria	C
GY38L01		<i>Rubus</i> sp. leaves, Sinyi, Nantou, Taiwan	ND

*Grouping based only on physiological data provided by H. Vishniac and N. Cadez (personal communication).

Phenotypic characterization of strains was achieved in liquid media employing standard methods (Yarrow, 1998). For comparison purposes, available strains of all known species of the *Holtermannia* clade were studied: *C. nyarrowii* CBS 8804^T and CBS 8805, *C. mycelialis* CBS 7712 and CBS 7743, *C. waticus* CBS 9496^T, *C. festucosus* CBS 10162^T and *Holtermannia corniformis* CBS 6979. These strains were included in selected phenotypic tests and were also included in PCR fingerprinting studies. Five additional available *Holtermannia corniformis* strains (CBS 7675, CBS 7676, CBS 7677, CBS 7678 and CBS 7088) were subjected to sequencing of the D1/D2 and ITS regions.

DNA extraction and sequencing of the D1/D2 region of the 26S rDNA and the ITS region was performed as described by Wuczkowski *et al.* (2003a, b). DNA extraction and rDNA sequencing of CBS 10506 were carried out as described by Passoth *et al.* (2007) and Olstorpe *et al.* (2008). PCR fingerprinting was carried out as described by Hong *et al.* (2005), with minor modifications. The primers PELF (5'-ATATCATCGAAGCCGC) and URPIF (5'-ATCCAAGGTCCGAGACAACC) were used. PCR was performed in 50 µl reactions, using 30.4 µl *Taq* buffer with KCl (Fermentas), 6.0 µl MgCl₂ (25 mM), 1.5 µl dNTPs (5 mM), 4 µl primers (10 pmol µl⁻¹), 0.75 µl BSA (10 mg ml⁻¹) and 0.4 µl *Taq* polymerase (5 U µl⁻¹). PCR was performed using the following parameters: 4 min at 95 °C followed by 35 steps of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C and a final extension for 8 min at 72 °C.

The novel genus *Holtermanniella*

Given the well-established polyphyletic state of the genus *Cryptococcus*, which represents one of the most complex taxonomic difficulties in yeast systematics, we propose the creation of the genus *Holtermanniella* gen. nov. to accommodate the anamorphic *Cryptococcus* species that are included phylogenetically in the *Holtermannia* clade, and the resulting description of four new combinations: *Holtermanniella nyarrowii* comb. nov., *Holtermanniella festucosa* comb. nov., *Holtermanniella mycelialis* comb. nov. and *Holtermanniella waticus* comb. nov. These species, together with *Holtermannia corniformis*, constitute a well-circumscribed and isolated monophyletic cluster within the Tremellomycetes (Fig. 1). Even though sexual structures have not been reported for any of these *Cryptococcus* species, we think that it is justified in this case to erect a separate genus and thus reduce the taxonomic complexity and polyphyletic status of *Cryptococcus*. The proposed taxonomic changes are intended to unify the classification scheme of the mitosporic and meiosporic taxa of dimorphic basidiomycetes, as addressed previously (Sampaio *et al.*, 2003), in order to restrict the anamorphic genera to coherent, phylogenetically related groups of species.

The members of the proposed genus *Holtermanniella* were found repeatedly in temperate to cold regions, and two of the five known species (*Holtermanniella waticus* and *Holtermanniella nyarrowii*) were originally collected from

Antarctica. Accordingly, most *Holtermanniella* species do not grow well above 25 °C. Moreover, three *Holtermanniella* species have been shown to possess significant amounts of polyunsaturated fatty acids (particularly linolenic acid) in comparison with *Holtermannia corniformis* and *Saccharomyces cerevisiae*, both of which lack this lipid (Thomas-Hall, 2004; Guffogg *et al.*, 2004). Such fatty acid compositions are typical of yeasts adapted to cold environments (McMurrough & Rose, 1973; Libkind *et al.*, 2008; Rossi *et al.*, 2009).

Holtermanniella species also differ from *Holtermannia corniformis* (the only cultured member of the genus *Holtermannia*) in the assimilation of *N*-acetylglucosamine and ribose, being positive in the former test and negative in the latter. In addition to these few physiological differences, true mycelium with clamp connections and haustoria develops after mating of compatible yeast cells in the case of *Holtermannia corniformis*. The sexual stage of the species now classified in *Holtermanniella* has not yet been found, but it is worth noting that single cultures of *Holtermanniella mycelialis* have the ability to form a monokaryotic mycelium with pseudoclamp connections and haustoria (Golubev & Golubev, 2003). The presence of haustoria suggests mycoparasitism (Bandoni, 1995), which may explain the difficulties in obtaining teleomorphic stages in the genus *Holtermanniella*.

Latin diagnosis of *Holtermanniella* Libkind, Wuczkowski, Turchetti et Boekhout gen. nov.

Fungi Tremellomycetum (Agaricomycotina, Basidiomycota) asexuales, sequentiis acidi nucleici ribosomalibus *Holtermanniae corniformis* affinis. Praeter *Holtermanniellam mycelialem* hyphas non formant. Coloniae cremae ad lutescentes vel erubescens. Fermentatio nulla. Inositolum et D-glucuronatum assimilantur, materia amyloidea iodophila formatur. Nitratum non assimilatur. Ad 30 °C non vel exigue crescunt. Reactiones Diazonium blue B et ureasi positivae. Species typica: *Holtermanniella takashimae* Wuczkowski, Passoth, Andersson, Turchetti, Prillinger, Boekhout et Libkind.

Description of *Holtermanniella* Libkind, Wuczkowski, Turchetti et Boekhout gen. nov.

Holtermanniella (Hol.ter.man.ni.el'la. N.L. fem. dim. n. *Holtermanniella* named after *Holtermannia*, the name of the related sexual genus).

Asexual fungi of the Tremellomycetes (Agaricomycotina, Basidiomycota), phylogenetically circumscribed from analysis of rDNA sequences and related to *Holtermannia corniformis*. With the exception of *Holtermanniella mycelialis*, mycelium is not produced. Colonies are cream to light yellow. Fermentation is absent. *myo*-Inositol and D-glucuronate are assimilated. Nitrate is not assimilated. Starch-like compounds are produced. At 30 °C, growth is weak. Diazonium blue B and urease reactions are positive. The type species is *Holtermanniella takashimae* Wuczkowski, Passoth, Andersson, Turchetti, Prillinger, Boekhout et Libkind.

Fig. 1. Phylogenetic placement of the genus *Holtermanniella* and the order Holtermanniales using sequence analysis of the D1/D2 region by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was carried out with 1000 replicates (Felsenstein, 1985); only values above 50 % are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Evolutionary distances were computed using Kimura's two-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Accession numbers of D1/D2 sequences available in GenBank are included. Bar, 0.02 substitutions per site.

Latin diagnosis of *Holtermanniella takashimae* Wuczkowski, Passoth, Andersson, Turchetti, Prillinger, Boekhout et Libkind sp. nov.

Fungus. Holtermannia clade (Tremellomycetes, Agaricomycotina). In statu unicellulari cellulae ovatae vel etiam longiores factae, 3–5 (4) × 2–4 (3) µm. Mycelium verum non formatur. Proprietates biochemicae physiologicaeque in Tabula 2 describuntur. Sequentiae acidi nucleici ITS et partium D1/D2 submonadis majoris ribosomalisi typi in

collectione sequentiarum acidi nucleici NCBI (<http://www.ncbi.nlm.nih.gov>) numeris FM246501 et FM242574 depositae sunt. Positio phylogenetica in Figura 1 illustratur. Cultura typica CBS 11174^T in collectione zymotica Centraalbureau voor Schimmelcultures (CBS), Utrecht, Hollandia, et HB 982^T in collectione zymotica Austrian Center of Biological Resources, Austria, et DBVPG 8012^T in collectione zymotica Industrial Yeasts Collection (DBVPG), Perugia, Italia, conservatur.

Table 2. Physiological and biochemical characteristics of strains of *Holtermanniella takashimae*

+, Growth; –, no growth; D, delayed growth; W, weak growth; ND, not determined. All strains were negative for glucose fermentation. All strains were positive for assimilation of D-glucose, D-galactose, D-fructose, D-mannose, D-glucosamine, D-ribose, D-xylose, L-arabinose, L-rhamnose, sucrose, maltose, α,α -trehalose, methyl α -D-glucoside, cellobiose, salicin, arbutin, melibiose, raffinose, melezitose, soluble starch, erythritol, D-glucitol, D-mannitol, galactitol, *myo*-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-glucuronate, succinate, citrate, ethanol, aesculin, turanose, D-arabinitol and *N*-acetyl-D-glucosamine and negative for assimilation of inulin, methanol, methyl α -D-mannoside, glycogen and methyl 3-D-xylopyranoside. All strains were positive for nitrogen assimilation from ethylamine, L-lysine and cadaverine and negative for nitrate, nitrite and imidazole. Positive, though mostly weak, growth of all strains was observed on L-arabinitol, amygdalin and gentiobiose. All strains grew at 25 and 30 °C (very weak) but were negative for growth at 35 °C. All strains were negative for growth in 50 % D-glucose and showed weak growth in 10 % NaCl. Growth in 0.1 % cycloheximide, urea hydrolysis and the Diazonium blue B reaction were positive for all strains.

Characteristic	Group A				Group B			Group C	
	HB 982 ^T	VTT C-04546	HB 1002	HB 1003	CRUB 1154	VTT C-04547	SLU J596	DBVPG 4685	HB 981
Carbon assimilation									
L-Sorbose	–	–	–	–	W	W	–	+	+
D-Arabinose	W	–	W	–	–	+	D	W	W
Lactose	W	W	W	–	+	+	+	+	+
Glycerol	–	–	–	–	–	W	–	W	–
Ribitol	W	W	W	–	–	W	D	W	–
Xylitol	–	W	W	–	–	W	W	W	W
D-Galacturonic acid	–	–	–	–	W	W	–	W	–
DL-Lactate	+	+	+	+	+	+	–	+	+
Succinate	+	W	+	W	+	W	+	W	+
Citrate	+	+	+	+	+	W	+	W	+
Ethanol	+	+	+	+	+	W	+	+	+
Propane-1,2-diol	+	W	W	W	W	W	ND	W	W
Butane-2,3-diol	W	W	W	+	W	W	ND	W	+
Nitrogen assimilation									
Creatine	W	–	–	–	–	–	–	–	–
Creatinine	W	–	–	–	–	–	–	–	–
D-Tryptophan	+	–	–	–	W	W	–	–	–
Growth with:									
1 % Cycloheximide	–	+	W	+	+	–	–	+	+
Vitamin-free medium	+	+	+	+	–	+	+	+	–
Additional tests									
Starch production*	+	+	W	–	–	+	+	W	W
L-Xylose	–	–	W	–	W	W	W	+	+
D-Lyxose	–	–	–	–	–	W	ND	W	–
D-Tagatose	–	–	W	–	–	–	ND	–	–
D-Fucose	–	–	–	–	W	–	ND	–	–
L-Fucose	–	–	–	–	W	–	ND	W	–

*Test performed at pH 4.5.

Description of *Holtermanniella takashimae* Wuczkowski, Passoth, Andersson, Turchetti, Prillinger, Boekhout et Libkind sp. nov.

Holtermanniella takashimae (ta.ka'shi.mae. N.L. gen. sing. fem. n. *takashimae* of Takashima, in honour of the Japanese yeast researcher Masako Takashima, for her valuable contributions to yeast systematics).

Anamorphic. Belongs to the subphylum Agaricomycotina, class Tremellomycetes, *Holtermannia* clade. Yeast cells after 5 days at 15 °C on potato dextrose agar (PDA) are oval to elongate, measuring 3–5 (4) × 2–4 (3) µm (Fig. 2). On PDA, colonies appear convex, circular, pale pink and pasty

in texture. Glucose is not fermented. All the strains of this species tested are anamorphic. Physiological and biochemical properties are depicted in Table 2; phylogenetic placement is presented in Fig. 1.

The type strain has been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, at the ACBR (Austrian Center of Biological Resources and Applied Mycology) and at the Industrial Yeast collection DBVPG (Dipartimento di Biologia Vegetale, Università di Perugia, Italy) as CBS 11174^T, HB 982^T and DBVPG 8012^T, respectively. The substrate of origin of the 15 known strains is shown in Table 1.

Fig. 2. Cells of *Holtermanniella takashimae* HB 982^T after 5 days at 15 °C on PDA, showing budding cells. Bar, 10 µm.

Novel combinations

***Holtermanniella festucosa* (Golubev & Sampaio) Libkind, Wuczkowski, Turchetti & Boekhout comb. nov.**

Basionym: *Cryptococcus festucosus* Golubev & Sampaio (Golubev *et al.*, *Can J Microbiol* 50, 1001–1006, 2004).

***Holtermanniella wattica* (Guffogg, Thomas-Hall, Holloway & Watson) Libkind, Wuczkowski, Turchetti & Boekhout comb. nov.**

Basionym: *Cryptococcus watticus* Guffogg, Thomas-Hall, Holloway & Watson (Guffogg *et al.*, *Int J Syst Evol Microbiol* 54, 275–277, 2004).

***Holtermanniella mycelialis* (Golubev & Golubev) Libkind, Wuczkowski, Turchetti & Boekhout comb. nov.**

Basionym: *Cryptococcus mycelialis* Golubev & Golubev (Golubev & Golubev, *Mikrobiologiya* 72, 822–827, 2003).

***Holtermanniella nyarrowii* (Thomas-Hall & Watson) Libkind, Wuczkowski, Turchetti & Boekhout comb. nov.**

Basionym: *Cryptococcus nyarrowii* Thomas-Hall & Watson (Thomas-Hall & Watson, *Int J Syst Evol Microbiol* 52, 1033–1038, 2002).

The new order Holtermanniales

According to analysis of partial 26S rDNA sequences, *Holtermannia corniformis* and *Holtermanniella* species form

a separate cluster (Scorzetti *et al.*, 2002; Golubev *et al.*, 2004), for which we propose the rank of order and the name Holtermanniales. As shown in Fig. 1, this cluster is related phylogenetically to the Tremellales. On the basis of ITS sequence analysis, Scorzetti *et al.* (2002) showed that the *Holtermannia* clade was closely related to the Tremellales, but they could not obtain a clear separation of the two taxa. However, at that time, sequences of only three species of the group were available. We repeated the analysis including all known species and using multiple sequences, including those obtained here for available cultures of *Holtermannia corniformis*, and the result confirmed the monophyletic condition of the *Holtermannia* clade and its close relationship to the order Tremellales (not shown). The definitive position of the *Holtermannia* clade as a well-separated lineage within the Tremellomycetes was less clear, probably because of difficulties in obtaining reliable alignments with the ITS region.

The relationship of the members of the Holtermanniales to the tremellaceous *Cryptococcus* species is in agreement with the lack of nitrate assimilation of both groups of species, a characteristic that distinguishes them from most filobasidiaceous *Cryptococcus* species. Furthermore, members of the Holtermanniales can be differentiated from members of the Trichosporonales by the tolerance of the latter to high concentrations of selenium (Golubev & Golubev, 2003).

Holtermannia, with type species *Holtermannia pinguis* (Holtermann) Saccardo & Traverso, was created by Saccardo & Traverso (1910) based on *Clavariopsis pinguis* Holtermann (1898), a name that turned out to be already in use. The monograph by Kobayashi (1937) described the genus in detail and also described among others *Holtermannia corniformis*, the species that was used in this study as a molecular reference. We investigated all available living cultures of this species by sequencing both the D1/D2 and ITS regions, and found relatively little variability. No nucleotide polymorphisms were detected in the former (not shown) and a single base mutation was seen in the latter region (Fig. 3).

We propose the order Holtermanniales to accommodate the species phylogenetically circumscribed in the *Holtermannia* clade. This proposal is based mainly on molecular data, given that hardly any specific phenotypic characters could be found for this group. However, it is well known that morphological characters may be poor indicators of fungal evolutionary relationships.

Latin diagnosis of Holtermanniales Libkind, Wuczkowski, Turchetti & Boekhout ord. nov.

Holtermanniales (N.L. nom. pl. fem. n. Holtermanniales referring to *Holtermannia*, type genus of the order; suff. -ales denoting an order).

Basidiomycetorum fungi (Tremellomycetes, Agaromycotina) *asexuales vel sexuales. Coloniae creamae in specieibus in asexuali (unicellulari) statu formantur. Fungi in hyphae*

Fig. 3. rDNA sequence analysis of *Holtermanniella takashimae* strains inferred using the ITS1–5.8S–ITS2 region and the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was carried out with 1000 replicates (Felsenstein, 1985); only values above 50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Evolutionary distances were computed using Kimura's two-parameter method (Kimura, 1980) and are in units of the number of base substitutions per site. A total of 454 positions were included in the final dataset (positions containing gaps and missing data were eliminated). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Groups A, B and C correspond to groups shown in Fig. 4 and Table 1. Bar, 0.02 substitutions per site.

statu, ubi presente, mycelium (cum) fibulis et haustoris frequenter formant. Sexuales species (Holtermannia species) robustas mollesque basidias cum simplicibus vel sermentosis convexionibus generant. Sequentiae acidi nucleici 26S ordinem distinctum decernunt, comprehensum in Tremellomycete et Tremellalis ordini affinem. Inositolum et D-glucuronatum assimilantur neque kalium nitricum. Materia amyloidea iodophila formatur. Genus typicus: Holtermannia Saccardo & Traverso.

Description of Holtermanniales Libkind, Wuczkowski, Turchetti & Boekhout ord. nov.

Basidiomycetous fungi of the Tremellomycetes (Agaricomycotina); asexual or sexual species with free-living yeast phase produce cream-coloured colonies. Filamentous stages, when present, usually consist of mycelium with clamp connections and haustoria. Sexual species (*Holtermannia* species) produce tough-gelatinous basidiocarps with simple to branching clavarioid lobes (Holtermann, 1898; Kobayashi, 1937; Boekhout *et al.*, 1998). Analysis of partial 26S rDNA sequences reveals that the order forms a monophyletic branch within the Tremellomycetes related to the order Tremellales. *myo*-Inositol and D-glucuronate are assimilated, nitrate is not utilized and starch-like compounds are produced. The type genus is *Holtermannia* Saccardo & Traverso (1910).

Phylogenetic placement and salient physiological markers of and ecological comments on the novel species *Holtermanniella takashimae*

Yeast isolates with phenotypic characteristics resembling those of most *Cryptococcus* species (i.e. absence of fermentation, Diazonium blue B and urease reactions positive, *myo*-inositol and D-glucuronate assimilated,

starch-like compounds produced) were isolated from various natural and artificial substrates (see Table 1) from different parts of the world (Austria, Argentina, Finland, Germany, Italy, USA, Slovenia and Sweden). These isolates were found to be conspecific, given their lack of nucleotide substitutions in the D1/D2 domain of the 26S rDNA sequence. The only exception was strain ZIM 629, which showed a single base substitution in this region when compared with the rest of the strains (Fig. 1). From a phylogenetic viewpoint, this group of strains is placed within the *Holtermannia* clade (now *Holtermanniales*) of the Tremellomycetes, with *Holtermanniella festucosa* (eight substitutions) and *Holtermanniella nyarrowii* (11 substitutions) being the closest known yeast species (Fig. 1). Taking into account the level of sequence variability in the D1/D2 region of the currently recognized species in *Holtermanniella*, and that of the closely related species *Holtermannia corniformis* (studied here), we conclude that this group of isolates represents a novel yeast species, *Holtermanniella takashimae* sp. nov. A D1/D2 sequence belonging to an isolate preliminarily identified as *Cryptococcus* sp. strain GY38L01, deposited in GenBank as accession no. FJ527085 (C.-F. Lee and C.-W. Hsieh, unpublished), shares 100% similarity to sequences from *Holtermanniella takashimae*, and the strain should be considered conspecific.

PCR fingerprinting studies on *Holtermanniella takashimae* isolates revealed intraspecific variability. Based on the similarity of DNA profiles, three groups were formed using both RAPD primers (Fig. 4). Four isolates, three from Austria (HB 982^T, HB 1002, HB 1003) and one from Finland (VTT C-04546), showed identical RAPD profiles and were labelled as group A. The second group (group B) included isolates CRUB 1154, VTT C-04547 and CBS 10506, while the third group (C) comprised isolates HB 981 and DBVPG 4685. All other species of the *Holter-*

mannia clade had DNA profiles different from those of the *Holtermanniella takashimae* strains. The two strains of *Holtermanniella nyarrowii* (CBS 8804 and CBS 8805^T) had identical PCR fingerprints, as did both strains of *Holtermanniella mycelialis* (CBS 7712 and CBS 7743).

ITS sequences of representative strains of each group were also polymorphic and correlated well with PCR-fingerprinting clustering (Fig. 3). Strains VTT C-04546 and HB 982^T from group A showed identical ITS sequences, differing by three to four mismatches from strains of groups B and C, which differed from each other in only one nucleotide. The exception was strain DBVPG 4685, which differed from the other member of group C in one substitution, although both strains shared the same polymorphic nucleotide that separated them from group B. The *Holtermanniella takashimae* strains of group A were phylogenetically closer to *Holtermanniella nyarrowii*, showing six nucleotide differences in the ITS region. Crosses among and within groups failed to produce sexual structures, and therefore the species is considered to be anamorphic. A few physiological differences were found between the three groups of *Holtermanniella takashimae* (Table 2); for example, L-sorbose was not assimilated by group A strains while it was assimilated weakly by strains of group B and was positive for group C. In addition, lactose was assimilated by groups B and C but was negative or weakly positive for group A, and L-xylose was only assimilated by group C strains, and was negative or weakly positive for the remaining two groups. No ITS sequences were available for strains CBS 11507 and ZIM 629 and they could not be properly grouped. However, both strains were preliminarily included in group A (see Table 1) given that they were L-sorbose-positive and lactose-negative. Salient physiological differences between *Holtermanniella takashimae* and closely related species of the Holtermanniales are shown in Table 3.

The strains of *Holtermanniella takashimae* were isolated from different kinds of substrates, making it difficult to draw specific conclusions about ecological aspects of this yeast species. The broad distribution suggests that additional isolates of *Holtermanniella takashimae* will probably appear in future studies. During a study of the fungal flora of indoor dust in Finland, a group B isolate of *Holtermanniella takashimae* was detected by culture-independent methods, based on its ITS sequence (GenBank accession no. AM901778; Pitkaranta *et al.*, 2008). Two of the isolates in this study come from Finland, one of them belonging to group B. Two isolates of *Holtermanniella takashimae* were obtained from plant samples from Taiwan (strain GY38L01) and grape berries from Slovenia (strain ZIM 629). Strains CBS 11581 and CBS 11582 were isolated from grassland soil in northern Germany, where a total of seven *Holtermanniella takashimae* isolates were found at two sampling sites separated by 30 km. Isolates from each site showed identical PCR fingerprints, but the fingerprints differed between sites. As shown in Fig. 3, representative isolates of each population showed different ITS sequences, indicating that one population belonged to group A and the other to group B. Based on an extensive yeast biodiversity study being carried out in Germany, the relative abundance of *Holtermanniella takashimae* in soil seems to be low (A. Yurkov, personal communication). Strain CBS 11507 was also collected from prairie soil, but in the USA. In this case, *Holtermanniella takashimae* again represented a very small fraction of the total yeasts recovered (H. Vishniac, personal communication). Soil is probably not the primary habitat of *Holtermanniella takashimae*.

Regarding the Patagonian *Holtermanniella takashimae* isolates, CRUB 1845 was recovered from the phylloplane of the endemic tree *N. pumilio* in Andean Patagonia (Argentina). Based on its ITS sequence, this isolate

Fig. 4. PCR fingerprints of *Holtermanniella takashimae* strains (groups A, B and C) and other species of the Holtermanniales using primer PELF. Reference species: 1, *Holtermanniella nyarrowii*; 2, *Holtermanniella festucosa*; 3, *Holtermanniella mycelialis*; 4, *Holtermanniella wattica*; 5, *Holtermannia corniformis*. Lane M, markers.

Table 3. Salient physiological differences between *Holtermanniella takashimae* and related species of the Holtermanniales

+, Growth; –, no growth; D, delayed growth; w, weak growth; v, variable. Data for *Holtermanniella takashimae* are consensus results from all studied strains. Physiological information for species other than *Holtermanniella takashimae* was obtained from this study or from the respective species descriptions (Thomas-Hall & Watson, 2002; Golubev & Golubev, 2003; Golubev *et al.*, 2004; Guffogg *et al.*, 2004).

Test	<i>Holtermanniella</i> species					<i>Holtermannia corniformis</i>
	<i>H. takashimae</i>	<i>H. nyarrowii</i>	<i>H. wattica</i>	<i>H. festucosa</i>	<i>H. mycelialis</i>	
Assimilation of:						
L-Arabinose	+	+	–	+	+	v
Melibiose	+	–	+	w/–	w	–
D-Gluconate	+	D/W	–	D	+	D
Erythritol	+	–	–	–	–	–
DL-Lactate	+	–	–	w	D	v
D-Glucono-1,5-lactone	+	+	–	+	w	–
Growth at:						
25 °C	+	–	w	D	+	+
30 °C	w	–	–	–	–	–

belonged to group B, as did the other Patagonian strain (Fig. 3). Interestingly, the other Patagonian isolate (CRUB 1154) was isolated from superficial water of a mountain lake surrounded by *N. pumilio* shrubs (Libkind *et al.*, 2009). Given that most of the strains have been isolated from soil or plant-related material, it is probable that the original source of CRUB 1154 was terrestrial and that it entered the lake through run-off. Such a plant-related origin is also in agreement with the ability of some of the strains (VTT C-04546 and VTT C-04547) to produce amylase and xylanase (weak), as well as showing intense degradation of cellulose (Laitila *et al.*, 2006). However, the rest of the strains have not been studied regarding extracellular enzyme production, so it is not known whether these characteristics are typical of *Holtermanniella takashimae*. Studies on strain CBS 10506 revealed relatively high growth rates at low temperatures (4 °C), showing metabolic adaptation to colder habitats (not shown). The above results suggest that *Holtermanniella takashimae* is a broadly distributed organism with a preference for plant-related substrates (probably involved in their biodegradation) in cold to temperate regions in both the Northern and Southern hemispheres.

Acknowledgements

This work was partially funded by the Universidad Nacional del Comahue (Project B143), granted to M. van Broock, and by MIUR (PRIN Project 2008), granted to P. Buzzini and B. Turchetti. The support of the Finnish Malting and Brewing Industry is acknowledged. This work was also supported by the thematic research program MicroDrive (Microbially Derived Energy; <http://microdrive.sl.se>) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS). Special thanks are given to A. Yurkov, H. Vishniac and N. Cadez, who kindly provided valuable data on their respective isolates. M. Muñoz is acknowledged given that he obtained strain CRUB 1845 which, coincidentally, was identified in the lab of Dr Masako Takashima (Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Japan).

References

- Bandoni, R. J. (1995). Dimorphic Heterobasidiomycetes: taxonomy and parasitism. *Stud Mycol* **38**, 13–27.
- Bandoni, R. J. & Boekhout, T. (1998). Tremelloid genera with yeast phases. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 705–717. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Boekhout, T., Bandoni, R. J., Fell, J. W. & Kwon-Chung, K. J. (1998). Discussion of teleomorphic and anamorphic genera of heterobasidiomycetous yeasts. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 609–625. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Buzzini, P., Gasparetti, C., Turchetti, B., Cramarossa, M. R., Vaughan-Martini, A., Martini, A., Pagnoni, U. M. & Forti, L. (2005). Production of volatile organic compounds (VOCs) by yeasts isolated from the ascocarps of black (*Tuber melanosporum* Vitt.) and white (*Tuber magnatum* Pico) truffles. *Arch Microbiol* **184**, 187–193.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Golubev, V. I. & Golubev, N. V. (2003). A new basidiomycetous yeast species, *Cryptococcus mycelialis*, related to *Holtermannia* Saccardo et Traverso. *Mikrobiologiia* **72**, 822–827 (in Russian).
- Golubev, W. I., Sampaio, J. P., Alves, L. & Golubev, N. W. (2004). *Cryptococcus festucosus* sp. nov. a new hymenomycetous yeast in the Holtermannia clade. *Can J Microbiol* **50**, 1001–1006.
- Guffogg, S. P., Thomas-Hall, S., Holloway, P. & Watson, K. (2004). A novel psychrotolerant member of the hymenomycetous yeasts from Antarctica: *Cryptococcus watticus* sp. nov. *Int J Syst Evol Microbiol* **54**, 275–277.
- Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M. & Lücking, R. (2007). A higher-level phylogenetic classification of the Fungi. *Mycol Res* **111**, 509–547.
- Holtermann, C. (1898). In *Mykologische Untersuchungen aus den Tropen*, pp. 85–86. Berlin: Gebr. Borntraeger (in German).

- Hong, S.-B., Go, S.-J., Shin, H.-D., Frisvad, J. C. & Samson, R. A. (2005). Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* **97**, 1316–1329.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kobayashi, Y. (1937). On the genus *Holtermannia* of Tremellaceae. *Sci Rep Tokyo Bunrika Daigaku Sect B* **3**, 75–81.
- Laitila, A., Wilhelmson, A., Kotaviita, E., Olkku, J., Home, S. & Juvonen, R. (2006). Yeasts in an industrial malting ecosystem. *J Ind Microbiol Biotechnol* **33**, 953–966.
- Libkind, D., Arts, M. T. & van Broock, M. (2008). Fatty acid composition of cold-adapted carotenogenic basidiomycetous yeasts. *Rev Argent Microbiol* **40**, 193–197.
- Libkind, D., Moliné, M., Sampaio, J. P. & van Broock, M. (2009). Yeasts from high-altitude lakes: influence of UV radiation. *FEMS Microbiol Ecol* **69**, 353–362.
- Lyberg, K., Olstorpe, M., Passoth, V., Schnürer, J. & Lindberg, J. E. (2008). Biochemical and microbiological properties of a cereal mix fermented with whey, wet wheat distillers' grain or water at different temperatures. *Anim Feed Sci Technol* **144**, 137–148.
- McMurrough, I. & Rose, A. H. (1973). Effects of temperature variation on the fatty acid composition of a psychrophilic *Candida* species. *J Bacteriol* **114**, 451–452.
- Olstorpe, M., Lyberg, K., Lindberg, J. E., Schnürer, J. & Passoth, V. (2008). Population diversity of yeasts and lactic acid bacteria in pig feed fermented with whey, wet wheat distillers' grains, or water at different temperatures. *Appl Environ Microbiol* **74**, 1696–1703.
- Passoth, V., Blomqvist, J. & Schnürer, J. (2007). *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* **73**, 4354–4356.
- Pitkaranta, M., Meklin, T., Hyvarinen, A., Paulin, L., Auvinen, P., Nevalainen, A. & Rintala, H. (2008). Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. *Appl Environ Microbiol* **74**, 233–244.
- Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U. M. & Matteuzzi, D. (2009). Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. *FEMS Microbiol Ecol* **69**, 363–372.
- Saccardo, P. A. & Traverso, J. B. (1910). *Holtermannia* Sacc. et Trav. (*Tremellaceae*). In *Sylloge Fungorum*, vol. XIX, p. 871. Patavii: Typis Seminarii.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sampaio, J. P. (2004). Diversity, phylogeny and classification of basidiomycetous yeasts. In *Frontiers in Basidiomycete Mycology*, pp. 49–80. Edited by R. Agerer, M. Piepenbring & P. Blanz. Eching, Germany: IHW-Verlag.
- Sampaio, J. P., Gadanho, M., Bauer, R. & Weiß, M. (2003). Taxonomic studies in the Microbotryomycetidae: *Leucosporidium golubevii* sp. nov., *Leucosporidiella* gen. nov. and the new orders Leucosporidiales and Sporidiobolales. *Mycol Prog* **2**, 53–68.
- Scorzetti, G., Fell, J. W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEM Yeast Res* **2**, 495–517.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thomas-Hall, S. R. (2004). *Physiological and biochemical characterisation of Antarctic yeast*. PhD thesis, University of New England, Armidale, Australia.
- Thomas-Hall, S. & Watson, K. (2002). *Cryptococcus nyarrowii* sp. nov., a basidiomycetous yeast from Antarctica. *Int J Syst Evol Microbiol* **52**, 1033–1038.
- Wuczkowski, M., Druzhinina, I., Gherbawy, Y., Klug, B., Prillinger, H. & Kubicek, C. P. (2003a). Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. *Microbiol Res* **158**, 125–133.
- Wuczkowski, M., Sterflinger, K., Kraus, G. F., Klug, B. & Prillinger, H. (2003b). Diversity of microfungi and yeasts in soils of the alluvial zone national park along the river Danube downstream of Vienna, Austria ("Nationalpark Donauauen"). *Bodenkultur* **54**, 109–118.
- Yarrow, D. (1998). Methods for the isolation and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.