Polyphasic re-examination of *Yarrowia lipolytica* strains and the description of three novel *Candida* species: *Candida oslonensis* sp. nov., *Candida alimentaria* sp. nov. and *Candida hollandica* sp. nov.

Ann Kristin Knutsen,¹ V. Robert,² G. A. Poot,² Wendy Epping,² Marian Figge,² A. Holst-Jensen,¹ Ida Skaar¹ and Maudy Th. Smith²

¹National Veterinary Institute, Ullevålsveien 68, PO Box 8156 Dep., 0033 Oslo, Norway

²Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Correspondence

V. Robert

robert@cbs.knaw.nl

The type strain of *Yarrowia lipolytica* and 38 strains identified as *Yarrowia lipolytica*, four strains of *Candida deformans*, including the type and two subcultures of the type, two strains of *Candida galli* and six unidentified strains that resembled *Y. lipolytica* were examined by PCR fingerprints using primers M13 and (GAC)5. The same strains, together with four strains of the recently introduced *Candida yakushimensis* nom. inval., were sequenced for the D1/D2 domain of the 26S rRNA gene and parts of the ITS domain and also studied for their physiological properties. Of the strains identified previously as *Y. lipolytica*, CBS 2076 had the same fingerprint as the type of *C. deformans* and strain CBS 4855 was distinct from all other strains. The six strains that resembled *Y. lipolytica* were separated into two groups distinct from any of the other clades. A total of six groups obtained by fingerprint and sequence data were evaluated by performing DNA reassociation reactions. Mating experiments among the 35 strains of *Y. lipolytica sensu stricto* showed that 15 strains represented one mating type and 16 strains represented the opposite mating type, while four strains were self-sporulating. Teleomorph states were not produced by *C. deformans*, *C. galli* or any of the unidentified isolates. However, positive mating reactions were rarely observed in crosses among *C. galli* and some strains of *Y. lipolytica* and *C. deformans*. Consequently, sharing the same mating type system, *C. deformans* and *C. galli* could be considered anamorphs of unnamed *Yarrowia* species. Results from PCR fingerprints, sequencing and mating studies support the grouping of the studied strains into *Y. lipolytica*, *C. galli*, *C. deformans*, *C. yakushimensis* nom. inval. and three novel species in the *Yarrowia* clade: *Candida oslonensis* sp. nov. (type strain CBS 10146^T = NRRL Y-48252^T; Mycobank number MB 510769), *Candida alimentaria* sp. nov. (type strain CBS 10151^T = NRRL Y-48253^T; Mycobank number MB 510770) and *Candida hollandica* sp. nov. (type strain CBS 4855^T = NRRL Y-48254^T; Mycobank number MB 510771).

INTRODUCTION

Physiologically, strains of the ascomycetous yeast species *Yarrowia lipolytica* (Wickerham, Kurtzman & Herman) van der Walt & von Arx have been found to be of

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 and ITS sequences obtained in this study are AM268436–AM268482 (D1/D2), AM279219–AM279270 (ITS) and EF405984 (D1/D2), as detailed in Fig. 2.

Results of DNA–DNA reassociation experiments and details of ITS sequence differences are available as supplementary material with the online version of this paper.

biotechnological importance. Although they assimilate only a few carbon compounds commonly tested in yeast classification (Barnett *et al.*, 2000; Yarrow, 1998), the strains are able to grow on various types of hydrocarbons (Wickerham *et al.*, 1970) and, therefore, are used to produce single-cell proteins from these compounds. Additionally, it has been observed that *Y. lipolytica* can secrete several kinds of metabolites in large quantities, such as organic acids (Franke-Rinker *et al.*, 1983; Furukawa *et al.*, 1982; Kapoor *et al.*, 1982; Matthey, 1992; Oogaki *et al.*, 1983; Tsugawa *et al.*, 1969) and extracellular proteins (Abdelal *et al.*, 1977; Ogrydziak, 1988; Ogrydziak & Scharf, 1982; Yamada & Ogrydziak, 1983). Among the latter, the lipases

are very significant (Fickers *et al.*, 2003; Hadeball, 1991; Kalle *et al.*, 1972; Novotný *et al.*, 1994). The reviews of Barth & Gaillardin (1996, 1997) contain additional references on these topics.

Y. lipolytica is the only known taxon in the teleomorph genus *Yarrowia* and has its anamorph in the genus *Candida* as *Candida lipolytica* (F. C. Harrison) Diddens & Lodder (van der Walt & von Arx, 1980; Kurtzman & Fell, 1998). Analyses of the 18S rRNA gene (Barns *et al.*, 1991) and the 26S rRNA gene (Kurtzman & Robnett, 1995, 1998) have shown that *Y. lipolytica* is taxonomically assigned to the Hemiascomycetes, a class that contains very divergent groups of yeasts, from which the species differs by several properties such as the relatively high G+C content of the nuclear DNA (nDNA) (Kurtzman & Fell, 1998) and the unique genomic organization of the rRNA genes (Fournier *et al.*, 1986). Reports on intraspecific differences in the genetic make-up of *Y. lipolytica* have also been published. Naumova *et al.* (1993) mentioned the presence of chromosomal polymorphism and, studying the triacylglycerol lipase gene family, Bigey *et al.* (2003) found sequence differences in the coding region of the lipase genes in *Y. lipolytica* and *Candida deformans*, the latter, by phenotypic comparison, considered as one of the synonyms of *Y. lipolytica* (Kurtzman & Fell, 1998; Barnett *et al.*, 2000). Therefore, Bigey *et al.* (2003) considered *C. deformans* to be a taxon separate from *Y. lipolytica*, and this view is supported by their finding of 14 nucleotide differences in domains 1 and 2 of the 26S rRNA gene (D1/D2). In view of the taxonomic conclusion of Bigey *et al.* (2003) and the biotechnological importance of *Y. lipolytica* strains, it seemed useful to re-examine the type strains of all 11 basionym species placed in synonymy with *Y. lipolytica* (Kurtzman & Fell, 1998; Barnett *et al.*, 2000) and to re-evaluate the classification of the genus. The methods and criteria applied here were PCR-mediated fingerprint determinations, whole-genome comparisons and sequence analyses of D1/D2 and the ITS (internal transcribed spacer) domains. The ability to produce ascospores was re-examined. Along with strains previously classified as *Y. lipolytica* or *C. deformans*, six strains isolated from spoiled food from different sources in Norway were included that showed similar physiological properties to *Y. lipolytica* but differed in molecular markers. In some parts of this study, strains were added of the species *Candida galli* Péter, Dlačny, Vasdinyei, Tornai-Lehoczki & Deák (Péter *et al.*, 2004) and *Candida yakushimensis*, the latter being introduced by Benno (2004) without a Latin diagnosis and therefore a *nomen invalidum*. These strains were isolated from poultry and termites, respectively, and showed similar physiological properties to *Y. lipolytica* and *C. deformans*.

METHODS

Strains. The strains studied, their origin and original denomination are listed in Table 1.

DNA extraction. For PCR-fingerprinting experiments and sequencing, DNA was extracted from yeast cultures grown on GPYA medium (4 % glucose, 0.5 % peptone, 0.5 % yeast autolysate, 2 % agar) for 1–3 days using the Bio 101 System and Fast DNA kit (Q Biogene). To determine the G+C content and to perform nDNA reassociations, DNA was extracted by hydroxyapatite column chromatography as described previously (Smith *et al.*, 1995) from cells grown in 2 l YM broth (Yarrow, 1998) for 2 days.

PCR-fingerprinting analysis. For PCR-fingerprinting analyses, the core sequence of the bacteriophage M13, 5'-GAGGGTGGCGTTCT-3' (Vassart *et al.*, 1987; Meyer *et al.*, 1997), and primer (GAC)5 (Baleiras Couto *et al.*, 1996) were used as single primers in PCR amplifications. The final reaction mixtures (50 µl) contained 200 µM each dNTP (Bioline), 1.5 mM MgCl₂ and 1 × NH₄ buffer supplied with 2 U BIOTAQ DNA polymerase (Bioline), 0.5 µM primer and 1 µl genomic DNA. The reactions were processed in a GeneAmp PCR System 9700 (Applied Biosystems) with the following program for the M13 primer: 94 °C for 120 s at the start, followed by 40 cycles of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 120 s and a final extension of 72 °C for 7 min. For the (GAC)5 primer, the annealing temperature was raised to 54 °C. The PCR products were separated on 1.5 % agarose gels in 1 × TAE buffer at 30 V for 20 min followed by 80 V for 2 h. Gels were stained with ethidium bromide and photographed in UV light. Group designations were made by visual inspection of the gels.

DNA G+C content and DNA–DNA reassociations. DNA G+C content was determined by the thermal denaturation (*T_m*) method of Marmur & Doty (1962) and levels of DNA–DNA reassociation were determined spectrophotometrically using the method of Seidler & Mandel (1971) as modified by Kurtzman *et al.* (1980). Reassociation experiments were performed twice. Strains used in reassociation experiments are indicated in Table 1.

rRNA gene sequencing and sequence analysis. To amplify the rDNA for sequencing, primers V9G (de Hoog & Gerrits van den Ende, 1998) and LR5 (Vilgalys & Hester, 1990) were used in the following reaction mixture (50 µl): 200 µM each dNTP (Bioline), 10 mM Tris/HCl (pH 9.5), 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, 0.5 U BIOTAQ DNA polymerase (Bioline), 0.5 µM each primer and 1 µl genomic DNA. The PCR was processed in a GeneAmp PCR System 9700 (Applied Biosystems). The following program was used: 94 °C for 5 min at the start followed by 35 cycles of 94 °C for 35 s, 55 °C for 50 s and 72 °C for 2 min and a final extension of 72 °C for 5 min. The specificity of the PCR product was checked on an agarose gel before it was purified by GFX columns (Amersham Pharmacia). To sequence both strands of the D1/D2 domain of the 26S rRNA gene, primers NL-1 and NL-4 (O'Donnell, 1993) were used, whereas primers ITS1 and ITS4 (White *et al.*, 1990) were used for the ITS domain (ITS 1, ITS 2 and the intervening 5.8S rRNA gene). Sequencing was performed using the BigDye terminator chemistry (Applied Biosystems) following the manufacturer's instructions. The final sequence reaction mixture (10 µl) contained 1 µl BigDye terminator ready reaction mix v. 3.1 (Applied Biosystems), 1 µl (5 µM) primer, 3 µl BigDye dilution buffer (Applied Biosystems), 4 µl MQ water (Millipore; product no. Biocel A 10) and 1 µl PCR product. The sequencing reactions were purified using G50 Superfine Sephadex columns (Amersham Pharmacia) and loaded on an ABI 3730 XL Genetic Analyzer (Applied Biosystems). Sequences of D1/D2 and the ITS domains were edited and trimmed using the BioEdit sequence alignment editor, version 7.0.2 (Hall, 1999). Some strains had double peaks at several positions for the ITS sequence, and these haplotype polymorphisms were determined by cloning. The D1/D2 and ITS sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997) and adjusted manually by visual inspection. Phylogenetic analyses were performed using a maximum-likelihood

Table 1. Strains of *Yarrowia lipolytica* and related species used in this study

Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; VI, National Veterinary Institute, Oslo, Norway; ZIM, Culture Collection of Industrial Microorganisms, Ljubljana, Slovenia; JCM, Japan Collection of Microorganisms, Wako, Japan. ND, Not determined/no data available. Standard deviations of G+C content measurements were <1.0 mol%.

Strain	Original species denomination	Origin	Sexual state*	G+C content (mol%)
<i>Yarrowia lipolytica</i>				
CBS 0599†	Type strain of <i>Mycotorula lipolytica</i> F. C. Harrison	Rancid margarine, The Netherlands	mt A	48.0
CBS 2070†	Type strain of <i>Monilia cornealis</i> Nannizzi	Corneal lesion, Italy	mt B	46.7
CBS 2072		Unknown	mt A	ND
CBS 2073†	Type strain of <i>Candida olea</i> van Rij & Verona	Olives, Italy	mt B	47.1
CBS 2074		Olives, Italy	mt A	ND
CBS 2075		Rancid margarine, The Netherlands	mt A	ND
CBS 2078		Soil, The Netherlands	mt A	ND
CBS 2787		Probably man, Germany	mt A	ND
CBS 5570		Lung, Argentina	mt B	ND
CBS 5589		Lung, Argentina	mt A	ND
CBS 5699		Hydrocarbons, France	mt B	ND
CBS 5919†		Unknown	mt B	48.8
CBS 6012		Unknown	mt B	ND
CBS 6114		Unknown	mt B	ND
CBS 6124†	Type strain of <i>Endomycopsis lipolytica</i>	Maize-processing plant, USA	SS	47.5
CBS 6124.1†		CBS 6124	mt A	47.5
CBS 6124.2†		CBS 6124	mt B	48.8
CBS 6125		Maize-processing plant, USA	mt A	ND
CBS 6303†	Type strain of <i>Candida paralipolytica</i> K. Yamada & Ota	Soil	mt A	48.0
CBS 6317		Probably dairy product, USA	mt A	ND
CBS 6331		Paraffin fermentation, UK	mt B	ND
CBS 6614		Wastewater, France	SS	ND
CBS 6659†	Type strain of <i>Candida lipolytica</i> var. <i>thermotolerans</i> Blagodatskaya & Kocková-Kratochvilová	Soil, Russia	mt B	47.7
CBS 6660†	Type strain of <i>Candida pseudolipolytica</i> Blagodatskaya & Kocková-Kratochvilová	Soil, Russia	mt A	48.6
CBS 7033	Type strain of <i>Candida petrophilum</i> Takeda, Tsuzuki & Nakano nom. nud.	Soil	mt B	ND
CBS 7034	Type strain of <i>Torulopsis petrophilum</i> Takeda, Tsuzuki & Nakano nom. nud.	Soil	mt A	ND
CBS 7133		Infected skin of offset-printer operator	mt A	ND
CBS 7311		Inbred mutant	SS	ND
CBS 7312		Inbred mutant	SS	ND
CBS 7326		Contaminated gas-oil fermentation, Germany	mt B	ND
CBS 7504		Soil	mt A	ND
CBS 8108†	Type strain of <i>Candida oleophila</i> Iizuka, Shimizu, Ishii & Nakajima	Kerosene aviation fuel	mt B	46.8
ZIM 39†		Unknown	mt B	46.7
CBS 10143 =VI 03208		Mayonnaise-based salad, Norway	–	ND
CBS 10150 =VI 03225		Mayonnaise-based salad, Norway	mt B	ND
CBS 10144 =VI 03463		Apple juice, Norway	mt B	ND
<i>Candida hollandica</i> sp. nov.				
CBS 4855†	Previously assigned to <i>Y. lipolytica</i> ; reassigned in this study	Back of cow, The Netherlands	–	47.9

Table 1. cont.

Strain	Original species denomination	Origin	Sexual state*	G + C content (mol%)
<i>Candida deformans</i>				
CBS 2076†	Previously assigned to <i>Y. lipolytica</i> ; reassigned in this study	Man, Germany	mt A	48.2
CBS 2071† = JCM 1694	Type strain of <i>Pseudomonilia deformans</i>	Fingernail, Austria	–	46.8
MS 59†	Type strain of <i>Pseudomonilia deformans</i>	Revived subculture of CBS 2071, lyophilized in 1979 at CBS	–	49.2
CBS 10250		Preserved fish, Japan	–	ND
G04-83†		Received from F. Bigey as subculture of CBS 2071	–	46.1
<i>Candida galli</i>				
CBS 9722†	Type strain of <i>Candida galli</i>	Chicken liver, Griffin, GA, USA	mt B	45.1
CBS 9723†		Chicken liver, Griffin, GA, USA	mt B	46.3
<i>Candida hispaniensis</i>				
CBS 9996		Larva of <i>Spondylus buprestoides</i> in a conifer forest in Spain	–	ND
<i>Candida yakushimensis</i> nom. inval.				
CBS 10252 = JCM 12781		Gut of Japanese termite (<i>Hodotermopsis sjoestedti</i>), Japan	–	ND
CBS 10253 = JCM 12780		Gut of Japanese termite (<i>H. sjoestedti</i>), Japan	–	ND
CBS 10254 = JCM 12782		Gut of Japanese termite (<i>H. sjoestedti</i>), Japan	–	ND
CBS 10255 = JCM 12783		Gut of Japanese termite (<i>H. sjoestedti</i>), Japan	–	ND
<i>Candida oslonensis</i> sp. nov.				
CBS 10145 = VI 03313		Yoghurt, Norway	–	45.7
CBS 10146† = VI 03316		Yoghurt, Norway	–	45.9
CBS 10147† = VI 03320		Yoghurt, Norway	–	45.8
CBS 10148† = VI 03329		Yoghurt, Norway	–	ND
<i>Candida alimentaria</i> sp. nov.				
CBS 10149† = VI 03347		Yoghurt, Norway	–	43.5
CBS 10151† = VI 03252		Cured ham, Norway	–	45.2

*SS, Self-sporulating; mt, mating type (designations in accordance with Wickerham *et al.*, 1970); –, no mating observed.

†Strains used in DNA–DNA reassociations.

phylogenetic approach based on PHYML software (Guindon & Gascuel, 2003; Guindon *et al.*, 2005) and also using the Bayesian Markov chain Monte Carlo method based on MrBayes software (Ronquist & Huelsenbeck, 2003) and were based on a concatenated alignment of the ITS and D1/D2 datasets. *Candida hispaniensis* CBS 9994 (Kurtzman, 2005) was used as the outgroup in all the analyses.

Cloning. Primers V9 and LR5 were used in a PCR to amplify the yeast DNA using conditions described previously. PCR products were purified using GFX columns (Amersham Biosciences) and ligation was carried out using the pGEM-T kit (Promega). Electroporation of electrocompetent *Escherichia coli* DH10B cells (Invitrogen) was done according to manufacturer's instructions.

Blue/white screening of transformants was performed by plating on selective Luria–Bertani (LB) agar plates containing 100 µg ampicillin ml^{−1} (Sigma), 40 µg X-Gal ml^{−1} (Promega) and 20 µg IPTG ml^{−1} (Sigma).

Single-colony transformants were transferred individually to vials of 15 µl LB broth containing 100 µg ampicillin ml^{−1}. A direct colony

PCR was performed by transferring 1 µl bacterial suspension from the previous transformant dilution into a PCR containing flanking primers T7 and SP6 (Promega).

The size of the insert was determined by electrophoresis on a 1% agarose gel.

Transformants carrying the pGEM plasmid containing the V9–LR5 insert were cultured in LB broth containing 100 µg ampicillin ml^{−1}. Preparation of plasmid DNA for sequencing purposes was carried out using a plasmid mini prep kit (Marligen) to obtain high-purity plasmid DNA. Of each strain, three transformants containing the V9–LR5 PCR product were sequenced using the procedure described above.

Ascospore formation. Ascospore production was tested by inoculating cultures, grown in 5 ml broth containing glucose (4%), tryptone (1%) and yeast extract (5%) for 1–2 days, on YM agar medium (Yarrow, 1998) alone or mixed with either the tester mating types A and B, CBS 6124.1 and CBS 6124.2, respectively, of *Y. lipolytica* (mating type designation adopted from Wickerham *et al.*,

1970) or with strains of known mating type. Ascospore formation was followed at regular intervals from 3 days up to 4 weeks.

Physiology. Physiological characteristics were determined in microplates (Kurtzman *et al.*, 2003) and processed using the BioloMICS system (Robert, 2003). All tests were replicated. Growth at different temperatures was determined by incubation on GPYA for 48 h.

RESULTS AND DISCUSSION

Molecular analysis

PCR fingerprinting. In order to select strains for rDNA reassociations, PCR fingerprints of all strains except CBS 10250 and strains of the species *C. hispaniensis* and *C. yakushimensis* nom. inval. (Table 1) were determined using the primers M13 and (GAC)5. Six different fingerprint types were obtained with each primer. Results obtained with each of the primers showed identical grouping of the strains. In Fig. 1, the fingerprints and groups obtained with M13 are shown. The majority of strains, including the type and the mating types of *Y. lipolytica* and 10 synonyms of the species, generated similar fingerprints. A group of four strains produced similar patterns that were different from the patterns of the majority of strains. This group is represented by CBS 2071 (the type of *C. deformans*), CBS 2076, labelled *Y. lipolytica*, strain G04-83, received from F. Bigey as a subculture of the type strain CBS 2071, and M59. The latter strain represented a subculture cultivated from the type of *C. deformans* lyophilized in 1979 at the CBS. Identical patterns were observed in the two strains of *C.*

galli. Strain CBS 4855, identified physiologically as *Y. lipolytica*, stood apart. The six unidentified strains showed two different patterns by which they were separated from the other groups of strains. The first group included CBS 10145, CBS 10146, CBS 10147 and CBS 10148; the second group included CBS 10149 and CBS 10151.

Based on the patterns obtained, 14 strains labelled *Y. lipolytica*, among them seven type strains of synonyms, three isolates representing *C. deformans*, two cultures of *C. galli* and five unidentified strains (marked in Table 1), were selected for whole-genome comparisons.

DNA base composition and DNA–DNA reassociation.

The DNA base compositions of the selected strains expressed as mol% G+C contents are presented in Table 1. The G+C content of strains labelled *Y. lipolytica* ranged from 46.7 to 48.8 mol% and that of strains of *C. deformans* ranged from 46.1 to 49.2 mol%. The G+C content of the five selected unidentified strains ranged from 43.5 to 45.9 mol%.

Intra- and interspecific reassociation values among the selected strains are shown in Supplementary Fig. S1 (available in IJSEM Online). The strains were separated into six groups, which coincided with the grouping based on fingerprints. The major group of 12 strains, including the type of *Y. lipolytica* and seven synonyms, showed DNA reassociation ranging from 92 to 100 %, which indicates that the strains of this group are conspecific. High reassociation values ranging from 84 to 100 % were observed among the two *C. deformans* strains and CBS

Fig. 1. PCR fingerprints of strains of *Y. lipolytica sensu lato* generated with primer M13. Lanes M, SmartLadder 200–10 000 bp (MW-170-10; Eurogentec). R, Strains selected for DNA–DNA reassociation experiments.

2076, labelled *Y. lipolytica*, which implies that the latter is indeed conspecific with *C. deformans* as observed from their fingerprints. The five selected unidentified strains could be subdivided into two reassociation groups; one group of three strains, CBS 10146, CBS 10147 and CBS 10148, and a second group of two strains, CBS 10149 and CBS 10151, showing reassociation values of 93–100 and 100 %, respectively. The two strains of *C. galli* showed a mutual reassociation value of 87 %. Strain CBS 4855 remained alone, showing reassociation values of 15–37 % with members of the other five groups.

The mean interspecific reassociation values among the six different reassociation clusters ranged from 15 to 50 %. By these values, the separate status of *C. deformans* from *Y. lipolytica*, as suggested by Bigey *et al.* (2003), is supported. Additionally, three distinct groups separate from *Y. lipolytica*, *C. deformans* and *C. galli* could be recognized.

Sequences and phylogeny. Among the strains of *Y. lipolytica*, sequences of the ITS revealed differences between individual clones generated from the same strain. This confirms that non-orthologous ITS sequences can be present within the same strain of some fungal species (O'Donnell & Cigelnik, 1997; Ko & Jung, 2002). The differences between haplotypes of CBS 6659, CBS 6660, CBS 7133 and CBS 10150 are presented in Supplementary Fig. S2. Additionally, strains CBS 2787, CBS 6114, CBS 6124.1, CBS 6303, CBS 6317 and CBS 6659 had either six or seven T repeats at nucleotide positions 255–261. Preliminary phylogenetic analysis containing all the haplotypes resulted in the same topology as it did when only one haplotype per strain was included. The trimmed alignment of the D1/D2 sequences resulted in 505 characters, whereas the ITS alignment yielded 329 characters. Table 1 presents the accession numbers of the strains included. The Bayesian phylogenetic analysis allowed the separation of the strains into six well-supported clades with 99 or 100 % probability (Fig. 2): I, *Y. lipolytica*; II, *C. yakushimensis* nom. inval.; III, CBS 10145, CBS 10146, CBS 10147 and CBS 10148 (*Candida oslonensis* sp. nov.); IV, *C. galli*; V, *C. deformans*; and VI, CBS 10149 and CBS 10151 (*Candida alimentaria* sp. nov.). Strain CBS 4855 was separated from all the clades. CBS 10149 and CBS 10151 diverged by nine characters in the D1/D2 sequences but only by one character in the ITS sequences. Most yeast species are more conserved in D1/D2 than in the ITS domain, and it has been suggested that strains showing more than 1 % difference or 3 nucleotide differences in this domain are likely to represent different species (Kurtzman & Robnett, 1998; Fell *et al.*, 2000). Others, however, have seen greater differences in D1/D2 between strains of the same species (Lachance *et al.*, 2003). Low sequence divergence in the ITS domain, fingerprinting results and DNA–DNA reassociation data support the hypothesis that the two strains CBS 10149 and CBS 10151 belong to the same species. The Bayesian and maximum-likelihood analyses (data not shown) of both domains support the distinction

between the three recognized species and three additional evolutionary groups that may represent novel species.

Mating studies

Crosses among strains assigned to *Y. lipolytica sensu stricto* by PCR-fingerprint patterns, DNA reassociations and sequence data resulted in the production of ascospores varying from very good (+ + +) to low (+). By applying various crossing combinations, 15 strains could be designated mating type A and 16 strains mating type B: four strains, including the type strain, were self-sporulating and one strain was not tested (Table 1). Intraspecific mating of strains of the *C. deformans* reassociation group with inclusion of CBS 2076 (labelled *Y. lipolytica*) did not result in the development of the teleomorph state in culture, neither did the mating within the species *C. galli* or within the other reassociation groups. However, asci with hat-shaped ascospores were observed in some interspecific crossings. Positive results, although low (+), were observed after mixing the two strains of *C. galli* with *Y. lipolytica* mating type A strains CBS 6124.1 and CBS 6303. Consequently, both isolates of the former species must belong to mating type B. Moderate (+ +) to low (+) ascospore formation was also observed by mixing the two strains of *C. galli* with strain CBS 2076 of the *C. deformans* reassociation group (Fig. 3). Consequently, strain CBS 2076 could be designated mating type A. Ascospores were not produced by the individual cultures.

The occurrence of mating among groups showing low DNA–DNA relatedness has been observed previously in polyphasic studies of the genera *Stephanoascus* (Ueda-Nishimura & Mikata, 2002), *Zygoascus* (Smith *et al.*, 2005) and *Arthroascus* (Naumov *et al.*, 2006). The latter study in particular showed by genetic analysis that recombinants were not produced, despite the occurrence of mating between taxa showing low DNA–DNA relatedness. Consequently, the presence of mating indicates close relatedness and not conspecificity per se. Molecular data reveal the extent of relatedness.

Physiology

The physiological characters of the novel species proposed are presented in Table 2. Reliable identification on the basis of physiological characters seems unlikely because of the few available isolates of each taxon. Therefore, molecular determinations using ITS and D1/D2 rDNA sequences are recommended.

Conclusions

Based on the total evidence from PCR fingerprinting, DNA–DNA reassociation studies, ITS and large-subunit rDNA phylogenies, we conclude that the strains included in the present study represent six distinct, although related, species. Three of these species are novel and are described formally below.

Fig. 2. Bayesian phylogenetic majority-rule consensus tree of the combined D1/D2 and ITS datasets. GenBank accession numbers for D1/D2 and ITS sequences are given in parentheses. Bar, 1 % distance.

Latin diagnosis of *Candida oslonensis* Knutsen, V. Robert & M. Th. Smith sp. nov.

In YM liquido post dies 3 ad 25 °C, cellulae vegetativae ovoidae aut globosae (3.0–6.0 × 3.0–9.0 µm), singulae, binae vel racemis parvis connexae, hyphae absunt. In YM agar post dies 3 ad 25 °C pseudohyphae et hyphae verae formantur. Charactera physiologica in Table 2.

Typus: Holotypus CBS 10146 (NRRL Y-48252) *lyophilus* in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Candida oslonensis* Knutsen, V. Robert & M. Th. Smith sp. nov.

Candida oslonensis (os.lo.nen'sis. N.L. fem. adj. *oslonensis* referring to the origin of the isolates, the city of Oslo in Norway).

Mycobank number MB 510769.

After 3 days at 25 °C in YM broth, cells are ovoid to globose, 3.0–6.0 × 3.0–9.0 µm in size. Vegetative reproduction is by multilateral budding and cells occur singly, in

Fig. 3. Hat-shaped ascospores after mixing of *Candida deformans* CBS 2076 with *Candida galli* CBS 9722 on V8 agar medium after 3 days at 25 °C. Bar, 10 µm.

pairs and in small clusters. No hyphal elements are produced. On Dalmau plate with YM agar, pseudohyphae and true hyphae are produced after 3 days at 25 °C. Physiological characters are presented in Table 2.

Type strain: the holotype CBS 10146^T (=NRRL Y-48252^T), isolated by A.K.K. from yoghurt, Norway, is deposited as a lyophilized preparation in the collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Latin diagnosis of *Candida alimentaria* Knutsen, V. Robert & M. Th. Smith sp. nov.

In YM liquido post dies 3 ad 25 °C, cellulae vegetativae ovoidae aut globosae (3.0–6.0 × 4.0–7.0 µm), singulae, binae vel racemis parvis connexae, hyphae absunt. In YM agaro post dies 3 ad 25 °C pseudohyphae et hyphae verae formantur. Charactera physiologica in Table 2.

Typus: Holotypus CBS 10151 (NRRL Y-48253) lyophilus depositus in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Candida alimentaria* Knutsen, V. Robert & M. Th. Smith sp. nov.

Candida alimentaria (a.li.men.ta'ri.a. L. fem. adj. *alimentaria* relating to food, because the specimens were isolated from different food products).

Mycobank number MB 510770.

After 3 days at 25 °C in YM broth, cells are ovoid to globose, 3.0–6.0 × 4.0–7.0 µm in size. Vegetative reproduction is by multilateral budding and cells occur singly, in pairs and in small clusters. No hyphal elements are

Table 2. Physiological characters of the three novel *Candida* species related to *Y. lipolytica*

Strains tested were CBS 10145, CBS 10146^T, CBS 10147 and CBS 10148 (*C. oslonensis* sp. nov.), CBS 10149 and CBS 10151^T (*C. alimentaria* sp. nov.) and CBS 4855^T (*C. hollandica* sp. nov.). w, Weak reaction; v, variable reaction. All strains tested were positive for growth in the presence of D-glucose, glycerol, D-gluconate, DL-lactate, succinate and galactaric acid as carbon sources, growth on L-lysine and cadaverine as nitrogen sources, growth with cycloheximide (0.1 %) and growth at 27 °C. All strains tested were negative for fermentation of D-glucose, growth on D-glucosamine, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α,α-trehalose, methyl α-D-glucoside, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, inulin, starch, xylitol, L-arabinitol, galactitol, myo-inositol, 5-keto-D-gluconate, D-glucuronate, D-galacturonate, butane 2,3 diol, quinic acid, D-glucarate, D-galactonate, laevulinate, L-tartaric acid, D-tartaric acid, meso-tartaric acid, ethylene glycol and Tweens 40, 60 and 80 as carbon sources, growth on nitrate, nitrite, creatine, creatinine, glucosamine and putrescine as nitrogen sources, growth without vitamins, growth on 1 % acetic acid, in 16 % NaCl and at pH 9.5, starch formation and growth at 35 °C.

Character	<i>C. oslonensis sp. nov.</i>	<i>C. alimentaria sp. nov.</i>	<i>C. hollandica sp. nov.</i>
Assimilation of carbon sources			
D-Galactose	+	w	+
L-Sorbose	+	v	+
D-Ribose	v	+	–
D-Xylose	v	–	–
Erythritol	v	+	+
Ribitol	+	–	w
D-Glucitol	+	–	–
D-Mannitol	–	–	w
D-Glucono-1,5-lactone	–	+	–
Citrate	+	v	+
Propane 1,2 diol	–	–	w
Palatinose	v	v	+
L-Malic acid	v	–	–
Uric acid	–	v	–
Gentiobiose	v	v	w
Assimilation of nitrogen sources			
Ethylamine	+	+	–
Imidazole	–	w	w
D-Tryptophan	–	–	w
D-Proline	–	+	–
Other tests			
Growth in 10 % NaCl	–	–	+
Growth at pH 3	+	v	+
Growth at 30 °C	+	–	+

produced. On Dalmau plate with YM agar, pseudohyphae and true hyphae are produced after 3 days at 25 °C. Physiological characters are presented in Table 2.

Type strain: the holotype CBS 10151^T (=NRRL Y-48253^T), isolated by A.K.K. from ham, Norway, is deposited as a lyophilized preparation in the collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Latin diagnosis of *Candida hollandica* Knutsen, V. Robert & M. Th. Smith sp. nov.

In YM liquido post dies 3 ad 25 °C, cellulae vegetativae ovoidae aut globosae (3.0–5.0 × 5.0–9.0 µm), singulae, binae vel racemis parvis connexae, hyphae absunt. *In* YM agar post dies 3 ad 25 °C pseudohyphae et hyphae verae formantur. Charactera physiologica in Table 2.

Typus: CBS 4855 (NRRL Y-48254) *lyophilus depositus in collectione zymotica* Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Candida hollandica* Knutsen, V. Robert & M. Th. Smith sp. nov.

Candida hollandica [hol.lan'di.ca. N.L. fem. adj. *hollandica* from Holland, referring to the country of origin of the isolate, The Netherlands (commonly referred to in English as Holland)].

Mycobank number MB 510771.

After 3 days at 25 °C in YM broth, cells are ovoid to globose, 3.0–5.0 × 5.0–9.0 µm in size. Vegetative reproduction is by multilateral budding and cells occur singly, in pairs and in small clusters. No hyphal elements are produced. On Dalmau plate with YM agar, pseudohyphae and true hyphae are produced after 3 days at 25 °C. Other physiological characters are presented in Table 2.

Type strain: CBS 4855^T (=NRRL Y-48254^T), isolated from the back of a cow in The Netherlands, is deposited as a lyophilized preparation in the collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

The formal description of *Candida yakushimensis* is left to Y. Benno and co-workers, who introduced this taxon as a provisional name without Latin diagnosis (Benno, 2004).

ACKNOWLEDGEMENTS

Arien van Ieperen and Mieke Starink-Willemsen are thanked for their technical assistance.

REFERENCES

- Abdelal, A. T. H., Kennedy, E. H. & Ahearn, D. G. (1977). Purification of a neutral protease from *Saccharomycopsis lipolytica*. *J Bacteriol* **130**, 1125–1129.
- Baleiras Couto, M. M., Hartog, B. J., Huis in't Veld, J. H. J., Hofstra, H. & van der Vossen, J. M. B. M. (1996). Identification of spoilage yeasts in a food-production chain by microsatellite polymerase chain reaction fingerprinting. *Food Microbiol* **13**, 59–67.

- Barnett, J. A., Payne, R. W. & Yarrow, D. (2000). *Yeasts: Characteristics and Identification*, 3rd edn. Cambridge: Cambridge University Press.
- Barns, S. M., Lane, D. J., Sogin, M. L., Bibeau, C. & Weisburg, W. G. (1991). Evolutionary relationships among pathogenic *Candida* species and relatives. *J Bacteriol* **173**, 2250–2255.
- Barth, G. & Gaillardin, C. (1996). *Yarrowia lipolytica*. In *Nonconventional Yeasts in Biotechnology: a Handbook*, pp. 313–388. Edited by K. Wolf. Berlin & London: Springer.
- Barth, G. & Gaillardin, C. (1997). Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol Rev* **19**, 219–237.
- Benno, Y. (2004). Microbe division (RIKEN BRC JCM). In *Annual Report of the Riken Biological Research Center*, pp. 905–914. Wako, Japan: RIKEN (in Japanese with English translation).
- Bigey, F., Tuery, K., Bougard, D., Nicaud, J. & Moulin, G. (2003). Identification of a triacylglycerol lipase gene family in *Candida deformans*: molecular cloning and functional expression. *Yeast* **20**, 233–248.
- de Hoog, G. S. & Gerrits van den Ende, A. H. (1998). Molecular diagnostics of clinical strains of filamentous Basidiomycetes. *Mycoses* **41**, 183–189.
- 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.
- Fickers, P., Nicaud, J. M., Destain, J. & Thonart, P. (2003). Overproduction of lipase by *Yarrowia lipolytica* mutants. *Appl Microbiol Biotechnol* **63**, 136–142.
- Fournier, P., Gaillardin, C., Persuy, M.-C., Klootwijk, J. & van Heerikhuizen, H. (1986). Heterogeneity in the ribosomal family of the yeast *Yarrowia lipolytica*: genomic organization and segregation. *Gene* **42**, 273–282.
- Franke-Rinker, D., Behrens, U., Nockel, E., Forner, C. & Portnowa, A. (1983). Joint utilization of glucose and n-alkanes in citric acid synthesis by *Saccharomycopsis lipolytica*. *Z Allg Mikrobiol* **23**, 9–16.
- Furukawa, T., Ogina, T. & Matsuyoshi, T. (1982). Fermentative production of citric acid from n-paraffins by *Saccharomycopsis lipolytica*. *J Ferment Technol* **60**, 281–286.
- Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696–704.
- Guindon, S., Lethiec, F., Duroux, P. & Gascuel, O. (2005). PHYML Online – a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res* **33**, W557–W559.
- Hadeball, W. (1991). Production of lipase by *Yarrowia lipolytica*. *Acta Biotechnol* **11**, 159–167.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Kalle, G. P., Gadkari, S. V. & Deshpande, S. Y. (1972). Inducibility of lipase in *Candida lipolytica*. *Indian J Biochem Biophys* **9**, 171–175.
- Kapoor, K. K., Chaudhary, K. & Tauro, P. (1982). Citric acid. In *Prescott & Dunn's Industrial Microbiology*, 4th edn, pp. 709–747. Edited by G. Reed. Westport, CT: AVI Publishing.
- Ko, K. S. & Jung, H. S. (2002). Three nonorthologous ITS1 types are present in a polypore fungus *Trichaptum abietinum*. *Mol Phylogenet Evol* **23**, 112–122.
- Kurtzman, C. P. (2005). New species and a new combination in the *Hyphopichia* and *Yarrowia* yeast clades. *Antonie van Leeuwenhoek* **88**, 121–130.

- Kurtzman, C. P. & Fell, J. W. (editors) (1998). *The Yeasts: a Taxonomic Study*, 4th edn. Amsterdam: Elsevier.
- Kurtzman, C. P. & Robnett, C. J. (1995). Molecular relationships among hyphal ascomycetous yeasts and yeastlike taxa. *Can J Bot* **73**, S824–S830.
- Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.
- Kurtzman, C. P., Smiley, M. J., Johnson, C. J. & Wickerham, L. J. (1980). Two closely related heterothallic species, *Pichia amylophila* and *Pichia mississippiensis*: characterization by hybridization and deoxyribonucleic acid reassociation. *Int J Syst Bacteriol* **30**, 206–216.
- Kurtzman, C. P., Boekhout, T., Robert, V., Fell, J. W., Yarrow, D. & Deak, T. (2003). Methods to identify yeasts. In *Yeasts in Food*, pp. 69–122. Edited by T. Boekhout & V. Robert. Hamburg: Behr's Verlag.
- Lachance, M.-A., Daniel, H. M., Meyer, W., Prasad, G. S., Gautam, S. P. & Boundy-Mills, K. (2003). The D1/D2 domain of the large-subunit rDNA of the yeast species *Clavispora lusitanae* is unusually polymorphic. *FEMS Yeast Res* **4**, 253–258.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of DNA from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Mattey, M. (1992). The production of organic acids. *Crit Rev Biotechnol* **12**, 87–132.
- Meyer, W., Latouche, G. N., Thanos, M., Mitchell, T. G., Yarrow, D., Schönian, G. & Sorrell, T. C. (1997). Identification of pathogenic yeasts of the imperfect genus *Candida* by polymerase chain reaction fingerprinting. *Electrophoresis* **18**, 1548–1559.
- Naumov, G. I., Naumova, E. S., Smith, M. Th & de Hoog, G. S. (2006). Molecular-genetic diversity of the ascomycetous yeast genus *Arthroascus*: *Arthroascus babjevae* sp. nov., *Arthroascus fermentans* var. *arxii* var. nov. and geographical populations of *Arthroascus schoenii*. *Int J Syst Evol Microbiol* **56**, 1997–2007.
- Naumova, E., Naumov, G., Fournier, P., Nguyen, H.-V. & Gaillardin, C. (1993). Chromosomal polymorphism of the yeast *Yarrowia lipolytica* and related species: electrophoretic karyotyping and hybridization with cloned genes. *Curr Genet* **23**, 450–454.
- Novotný, C., Doležalová, L. & Lieblová, J. (1994). Dimorphic growth and lipase production in lipolytic yeasts. *Folia Microbiol (Praha)* **39**, 71–73.
- O'Donnell, K. (1993). *Fusarium* and its near relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*, pp. 225–233. Wallingford, UK: CAB International.
- O'Donnell, K. & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* **7**, 103–116.
- Ogrydziak, D. M. (1988). Production of alkaline extracellular protease by *Yarrowia lipolytica*. *Crit Rev Biotechnol* **8**, 177–187.
- Ogrydziak, D. M. & Scharf, S. J. (1982). Alkaline extracellular protease produced by *Saccharomycopsis lipolytica* CX161-1B. *J Gen Microbiol* **128**, 1225–1234.
- Oogaki, M., Nakahara, T., Uchiyama, H. & Tabuchi, T. (1983). Extracellular production of D-(+)-2-hydroxyglutaric acid by *Yarrowia lipolytica* from glucose under aerobic, thiamine-deficient conditions. *Agric Biol Chem* **47**, 2619–2624.
- Péter, G., Dlačny, D., Vasdinyei, R., Tornai-Lehockzi, J. & Deak, T. (2004). *Candida galli* sp. nov., a new yeast from poultry. *Antonie van Leeuwenhoek* **86**, 105–110.
- Robert, V. (2003). Data processing. In *Yeasts in Food*, pp. 139–170. Edited by T. Boekhout & V. Robert. Hamburg: Behr's Verlag.
- Ronquist, F. & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.
- Seidler, R. J. & Mandel, M. (1971). Quantitative aspects of deoxyribonucleic acid renaturation: base composition, site of chromosome replication, and polynucleotide homologies. *J Bacteriol* **106**, 608–614.
- Smith, M. T., de Cock, A. W. A. M., Poot, G. A. & Steensma, H. Y. (1995). Genome comparisons in the yeastlike fungal genus *Galactomyces* Redhead et Malloch. *Int J Syst Bacteriol* **45**, 826–831.
- Smith, M. Th., Robert, V., Poot, G. A., Epping, W. & de Cock, A. W. A. M. (2005). Taxonomy and phylogeny of the ascomycetous yeast genus *Zygoascus*, with proposal of *Zygoascus meyeriae* sp. nov. and related anamorphic varieties. *Int J Syst Evol Microbiol* **55**, 1353–1363.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tsugawa, R., Nakase, T., Koyabashi, T., Yamashita, K. & Okumura, S. (1969). Fermentation of n-paraffins by yeast. Part III. α -Ketoglutarate productivity of various yeasts. *Agric Biol Chem* **33**, 929–938.
- Ueda-Nishimura, K. & Mikata, K. (2002). Species distinction of the ascomycetous heterothallic yeast-like fungus *Stephanoascus ciferrii* complex: description of *Candida allociferrii* sp. nov. and reinstatement of *Candida mucifera* Kocková-Kratochvílová et Sláviková. *Int J Syst Evol Microbiol* **52**, 463–471.
- van der Walt, J. P. & von Arx, J. A. (1980). The yeast genus *Yarrowia* gen. nov. *Antonie van Leeuwenhoek* **46**, 517–521.
- Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarré, A. S. & Christophe, D. (1987). A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. *Science* **235**, 683–684.
- Vilgalys, R. & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* **172**, 4238–4246.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Edited by N. Innis, D. Gelfand, J. Sninsky & T. White. New York: Academic Press.
- Wickerham, L. J., Kurtzman, C. P. & Herman, A. I. (1970). Sexuality in *Candida lipolytica*. In *Recent Trends in Yeast Research (Spectrum, vol. 1)*, pp. 31–92. Edited by D. G. Ahearn. Atlanta: Georgia State University.
- Yamada, T. & Ogrydziak, D. M. (1983). Extracellular acid proteases produced by *Saccharomycopsis lipolytica*. *J Bacteriol* **154**, 23–31.
- Yarrow, D. (1998). Methods for the isolation, maintenance 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.