CHAPTER 2

Materials and Methods

Materials

Chemicals

All chemicals were analytical grade or higher. A list of specific chemicals and their respective suppliers are listed below:

Amersham-Pharmacia (Baulkham Hills, Sydney, NSW):

Cellophane sheets, Enhanced chemiluminescence (ECL™) western blotting detection kit, Ficoll-Paque® Plus, nitrocellulose membrane (Hybond-C super), Hyperfilm-MP film

Merck-BDH (Kilsyth, Melbourne, Victoria):

Acetic acid, acrylamide, ammonium peroxydisulphate (AMPS), boron-trifluoride in methanol, bromophenol-blue, β-mercaptoethanol, congo red, disodium hydrogen orthophosphate, ethanol, ethidium bromide, glycerol, gum arabic, hydrochloric acid (HCl), isopropanol, magnesium chloride, methanol, potassium chloride, potassium dihydrogen orthophosphate, sodium chloride, sodium hydroxide, N,N,N',N'-Tetramethylenediamine (TEMED), trichloroacetic acid (TCA)

Bio-Rad (Regents Park, Sydney, NSW):

Cellophane membrane backing, filter backing paper, kaleidoscope prestained standards, Quantum Prep. AquaPure Genomic DNA Isolation Kit, SDS-PAGE molecular weight standards (low range), silver stain kit

Boehringer Mannheim (Castle Hill, Sydney, NSW):

Chloramphenicol, phenylmethylsulphonylfluoride (PMSF)

Total Antioxidant Status Assay kit

CSL Biosciences (Parkville, Melbourne, Victoria):

Foetal bovine serum

CSR (Pyrmont, Sydney, NSW):

Absolute ethyl alcohol (99.8%), methyl alcohol

Difco Laboratories (Bioscientific, Gymea, NSW):

Yeast nitrogen base without amino acids, yeast carbon base, vitamin-free yeast base, malt agar, cornmeal agar.

Diploma (Melbourne, Victoria):

Instant skim milk powder

Fisher Biotech (Perth, WA):

Fast Blue protein stain

Gibco BRL (Melbourne, Victoria):

RPMI methionine-deficient medium

Gradipore Ltd. (Frenchs Forest, Sydney, NSW):

10% Pre-cast polyacrylamide electrophoresis gels (iGels)

Greiner Bio-One GmbH (Kremsmuenster, Austria):

9 ml Lithium Heparin Vacuette® blood collection tubes

Millipore (Waters Associates, Lane Cove, Sydney, NSW):

Isopropyl alcohol, hexane

ICN Biomedicals (Seven Hills, Sydney, NSW):

Acrylamide (electrophoresis grade), ammonium persulphate (AMPS), arbutin, β-nicotinamide adenine dinucleotide phosphate reduced (NAD(P)H), 2,6-dichloroindophenol (DCPIP), 2,4'-dinitrophenyl hydrazine (DNPH), dithiothreitol (DTT), ethyl acetate, glycine, isopropyl alcohol, hexane, N,N'-methylenebisacrylamide, sodium dodecyl sulphate (SDS), sucrose,

ICN Radiochemicals (Seven Hills, Sydney, NSW):

Trans ^{35}S-Label™ ([^{35}S] labelling reagent, containing 70% L-Methionine, [^{35}S], and 15% L-Cysteine [^{35}S])

Integrated Sciences (Willoughby, Sydney, NSW):

Kodak Biomax MR film, Kodak GBX liquid X-ray developer and replenisher, Kodak GBX liquid X-ray fixer and replenisher

Oxoid Ltd (West Heidelberg, Melbourne, VIC):

Agar No. 1, bacteriological peptone, yeast extract, meat extract (lab lemco), tryptone

Pierce (Laboratory Supply, Marrickville, Sydney NSW):

Coomassie G-250 protein assay kit

Sigma-Aldrich Incorporated (Castle Hill, Sydney, NSW):

α-methyl-D-glucoside, ampicillin, carboxymethyl cellulose, cellobiose, citrate, D-arabinose, D-glucosamine, D-glucitol, D-gluconate, D-glucoronate, DL-lactate, D-mannitol, D-ribose, D-xylose, erythritol, fatty acid standards (189-1, 189-2, 189-3), galactose, galactitol, glass beads (0.5 mm), hexadecane, L-arabinose, L-sorbose, L-rhamnose, maltose, melibiose, melezitose, *N*-acetyl-D-glucosamine, nitrate, olive oil, ribose, raffinose, soluble starch, salicin, succinate, trehalose, urease, ampicillin, ethylenediamine tetraacetic acid (EDTA), trizma base (Tris), fast blue B salt, glutathione (reduced), ployoxyethylene-sorbitan monolaurate (Tween-20), Triton X-100

StressGen Biotechnologies Corporation (Victoria, British Columbia, Canada):

StressXpress^{TM} Hsp70 ELISA kit (EKS-700), mouse monoclonal anti-human hsp70 antibody (SPA-810)

Wako Pure Chemical Industries (Osaka, Japan):

2,2'-Azobis-(2-amidinopropane) dihydrochloride (AAPH)

Media

General

All solutions and media were prepared using water treated with the Millipore Milli-Q filtration system (Millipore, Lane Cove, NSW). Solutions and media were autoclaved at 121°C for 15 min as required. All media were made up to 1 L unless otherwise stated.

Yeast Extract Peptone (YEP): 5 g bacteriological peptone, 2% w/v glucose, 3 g KH_2PO_4 (potassium dihydrogen orthophosphate), 3 g $[NH_4]_2SO_4$ (ammonium sulphate), 5 g yeast extract. Agar plates were prepared from YEP with the addition of 1.5% w/v agar to the media before autoclaving.

Yeast Nitrogen Base (YNB): 0.67% yeast nitrogen base, 0.3% KH_2PO_4 and 2% glucose, all w/v.

Malt extract and corn meal agar plates were used to encourage sporulation and sexual reproduction of the yeast strains and were made according to manufacturer's instructions.

Cellulase plates: 1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.4% CMC, 4% congo red, all w/v.

Protease plates: positive controls = 1% yeast nitrogen base, 2% glucose, 0.3% ammonium sulphate, 1% skim milk, 2% agar, all w/v; remove either the carbon or nitrogen source for assay.

Lipase plates: 1% tryptone, 0.3% yeast extract, 0.5% meat extract, 0.5% NaCl, 0.7% KH_2PO_4, 1.5% agar, all w/v, 50 ml olive oil emulsion [100 ml olive oil, 100 g gum Arabic].

Glycerol stocks

Stocks of yeast strains were stored in 15% v/v glycerol at –80°C.

Solutions

Protein lysis buffer: 0.1% v/v TritonX-100, 100 mM KCl, 8 mM $MgCl_2$, 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM PMSF.

3 X SDS loading buffer: 150 mM Tris-HCl pH 6.8, 300 mM DTT, 6% w/v SDS. 0.3% w/v bromophenol blue, 30% v/v glycerol.

SDS-PAGE Running buffer: 6.0 g Tris base, 37.6 g glycine, 2.0 g SDS. Made up to 2 L with ddH_2O.

4% Stacking gel: 1.3 ml 30% w/v acrylamide 0.8% w/v bisacrylamide, 1.25 ml Tris-HCl pH 6.8, 7.24 ml ddH_2O, 100 µl 10% SDS, 10 µl TEMED, 100 µl 10% w/v AMPS.

10% Stacking gel: 13.32 ml 30% w/v acrylamide 0.8% w/v bisacrylamide, 10 ml Tris-HCl pH 8.8, 16.1 ml ddH_2O, 400 µl 10% SDS, 40 µl TEMED, 140 µl 10% w/v AMPS.

Protein gel fixative: 45% v/v methanol, 10% v/v acetic acid.

Phosphate buffer (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4.

Continuous transfer buffer: 39 mM glycine, 48 mM Tris, 20% (v/v) methanol, 0.0375% (w/v) SDS.

PBS-T: 100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4, 0.1% (v/v) Tween-20 incorporating 5% (w/v) skim milk powder.

TCA-TBA-HCl: 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid in 0.25 M HCl.

Methods

Yeast maintenance

Antarctic yeast were isolated from soil and related material as detailed by Thomas-Hall and Watson (2002). Yeast strains were maintained on YEP agar plates containing 2% w/v glucose 5 g bacteriological peptone, 5 g yeast extract, 3 g KH_2PO_4, 3 g $(NH_4)_2SO_4$. These plates were stored at 6°C and were re-plated every 2-3 months. For long-term storage, cultures were inoculated into 15% glycerol stocks and stored at –80°C. All cultures were maintained at the University of New England, Armidale as part of the Antarctic yeast culture collection.

Yeast cultures

Starter cultures (20 ml) were used to inoculate (1 ml inoculum) 100 ml of YEP media. Cultures for analyses were aerobically grown at 15°C on an orbital shaker [Paton Industries, Victor Harbour, South Australia] at 180 o.p.m. Cells were generally grown to stationary phase defined as glucose exhaustion, as tested by glucose indicator strips (Diabur-Test 500, Boehringer Mannheim). Stationary phase was reached in about 5 days at 15°C.

Protein Analysis

Protein extraction

The method described by McAlister *et al.,* (1979) was employed for protein extraction with a few modifications. Yeasts were inoculated into 100 ml of YEP in 250 ml conical flasks and incubated on an orbital shaker (180 o.p.m.) at 15°C until stationary phase was reached (~5 days). Cells were centrifuged for 2 min at 13,000 x *g* in a swinging bucket rotor and the supernatant discarded. The pellet was then vortexed and transferred to a microfuge tube and the samples were again centrifuged and washed with H_2O (2 times), discarding the supernatant each time. Cells were then lysed by vortexing for 30 sec in the presence of an equal volume of 0.5 mm acid-washed glass beads and 120 µl of protein lysis buffer (solutions, p.56). The mixture was then put on ice and vortexing and cooling was repeated 6 more times. Samples were then centrifuged at 900 x *g* for 4 min to pellet cell debris and the remaining supernatant

100 dilution) for determination of the protein concentration. To the remaining supernatant, an equal volume of sodium dodecyl sulphate (SDS) sample buffer was added plus 15 µl of 0.1% w/v bromophenol blue loading dye. The samples were then placed in boiling water for 3 min to ensure protein denaturation. Samples were then stored at –20°C until required for SDS-polyacrylamide gel electrophoresis.

Protein assay

The Pierce Coomassie G-250 protein assay kit was employed to determine the protein concentration of the samples. Based on the Bradford method (Bradford, 1976), the blue dye binds to protein thus initiating a colour change from red-brown to blue, the intensity of the blue is directly proportional to the concentration of protein in the sample. One ml of Coomassie protein assay reagent was added to 1 ml of yeast protein sample (1:100 dilution from the protein extracts). The absorbancies of both samples and bovine serum albumin (BSA) standards were measured against a reagent blank using a spectrophotometer at 595 nm. Protein content was calculated by interpolation from the constructed BSA standard curve.

^{35}S-methionine labelling of proteins in yeast

For yeast cells, 40 ml of YEP culture was centrifuged at 3000 x *g* for 5 min. The cells were washed 1 X in ddH_2O and resuspended in 2 ml of YNB (0.67% yeast nitrogen base, 0.3% KH_2PO_4 and 2% glucose) medium without amino acids. 100 µCi/ml of ^{35}S-methionine was added to control, heat and UVA shocked samples.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels were used to separate proteins, essentially as outlined by Lammeli (1970) and Lammeli and Favre (1973). A two-gel system was employed where a stacking gel (on top) of 4% acrylamide was used to condense the proteins as they approached the separating gel. The differences in pH from the stacking gel and the buffer result in a localized increase in the electric field, which causes an ion deficiency. Due to this deficiency, the proteins rapidly migrate through the gel until they reach the separating gel at which point the deficiency no longer exists. The proteins slow down at this point due to the filtration effects and separation takes place. Polyacrylamide separating gels (7, 10 or 12% w/v) were

used and electrophoresis was run on a Protean™ II Slab Cell (Bio-Rad) essentially as described in the instruction manual.

Samples were loaded with an adequate volume to give a concentration of 10 μg of protein along with pre-stained molecular weight markers (Bio-Rad). The advantage in using a two-gel system is the compactness of the proteins as they enter the separating gel resulting in greater resolution of the bands. Gels were run at 10°C at 25-30 mA and were terminated when the bromophenol blue dye ran off the gel, generally 5-6 hr in duration.

SDS-polyacrylamide gel silver staining

Gels were fixed using protein gel fixative 1 followed by fixative 2. Staining was then achieved using a silver stain kit from Bio-Rad that utilizes the principles of the binding of reduced silver ions to amino acid side chains. The procedure is described in full in the instruction manual. Staining was terminated using 5% v/v acetic acid.

Table 2.1: Bio-Rad molecular weight protein standards (Da)

Protein	Source	Molecular Weight Standards	Prestained Standards	Kaleidoscope Prestained Standards	
		Low Range	Broad Range	Colour	Size
Myosin	Rabbit skeletal muscle	-	200 000	Blue	192 000
B-galactosidase	*E.coli*	-	113 000	Magenta	127 000
Phosphorylase b	Rabbit muscle	97 400	-	-	-
Bovine serum albumin	Bovine plasma	66 200	82 000	Green	73 000
Ovalbumin	Chicken egg white	45 000	49 200	-	-
Carbonic anhydrase	Bovine erythrocytes	31 000	34 800	Violet	43 000
Trypsin inhibitor	Soybean	21 500	29 400	Orange	32 300
Lysozyme	Chicken egg white	14 400	20 900	Red	17 000
Aprotinin	Bovine pancreas	-	7 400	Blue	6 600

SDS-polyacrylamide gel Fast Blue staining

Gels were washed twice with fixative (45% w/v methanol/10% w/v acetic acid) for 10 min with gentle agitation. Gels were then submerged in dilute FAST Blu (8 ml FAST Blu (Fisher Biotech, 32 ml ddH_2O, 10 ml 45% v/v methanol/10% v/v acetic acid solution) and

gently agitated until bands were clearly visible (approx. 20 min). Gels were then destained in 10% v/v acetic acid overnight before drying.

Polyacrylamide gel drying

Stained gels were dried for 60 - 70 min at 72°C in a Bio-Rad Model 543 Gel Slab Dryer as per the manufacturer's instruction.

Autoradiography

Dried gels were exposed to Biomax MR film (Kodak) at –80°C for a time period of 8 to 14 days. Following incubation, films were brought back to room temperature and developed under infrared (1-2 min in Kodak X-ray developer, rinsed under running water 1 min, immersed in Kodak X-ray fixer for 1 min, rinsed under running water 30 sec).

Fatty acid analysis

Fatty acid extraction

A modification of the method described by Rule (1976) was employed for the fatty acid extractions and methylations. Cells were inoculated into 100 ml of YEP broth in 250 ml conical flasks and left at 15°C on an orbital shaker (180 o.p.m.) until stationary phase was reached (~5 days). Cells were then centrifuged at 13,000 x *g* and the pellet suspended in 2 ml of boron trifluoride in methanol (BTM) and 2 ml of methanol, in that order. The mixture was then transferred to a 5 ml glass tube with a screw cap and gassed with N_2 to remove any oxygen before being placed in an 80°C water bath for 2 hr. Tubes were vigorously shaken every 15 min. Tubes were then allowed to cool before adding 3 ml of water followed by 3 ml of hexane. Tubes were then vortexed and centrifuged at 13,000 x *g* to induce separation. The top hexane layer containing the methylated fatty acids was transferred into 1.5 ml vials and concentrated down to a small volume (< 0.2 ml) using N_2 gas. Samples were stored frozen at –20°C until required.

Fatty acid Analysis – Gas chromatography

Gas liquid chromatography was employed to analyse the fatty acid content of the concentrated samples. Samples were analysed with a Hewlett Packard 5890 series II (Hewlett Packard Company, Avondale, Pennsylvania, USA) gas chromatograph equipped with a flame ionisation detector. The fatty acid methyl esters (0.1-0.2 µl) were separated on a 30 m x 0.53 mm FFAP (polyethylene glycol ester; Alltech Australia, Homebush, Australia) column using helium as the carrier gas (flow rate 10 ml min^{-1}). The column was located inside an oven preset at 200°C. Injector and detector were set at 220°C. As the sample passed through the column, individual fatty acids were eluted on the basis of their relative volatility and charge. The more volatile and less charged the fatty acid – the earlier it will elute. A standard was run at the start and finish of each session so that an elution profile could be established (time elapsed / volts generated). By comparing the elution profile of the unknown samples with appropriate standards (Sigma), individual fatty acids were identified. Histograms were generated so that comparison assays were made more comprehensible.

rDNA Sequencing

DNA extraction

The AquaPure Genomic DNA Isolation Kit from Bio-Rad was employed to extract DNA from the yeast isolates. PCR products were obtained utilising the Qiagen Hotstart PCR kit [2 µl purified DNA, 0.5 µl forward primer (5'-TCCGTGTTTCATGAACCTGCGG-3'), 0.5 µl reverse primer (5'-TCCTCCGCTTATTGATATGC-3'), 21.5 µl MilliQ water, 2.5 µl Q-solution, 25 µl HotStart Mastermix] followed by thermal cycling using the following conditions: 95°C for 15 min followed by 30 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min with a final 10 min at 72°C. PCR products were purified with the Prep-a-gene-clean system. The Beckman CEQ2000 Dye Terminator Quick Start kit was used for the sequence reaction of the D1/D2 domain [purified PCR product, 2 µl primer (forward primer MLF 5'-GCATATCAAGCGGAGGAAAAG-3', reverse primer MLR 5'-GGTCCGTGTTTCAAGACGG-3'), 4 µl Beckman CEQ DTCS Quickstart mix, 14 µl MilliQ water, total volume of 20 µl], placed in the thermal cycler for 30 cycles of 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min. Sequences were obtained with the Beckman CEQ2000 sequencer and visually aligned with BioEdit.

Phylogenetic analysis

Species identification was determined by performing an NCBI Blast search. For those that did not find a match, the closest species was used to compare sequences in BioEdit. Species that were found to be closely related in other publications were also included in the phylogenetic analysis (Kurtzman and Robnett, 1998; Fell *et al.*, 2000). Phylogenetic relationships were aligned with MegAlign (DNAStar). Phylogenetic analyses employed the maximum parsimony program of PAUP 4.0 (Sinauer Associates) using a parsimonious heuristic search with random addition of sequences (100 replicates) and neighbour-joining analysis. Bootstrap values were determined using PAUP, values less than 50% were not included in tree figures.

Assimilation Tests

Procedure

Assimilation tests have been traditionally used to assess the ability of yeast strains to utilize certain organic compounds in methods first outlined by Wickerham and Burton (1948) and Whickerham (1951) [Table 2.2]. The following solutions were prepared:

A 10 X solution of a particular carbon source (6.7 g yeast nitrogen base, 5 g carbon source in 100 ml ddH_2O), for the raffinose test 10 g was used instead of 5 g.; 10 X nitrogen source (11.7 g yeast carbon base, 0.78 g potassium nitrate in 100 ml ddH_2O); 10 X vitamin-free media with required vitamins (16.7 g vitamin-free yeast base, 20 µg biotin, 2 mg calcium pantothenate, 2 µg folic acid, 10 mg inositol, 400 µg niacin, 200 µg *p*-aminobenzoic acid, 400 µg pyridoxine hydrochloride, 200 µg riboflavin and 400 µg thiamine hydrochloride in 100 ml ddH_2O); 50% w/v glucose agar (13 g agar, 500 g glucose, 6.7 g yeast nitrogen base made up to 1 L with ddH_2O); Christensen's urea media (1 g peptone, 5 g NaCl, 2 g dihydrogen phosphate, 12 µg phenol red made up to 1 L ddH_2O); gelatin media (100 g gelatin, 5 g glucose, 6.7 g yeast nitrogen base made up to 1 L ddH_2O) was prepared and filter sterilized before being stored at – 20 °C. When required, appropriate dilutions were made and 1 ml aliquots were dispersed into a 1.5 ml microfuge tubes under sterile conditions.

Table 2.2: Biochemical tests used in yeast characterisation

glucose	erythritol	melibiose	nitrate
galactose	ribitol	raffinose	vitamin-free
L-sorbose	galactitol	melezitose	citrate
sucrose	D-mannitol	inulin	inositol
maltose	D-glucitol	soluble starch	hexadecane
cellobiose	succinate	D-xylose	gelatin liquification
trehalose	salicin	L-arabinose	N-acetyl-D-glucosamine
lactose	D-gluconate	D-arabinose	10%NaCl 5%glucose
D-ribose	D-glucosamine	starch formation	α-methyl-D-glucoside
D-glucoronate	urease	DL-lactate	50%(w/w) glucose-yeast extract agar
methanol	glycerol	biotin-free	
ethanol	L-rhamnose	thiamine-free	

Cells taken (loopful) from single colonies on a plate were inoculated into 20 ml of YEP broth in 50 ml conical flasks and grown at 15°C on a rotary shaker (180 o.p.m.) until stationary phase was reached. An aliquot (25 µl) of this culture was inoculated into individual test tubes. In the case of solid media, streak inoculums were made across the agar surface. Tubes were stored at 15°C on a rotary shaker for 2 weeks and results recorded as positive, weak or negative according to whether any growth had occurred. Test results were recorded again at 4 weeks. Results indicating a positive reaction at 4 weeks following a negative result at 2 weeks were recorded as delayed. This recording procedure is outlined in '*The Yeasts, a Taxonomic Guide*' by Yarrow (1998), with the following distinctions: tubes were placed in front of white cardboard with black lines 0.75 mm thick and 5 mm apart. Results were recorded as positive if the lines are completely obscured or appear as diffuse bands; weak if lines were visible but fuzzy and negative if the lines appeared sharp. Glucose was used as the positive control and all strains were normalised accordingly. All tests were done in triplicate for each strain. Soluble starch and inulin needed to be scored microscopically to check for the presence of active growth (budding). Formation of starch-like compounds was determined by adding 20 µl of Lugol's iodine to the glucose test tube (positive control). A dark blue-green reaction was recorded as positive. Hydrolysis of urea was recorded as positive if media turned pink/red.

Diazonium Blue B (DBB) test

Cells were inoculated into 5 ml of yeast nitrogen base with 0.5% glucose in 25 ml conical flasks and left at 15°C on an orbital shaker (180 o.p.m.) for 5 days. Cells were harvested by centrifugation at 13,000 x *g* and supernatant discarded. Cells were resuspended in 0.5 ml 0.05 M KOH and the tubes placed in boiling water for 10 min. Once cooled, 2.5 ml of 95% ethanol was added and mixed well followed by centrifuging briefly and the supernatant discarded. Cells were then resuspended in 0.3 ml of freshly prepared and chilled DBB reagent. A positive result was recorded if a violet colour developed.

Colony photographs and micrographs

All colony photographs were taken with the Nikon Zoom Digital Camera, COOLPIX950. Labels and bars found on each photograph were added using Photoshop 6.0 (Adobe Systems Inc.).

Ubiquinone analysis

Ubiquinone extraction

Cells were inoculated into 100 ml of YEP broth in 250 ml conical flasks and left at 15°C on an orbital shaker (180 o.p.m.) until stationary phase was reached (approximately 5 days). Cells were then harvested by centrifugation at 13,000 x *g* and kept at –80 °C until required. Packed cells (100 µl) were resuspended in 1 ml of 20 mM PBS in a 1.5 ml microfuge tube, centrifuged at 10,000 x *g* for 1 min and the supernatant removed by vacuum. Two hundred µl of EtoAc/IPA [1:1] (ethyl acetate/isopropanol alcohol) was then added to the cells, vigorously vortexed for 1 min, centrifuged and the supernatant removed to a new microfuge tube. This process was then repeated (thus two extractions were performed) (Dunlap *et al.*, 2002).

Ubiquinone analysis – High performance liquid chromatography (HPLC)

Relative CoQ and $CoQH_2$ concentrations were determined immediately by HPLC analysis using the method described by Yamamoto *et al.*, (1998). Twenty-five µl of the EtoAc/IPA fraction was then immediately injected onto a Phenomenex ODS (2) column followed in-line by a platinum reduction column (Type RC – 10, Irica), with amperometric electrochemical detection (Model Σ 985, Irica) operating at an oxidation potential of +600mV (vs $Ag/AgCl_2$) on a glassy carbon electrode. The mobile phase consisted 50 mM sodium perchlorate in methanol/isopropanol (60/40 v/v) delivered at a flow rate of 1.0 ml/min.

NAD(P)H: quinone oxidoreductase activity

NAD(P)H: quinone oxidoreductase activity was determined using the method outlined by Lind *et al.* (1990). Cells exposed to UVA-irradiation (1 ml) were periodically harvested and washed twice in ddH_2O. An equal volume of glass beads (0.5 mm diameter) was then added as well as 200 µl of buffer (0.1% Triton X100, 50 mM Tris-HCl pH 7.4). Samples were then interchangeably vortexed for 20 sec and rested on ice for 20 sec a total of 7 times, centrifuged and supernatant transferred to a fresh microfuge tube. Enzyme activity was measured spectrophotometrically using NAD(P)H as the electron donor and 2,6-dichlorophenolindophenol (DCPIP) as the electron acceptor. Forty µl of 1 mM DCPIP and 5 µl 0.1M NADPH was added to 950 µl of Tris-HCl buffer, the reaction was initiated by the addition of the 100 µl of the enzyme. The reduction of DCPIP was measured at 600 nm ($\varepsilon = 21$ mM^{-1} cm^{-1}).

Stress response

Thermotolerance

All yeast samples (*Rhodotorula mucilaginosa*) were grown over night at 25°C in 2% glucose YEP medium (20 ml) to stationary phase then washed and resuspended in YNB. This was necessary to eliminate methionine from the medium so that ^{35}S-methionine incorporation into protein could be enhanced. Intrinsic thermotolerance was measured by rapidly increasing the temperature of cells to the heat stress temperature under hot running water transferring

them to an oscillating water bath set at the desired temperature, in this case 52°C. Induced thermotolerance was measured by exposing the cells to a mild heat shock of 37°C for 1 hr prior to a heat stress of 52°C for 3 hr. Samples (0.5 ml) were taken at appropriate time intervals and spread onto YEP plates, in duplicate, and incubated at 15°C for 3 – 5 days. Stress tolerance was estimated by visible plate count, determined as cfu ml^{-1}, and presented as survivors after stress divided by survivors before stress, expressed as a percentage.

UV-irradiation

A small loopful of cells from a stock YEP agar plate was inoculated into 100 ml YEP broth. Antarctic yeasts were grown at 15°C and mesophilic yeasts at 25°C in an orbital shaker operating at 180 o.p.m. (Paton Industries, Adelaide) to late exponential phase (exhaustion of glucose). This generally corresponded to about 24 hr growth for the mesophilic yeasts and to between 48 to 60 hr for the Antarctic yeasts. Appropriate serial dilutions (10^0 to 10^{-5}) were made and 0.1 ml was spread on YEP plates in duplicate. Plates were exposed to twin 20 watt UVA fluorescent lamps (Phillips TL-20W/05) at a distance of 5 cm for 2, 3 and 4 hr. The amount of UVA exposure at that distance was found to be 4.58×10^{-1} J cm^{-2} s^{-1} at the agar surface.

Statistical analysis

All experiments were performed in duplicate or in some cases triplicate. Representative results are presented from typical experiments while data were presented as the mean ± standard deviation (SD) or alternatively, mean ± standard error (SE) of individual experiments. Statistical significant differences were determined by Student's *t*-test using Data Analysis in the Microsoft Excel program.

CHAPTER 3

The Basidiomycetes

Introduction

The Basidiomycetes are a diverse group of fungi and are divided into three classes: the Urediniomycetes, Ustilaginomycetes and the Hymenomycetes. These classes are based on sequence analyses of the 18S rRNA (Swann and Taylor, 1995) and sequencing analysis of the D1/D2 region of the 26S rDNA which indicates similar patterns (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). Many basidiomycetes occur as yeasts (unicellular fungi) during all, or part of, their entire life cycle. There are two morphologically distinct sexual states; one where teliospores are formed to produce a basidium that goes on to produce basidiospores and a second state which develop basidium on the hyphae without the formation of a teliospore (Kurtzman and Blanz, 1998). Asexually reproducing basidiomycetous yeasts are found throughout the classes and are based on to a number of physical properties including a positive reaction to Diazonium Blue B, urease production, presence of xylose in extracellular carbohydrates and enteroblastic budding (Boekhout *et al.*, 1998). Some basidiomycetous yeasts produce ballistospores and/or carotenoid pigment which are absent in ascomycetous yeasts (Bandoni, 1987). Walker and Doolittle (1983) first introduced molecular studies for taxonomic purposes utilizing the 5S ribosomal RNA region. Molecular techniques have provided a powerful taxonomic tool for phylogenetic purposes and comprehensive studies applying these techniques to the classification of basidiomycetous yeast have been published (Kurtzman, 1994; Swann and Taylor, 1995; Fell *et al.*, 2000; Fonseca *et al.*, 2000; Hamamoto *et al.*, 2002).

Urediniomycetes

The class Urediniomycetes is comprised of the three subclasses; Microbotryomycetidae, Agaricostilbomycetidae and Urediniomycetidae. Within the Microbotryomycetidae are the teleomorphic genera *Microbotryum, Rhodosporidium,*

Leucosporidium, Colacogloea and *Heterogastridium*. Fell *et al.* (2000) and Scorzetti *et al.* (2002) have produced phylogenetic trees from their analyses of typical basidiomycetous yeast species which indicated a number of anamorphic species of the genera *Rhodotorula* and *Sporobolomyces* as belonging to the same phylum. Recently, Sampaio *et al.* (2003) proposed the order Leucosporidiales to incorporate *Leucosporidium scottii* and closely related species, both sexual and asexual (Sampaio *et al.*, 2003). Species belonging to the subclass Agaricostilbomycetidae are dimorphic and include the genera *Kurtzmanomyces, Sterigmatomyces* and the majority of the *Bensingtonia* (Fell *et al.*, 2000; Scorzetti *et al.*, 2002). The third subclass, Uredinomycetidae, is mainly made up of plant parasites with the most prominent order being the Uredinales, which comprise the rusts. At present, the only known species to have a yeast stage in this subclass belong to the genus *Septobasidium*.

Due to limited data for all species belonging to the Urediniomycetes, there are several unnamed clades including species from the genera *Naohidea, Occultifur, Rhodotorula, Sporobolomyces, Erythrobasidium* and *Bannoa* (Sampaio *et al.*, 1999).

Ustilaginomycetes

The Ustilaginomycetes primarily consist of plant parasites. There are three sub-classes namely: Enterrhizomycetidae, Ustilaginomycetidae and Exobasidiomycetidae. Yeast species are found in the Ustilaginomycetidae in the order Ustilaginales and the Exobasidiomycetidae in the orders Microstromatales and Malasseziales (Fell *et al.*, 2000). The cell walls of the Ustilaginomycetes have a predominance of glucose and lack xylose, like the Hymenomycetes they have a type B secondary structure in the 5S rRNA and lack parenthesomes at the septal spores like the Urediniomycetes (Bauer *et al.*, 1997).

Hymenomycetes

The Hymenomycetes are divided into two sub-classes, the Hymenomycetidae consisting of the mushrooms and puff-balls and the Tremellomycetidae. Glucose is the major cell wall carbohydrate and, unlike the Urediniomycetes and the Ustilaginomycetes, xylose is present (Swann and Taylor, 1995). There are four major clades within the Tremellomycetidae: the Tremellales, the Trichosporonales, the Filobasidiales and the Cystofilobasidiales. The genus *Cryptococcus* is polyphyletic occurring in all four clades. The Tremellales clade consist

of the genera *Bullera, Bulleromyces, Fellomyces, Filobasidiella, Kockovaella* and *Tsuchiyaea*. The representative of the Tremellales is the teleomorphic species *Tremella* although other teleomorphic species such as *Bulleromyces* and *Filobasidiella* are also present. *Bullera* is the anamorphic genera of the *Bulleromyces* (Boekhout and Nakase, 1998) and can be distinguished from *Cryptococcus* by the production of ballistoconidia. *Holtermannia corniformis* and the closely related strains *Cryptococcus nyarrowii, Cr. watticus, Cryptococcus* species KCTC 17061, KCTC 17062, KCTC 17063, CBS 7712, CBS 7713, CBS 7743 and CBS 8016 appear in a separate cluster and have been suggested to represent a separate lineage (Scorzetti *et al.*, 2002). The Trichosporonales consist of all the species of *Trichosporon* with the exception of *Trichosporon pullulans* which belongs to the Cystofilobasidiales. The genus *Trichosporon* produce true hyphae, septa with dolipores with or without parenthesomes, the major coenzyme system is CoQ_9 or CoQ_{10} and the cell walls contain xylose. The only teleomorphic genus in the Filobasidiales clade is *Filobasidium*. The Filobasidiales are comprised of four clusters, one of which includes many Antarctic species such as *Cr. antarcticus, Cr. adeliensis, Cr. albidosimilis, Cr. friedmannii* and *Cr. vishniacii* (Fell *et al.*, 2000). *Cr. gilvescens* and *Cr. gastricus* are clustered together and both of these strains have been isolated from Antarctica, as reported in the current study. The Cystofilobasidiales consist of the teleomorphic genera *Mrakia* and *Cystofilobasidium*. Both genera produce teliospores, a characteristic not found among other teleomorphic genera of the Hymenomycetes. Anamorphic genera include the *Udeniomyces* as well as several *Cryptococcus* species (Fell *et al.*, 2000).

Figure 3.1. Classification of the Basidiomycetes

BASIDIOMYCETE [subdivision]
UREDINIOMYCETES [class]
MICROBOTRYOMYCETIDAE [sub-class]
Cryptomycocolacales [order]
Crypomycocolacaceae [family]
Cryptomycocolax
Colacosiphon
Heterogastridiales [order]
Heterogastridiaceae [family]
Heterogastridium
Leucosporidiales [order]
Leucosporidium
Leucosporidiella
Mastigobasidium
Sporidiobolales [order]
Sporidiobolaceae [family]
Sporidiobolus
Sporobolomyces
Rhodosporidium
Rhodotorula
Microbotryales [order]
Microbotryaceae [family]
Bauerago
Liroa
Microbotryum
Sphacelotheca
Zundeliomyces
Ustilentylomataceae [family]
Aurantiosporium
Fulvisporium
Ustilentyloma

Taxa of Microbotryomycetidae not belonging to any order
Krieglsteineraceae [family]
Krieglsteinera
Camptobasidiaceae [family]
Camptobasidium
Atractocolax
Bensingtonia
Colacogloea
Kriegeria

AGARICOSTILBOMYCETIDAE [sub-class]

Agaricostilbales [order]

Agaricostilbaceae [family]

Agaricostilbum

Chionosphaeraceae [family]

Chionosphaera
Stilbum
Fibulostilbum

Genera of the Agaricostilbomycetidae not belonging to any order

Bensingtonia
Kondoa
Kurtzmanomyces
Mycogloea
Sporobolomyces
Sterigmatomyces
Spiculogloea
Zygogloea

UREDINIOMYCETIDAE [sub-class]

Uredinales [order]

14 Families, 164 genera, approx. 7000 species

Septobasidiales [order]

Septobasidiaceae [family]

Auriculoscypha
Coccidiodictyon
Ordonia
Septobasidium
Uredinella

Pachnocybaceae [family]

Pachnocybe

Platygloeales [order]

Platygloea

Taxa of the Urediniomycetidae not belonging to any order

Eocronartiaceae

Eocronartium
Helicobasidium
Herpobasidium
Insolibasidium
Jola
Platycarpa
Ptechetelium

Groups belonging to the Urediniomycetes for which higher rank is incomplete

Atractiellales [order]
Hoehnelomycetaceae [family]
Atractiella
Phleogenaceae [family]
Phleogena
Helicogloeaceae [family]
Helicogloea
Saccoblastia

Classiculales [order]
Classiculaceae [family]
Classicula
Jaculispora

Cystobasidiales [order]
Bannoa
Cystobasidium
Naohidea
Occultifur
Sakaguchia
Erythrobasidium
Rhodotorula
Sporobolomyces
Mixiaceae [family]
Mixia

USTILAGINOMYCETES [class]

ENTORRHIZOMYCETIDAE [sub-class]

Entorrhizales [order]
Entorrhizaceae[family]
Entorrhiza

USTILAGINOMYCETIDAE [sub-class]

Urocystales [order]
Melanotaeniaceae [family]
Exoteliospora
Melanotaenium
Yelsemia
Doassansiopsaceae [family]
Doassansiopsis
Urocystaceae [family]
Mundkurella
Urocystis
Ustacystis
Vankya

Ustilaginales [order]
- Ustilaginaceae [family]
  - *Anthracoidea*
  - *Cintractia*
  - *Cintractiella*
  - *Clintamra*
  - *Dermatosorus*
  - *Farysia*
  - *Farysporium*
  - *Franzpetrakia*
  - *Geminago*
  - *Heterotolyposporium*
  - *Kuntzeomyces*
  - *Leucocintractia*
  - *Macalpinomyces*
  - *Melanopsichium*
  - *Moesziomyces*
  - *Moreaua*
  - *Orphanomyces*
  - *Pericladium*
  - *Planetella*
  - *Pseudozyma*
  - *Rhodotorula*
  - *Schizonella*
  - *Sporisorium*
  - *Stegocintractia*
  - *Testicularia*
  - *Tolyposporium*
  - *Tranzscheliella*
  - *Trichocintractia*
  - *Uleiella*
  - *Ustanciosporium*
  - *Ustilago*
  - *Websdanea*
- Glomosporiaceae [family]
  - *Glomosporium*
  - *Thecaphora*
  - *Tothiella*
- Mycosyringaceae [family]
  - *Mycosyrinx*

EXOBASIDIOMYCETIDAE [sub-class]

Malasseziales [order]

Malassezia

Georgefischeriales [order]

Georgefischeriaceae [family]

Georgefischeria

Jamesdicksonia

Tilletiariacea [family]

Phragmotaenium

Tilletiaria

Tilletiopsis

Tolyposporella

Eballistraceae [family]

Eballistra

Tilletiales [order]

Tilletiaceae [family]

Conidiosporomyces

Erratomyces

Ingoldiomyces

Neovossia

Oberwinkleria

Tilletia

Microstromatales [order]

Microstromataceae [family]

Cerinosterus

Microstroma

Rhodotorula

Sympodiomycopsis

Volvocisporaceae [family]

Volvocisporium

HYMENOMYCETES [class]

TREMELLOMYCETIDAE [sub-class]

Cystobasidiales [order]

Itersonilia

Cryptococcus

Cystofilobasidium

Mrakia

Tausonia

Trichsporon

Udeniomyces

Xanthophyllomyces

Trichosporonales [order]

Cryptococcus

Trichosporon

Filobasidiales [order]
- Filobasidiaceae [family]
  - *Cryptococcus*
  - *Filobasidium*
- Carcinomycetaceae [family]
  - *Carcinomyces*
  - *Christiansenia*
  - *Syzygospora*

Tremellales [order]
- Cuniculitremaceae [family]
  - *Cuniculitrema*
  - *Fellomyces*
  - *Kockovaella*
  - *Sterigmatosporidium*
- Phragmoxenidiaceae [family]
  - *Phragmoxenidium*
  - *Phyllogloea*
- Sirobasidium [family]
  - *Fibulobasidium*
  - *Sirobasidium*
- Tremellaceae [family]
  - *Bullera*
  - *Bulleribasidium*
  - *Bulleromyces*
  - *Cryptococcus*
  - *Dioszegia*
  - *Filobasidiella*
  - *Holtermannia*
  - *Papiliotrema*
  - *Sirotrema*
  - *Tremella*
  - *Tremellina*
  - *Trimorphomyces*
  - *Tsuchiyaea*
  - *Xenolachne*
- Tetragoniomycetaceae [family]
  - *Tetragoniomyces*
- Rhynchogastremataceae [family]
  - *Rhynchogastrema*

Table 3.1. Basidiomycetous yeasts examined in the D1/D2 and internal transcribed spacer rDNA regions

Species	26S GenBank accession no.	ITS1-5.8S-ITS2 GenBank accession no.	CBS no.
Bullera dendrophilia	AF189870	AF444443	6074^T
Bullera oryzae	AF075511	AF444413	7194^T
Bullera sinensis	AF189884	AF444468	7238^T
Bullera unica	AF075524	AF444441	8290^T
Cryptococcus albidus	AF075474	AF145321	142^T
Cryptococcus antarcticus	AF075488	AF145326	7687^T
Cryptococcus aquaticus	AF075470	AF410469	5443^T
Cryptococcus curvatus	AF189834	AF410467	570^T
Cryptococcus dimennae	AF075489	AF410473	5770^T
Cryptococcus friedmannii	AF075478	AF145322	7160^T
Cryptococcus gastricus	AF137600	AF145323	2288^T
Cryptococcus gilvescens	AF181547	AF444380	7525^T
Cryptococcus heveanensis	AF075467	AF444301	569^T
Cryptococcus humicola	AF189836	AF410470	571^T
Cryptococcus laurentii	AF075469	AB035049	139^T
Cryptococcus nyarrowii	AY006480	AY006481	8804^T
Cryptococcus victoriae	AF363647	AF444469	8685
Cryptococcus vishniacii	AF075473	AF145320	7110^T
Cryptococcus watticus UNE41b	AY138478		9496^T
Cryptococcus sp.	AJ311450	AF408417	7712
Cryptococcus sp.	AF360844		8016
Cryptococcus sp. KCTC 17061	AF459677		
Cryptococcus sp. KCTC 17062	AF459678		
Cryptococcus sp. KCTC 17063	AF459679		
Dioszegia crocea	AF075508	AF444406	6714^T
Dioszegia hungarica	AF075503	AF444379	4214^T
Filobasidium capsuligenum	AF075501	AF444369	4736
Filobasidium elegans	AF181548	AF190006	7640
Filobasidium floriforme	AF181507	AF190007	6241
Filobasidium uniguttulatum	AF075468	AF444302	1730^T
Holtermannia corniformis	AF189843	AF410472	6979
Leucosporidium antarcticum	AF189906	AF444529	5942^T
Leucosporidium fellii	AF189907	AF444508	7287^T
Leucosporidium scottii	AF070419	AF444495	5930^T
Mrakia gelida	AF189831	AF144485	5272^T
Rhodotorula araucariae	AF070427	AF444510	6031^T

Rhodotorula diffluens	AF075485	AF444533	5233^T
Rhodotorula glutinis	AF070430	AF444539	20^T
Rhodotorula lactosa	AF189936	AF444540	5826^T
Rhodotorula marina	AF189944	AF444504	2365^T
Rhodotorula minuta	AF189945	AF190011	319^T
Rhodotorula mucilaginosa	AF070432	AF444541	316^T
Rhodotorula muscorum	AF070433	AF444527	6921^T
Rhodotorula yarrowii	AF189971	AF444628	7417^T
Sporobolomyces elongatus	AF189983	AF444561	8080^T
Sporobolomyces lactophilus	AF177411	AF444545	7527^T
Sporobolomyces ruberrimus	AF070442	AY015439	7500^T
Sporobolomyces singularis	AF189996	AF444600	5109^T
Sporobolomyces taupoensis	AF177413	AF444592	7898^T
Tremella cinnabarina	AF189866	AF444430	8234
Tremella encephala	AF189867	AF410474	6968
Tremella mesenterica	AF075518	AF444433	6973
Trichosporon aquatile	AF075520	AF410475	5973^T
Trichosporon brassicae	AF075521	AF444436	6382^T
Trichosporon cutaneum	AF075483	AF444325	2466^T

Table 3.2. UNE yeast strains examined in the D1/D2 and internal transcribed spacer rDNA regions

Species	**UNE Reference No.**
Cryptococcus gastricus	25b
Cryptococcus gilvescens	110c 124g 1026b 1028e
Cryptococcus nyarrowii	64a 73a 116b 125a 157a 172a 180e 182c
Cryptococcus victoriae	76c 98a 151b 190b
Cryptococcus watticus	22c 41b
Cryptococcus sp. KCTC 17063	22d
Holtermannia corniformis	97b
Leucosporidium antarcticum	174b
Rhodotorula laryngis	1266b
Rhodotorula minuta	17b
Rhodotorula mucilaginosa	1130a
Rhodotorula sp.	1028e

Cryptococcus species

The genus *Cryptococcus* is polyphyletic and is distributed throughout the Hymenomycetous yeasts and is represented in the Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales orders [Fig 3.1]. The *Cryptococcus* genus is restricted to anamorphic heterobasidiomycetous yeasts and yeast-like fungi. Characteristics of *Cryptococcus* species are: enteroblastic budding; parenthesomes may be present; cell hydolyzates contain xylose; usually assimilate myo-inositol and D-glucoronate; starch compounds are sometimes produced; negative reaction to fermentation; positive reaction to both Diazonium Blue B and urease and lack of red pigmentation (Barnett *et al.*, 1990; Boekhout *et al.*, 1998; Montes *et al.*, 1999).

In the present study, two collections, one from the summer 1997/98 and the second from the summer 1994/95, resulted in the isolation of 22 *Cryptococcus* species. 1D-SDS-PAGE was used to rapidly identify identical or closely related strains [Figs 3.6 and 3.7]. The protein profiles for the *Cr. nyarrowii* strains [Fig 3.6, lanes 1-8] were very similar and this observation was confirmed by sequencing data from the D1/D2 and ITS regions of the 26S ribosomal DNA [Fig 3.2]. Similarly, the protein profiles of the *Cr. victoriae* strains [Fig 3.6, lanes 10-14] agreed with the sequencing data with the exception of *Cr. victoriae* UNE76c in which the protein profile [Fig 3.6, lane 10] differed in some respects to the other strains. In the case of *Cr. gilvescens*, UNE110c, UNE1028a and to a lesser extent UNE1026b [Fig 3.7, lanes 1-3] showed similar protein profiles but strain UNE124g [Fig 3.7, lane 4] differed somewhat to the other three strains. On the other hand, all three *Cr. watticus* strains showed very similar protein profiles [Fig 3.7, lanes 7-9]. Following this initial screening process, further categorizations were made utilising morphological and physiological characteristics including biochemical assimilations, fatty acid analysis and coenzyme Q analysis.

Cryptococcus watticus sp. nov.

Isolates UNE41b and UNE22c were isolated from soil and snow samples collected in 1997, while isolate UNE1026a came from a 1994 collection. UNE41b was chosen as the type strain. Phylogenetic analysis placed UNE41b in a clade with *Cryptococcus nyarrowii*, also isolated from the Vestfold Hills, Antarctica (Thomas-Hall and Watson, 2001), three Korean

strains, KCTC 17061, KCTC 17062 and KCTC 17063 isolated from tundra soils, *Cryptococcus species* CBS 7712 isolated from Antarctic soil (Golubev, 1977), *Cryptococcus species* CBS 8016 isolated in water, Sweden and *Holtermannia corniformis* CBS 6979 (Kobayasi, 1937).

Description of *Cryptococcus watticus* Guffogg, Thomas-Hall, Holloway & Watson sp. nov. *Cryptococcus watticus* (watticus referring to Watts Lake, Antarctica from where the isolate originated).

Growth in YEP broth after 3 days at 15°C, cells are ovoidal and occur singly or in pairs [Fig 3.5]. Budding is polar. Aerobic growth results in pink convex circular colonies with an entire margin. Colonies are viscous in consistency. No sexual state was observed from mixed or pure cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. Growth on Yeast Nitrogen Base agar and cornmeal agar is slower than on YEP or malt agar. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, sucrose, trehalose, lactose, melibiose, raffinose, inulin, D-xylose, D-ribose, L-rhamnose, *N*-acetyl-D-glucosamine, D-mannitol, α-methyl-D-glucoside, salicin, succinate, citrate, inositol and D-glucoronate; negative or weak reaction for D-arabinose, D-glucosamine, glycerol, erythritol, ribitol, D-glucitol, hexadecane, L-sorbose, maltose, cellobiose, melezitose, L-arabinose, methanol, ethanol, galactitol, D-gluconate, DL-lactate. Assimilation of soluble starch is weak. Assimilation of nitrate is positive. Growth in vitamin-free medium, 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose is negative. Growth is positive in both biotin-free and thiamine-free media. Gelatin liquefication and urease reactions are positive. Diazonium Blue B reaction is positive. Starch formation is negative. Growth at 25°C is weak, no growth at 30°C. These strains were isolated from soil [UNE41b] and stromatolite and shell [UNE22c], Watts Lake, Vestfold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica. The type strain is UNE41b. It was deposited in the Centraalbureau voor Schmmelcultures, Utrecht, The Netherlands, as CBS 9496^T (NRRL Y-27556).

Phylogenetic analyses [Figs 3.2 and 3.3] of *Cr. watticus* placed it among other established Antarctic yeasts including strains CBS 7712, CBS 7713, CBS 7743 (Golubev, 1977) and *Cr. nyarrowii* (Thomas-Hall & Watson, 2002). The closest phylogenetic relatives, *Cryptococcus sp.* KCTC 17061, KCTC 17062 and KCTC 17063, differed by 2 nucleotides

from the newly isolated yeast. However, no physiological data for these strains were available, therefore, other relationships could not be studied. All strains utilised in the phylogenetic analyses are listed in Table 1. Protein profiles [Fig 3.7] for *Cr. watticus* strains UNE41b, UNE22c were obtained by 1D-SDS-PAGE and comparative protein banding patterns indicated these strains to have identical 1D-protein profiles, with similar protein patterns to closely phylogenetically related *Cr. nyarrowii*. Fatty acid analysis of strain UNE41b [Fig 3.4] revealed oleic acid ($C_{18:1}$) to be the predominant fatty acid present (61%) with only trace amounts of the polyunsaturated fatty acids linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$). It was noteworthy that strains UNE22d and UNE22c, although containing high amounts of $C_{18:1}$, also showed substantial concentrations of the polyunsaturated fatty acids $C_{18:2}$ and $C_{18:3}$ [Fig 3.4]. *Cr. watticus* was found to contain predominately $CoQH_2$-10 and CoQ-10 in the ratio 76% to 24% respectively [see Table 6.2]. The new yeast isolate has been placed in the genus *Cryptococcus* as no sexual state has been observed under a variety of conditions.

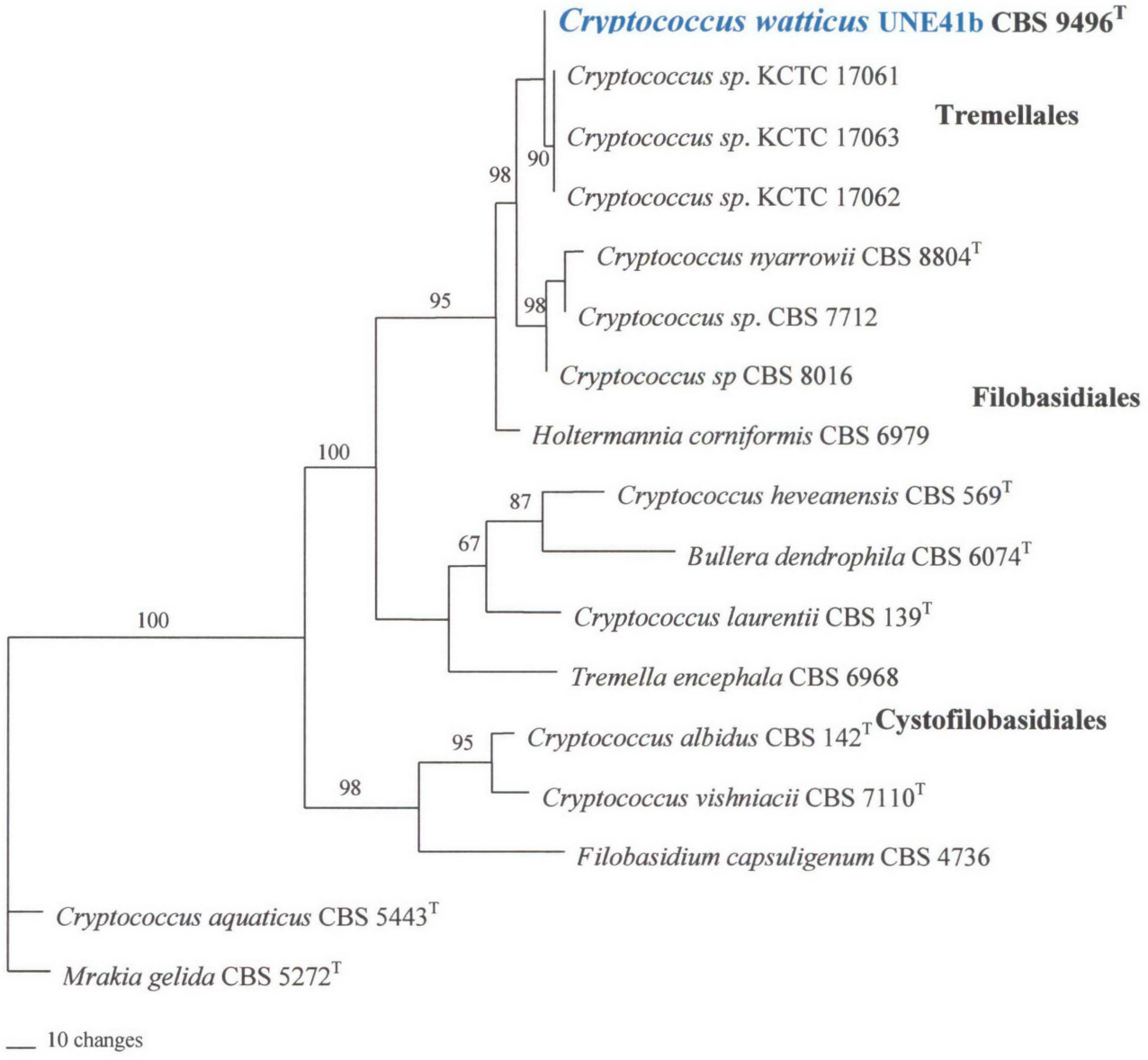

Figure 3.2. Phylogenetic tree showing placement of *Cryptococcus watticus* among related basidiomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Cryptococcus aquaticus* and *Mrakia gelida* representing the Cystofilobasidiales are the designated out-group species in this analysis. Labelled clades have representative species only.

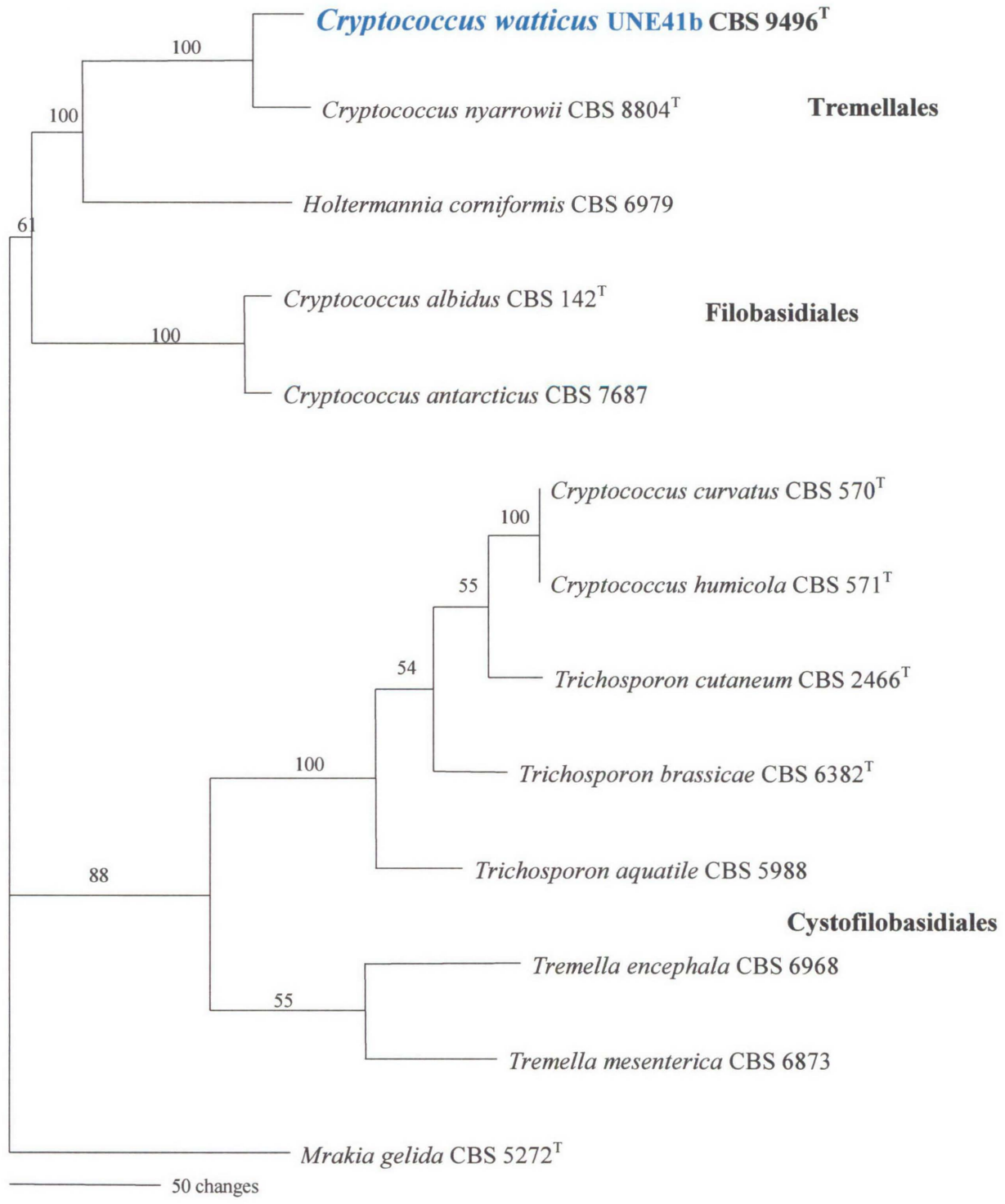

Figure 3.3. Phylogenetic tree showing placement of *Cryptococcus watticus* among related basidiomycetous yeasts derived from maximum parsimony analysis of the ITS1-5S-ITS2 region. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Mrakia gelida* representing the Cystofilobasidiales is the designated out-group species in this analysis. Labelled clades have representative species only.

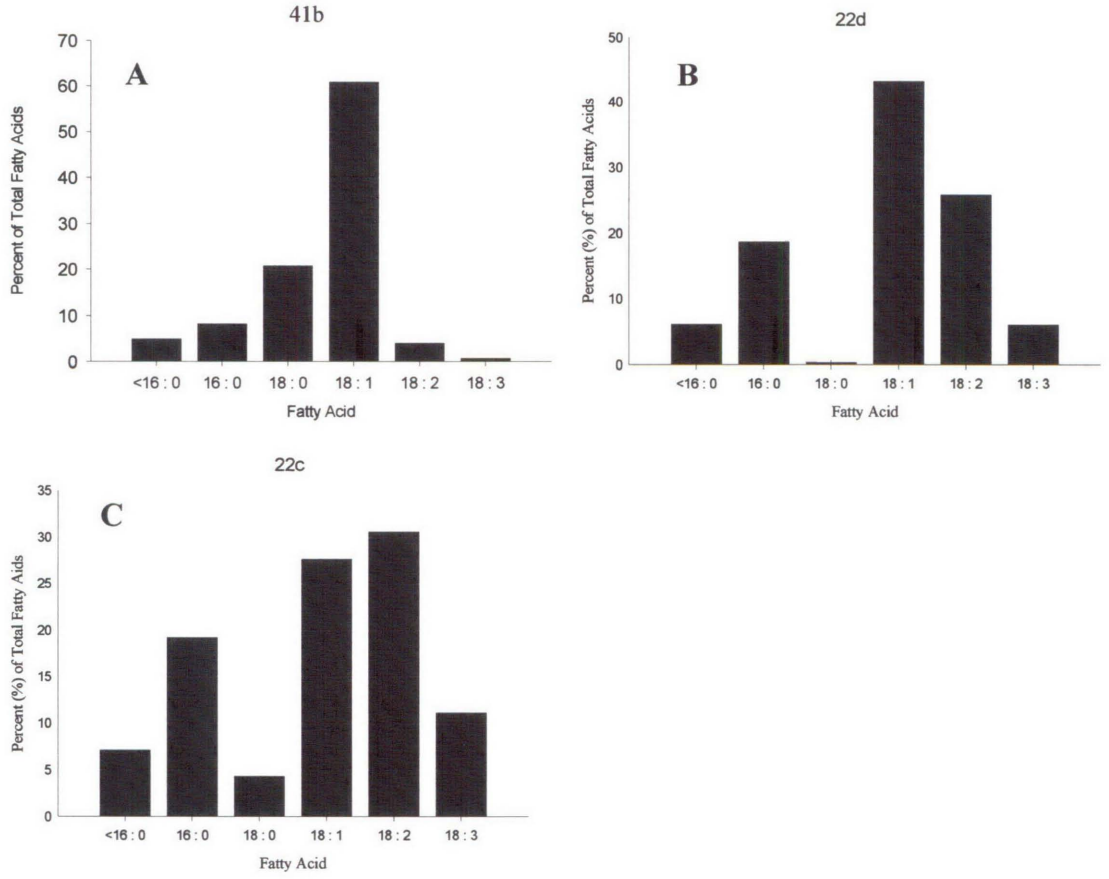

Figure 3.4. Fatty acid profiles for *Cryptococcus watticus* [A] strain UNE41b; [B] strain UNE22d and [C] strain UNE22c.

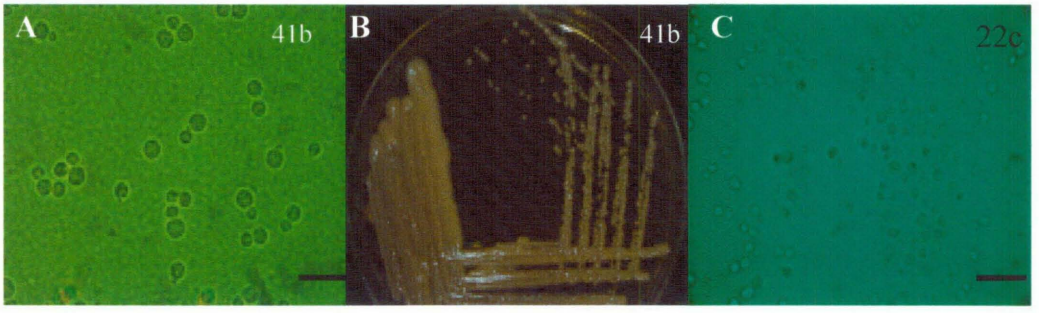

Figure 3.5. *Cryptococcus watticus* after 3 days at 15°C in YEP broth showing budding cells [A & C]. Bar = 10 μm. [B] Colony formation of *Cr. watticus* after 14 days at 6°C on YEP.

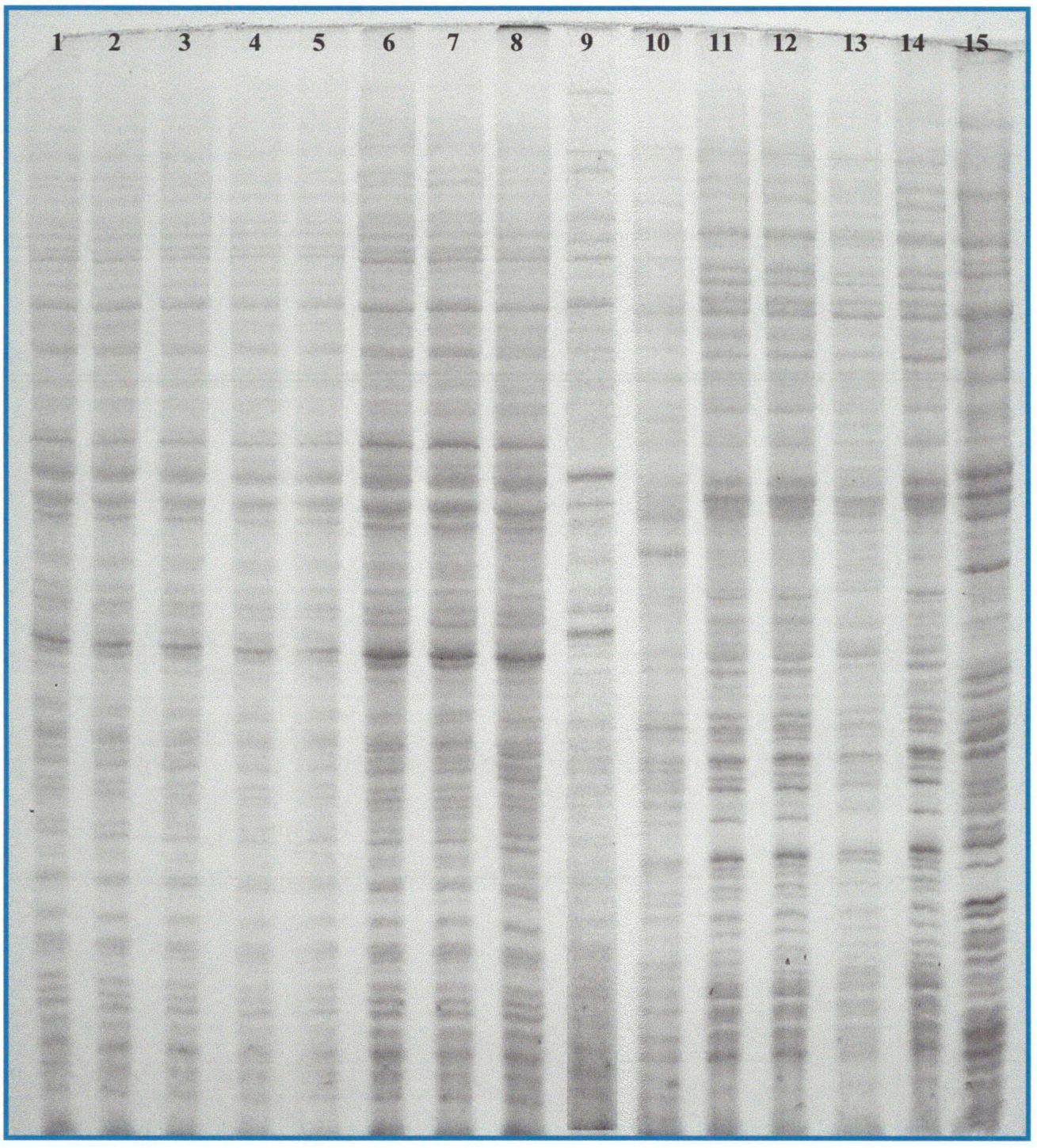

Figure 3.6. 1D-SDS-PAGE of whole cell proteins from various Antarctic yeasts

Lanes 1 through 8 – *Cr. nyarrowii* [1080c, 182c, 125a, 64a, 116b, 73a, 172a, 157a]
Lane 9 - KCTC 17063 [22d]
Lanes 10 through 14 – *Cr. victoriae* [76c, 98a, 151b, 190b, CBS 8685]
Lane 15 - *Mrakia gelida* [CBS 5272]

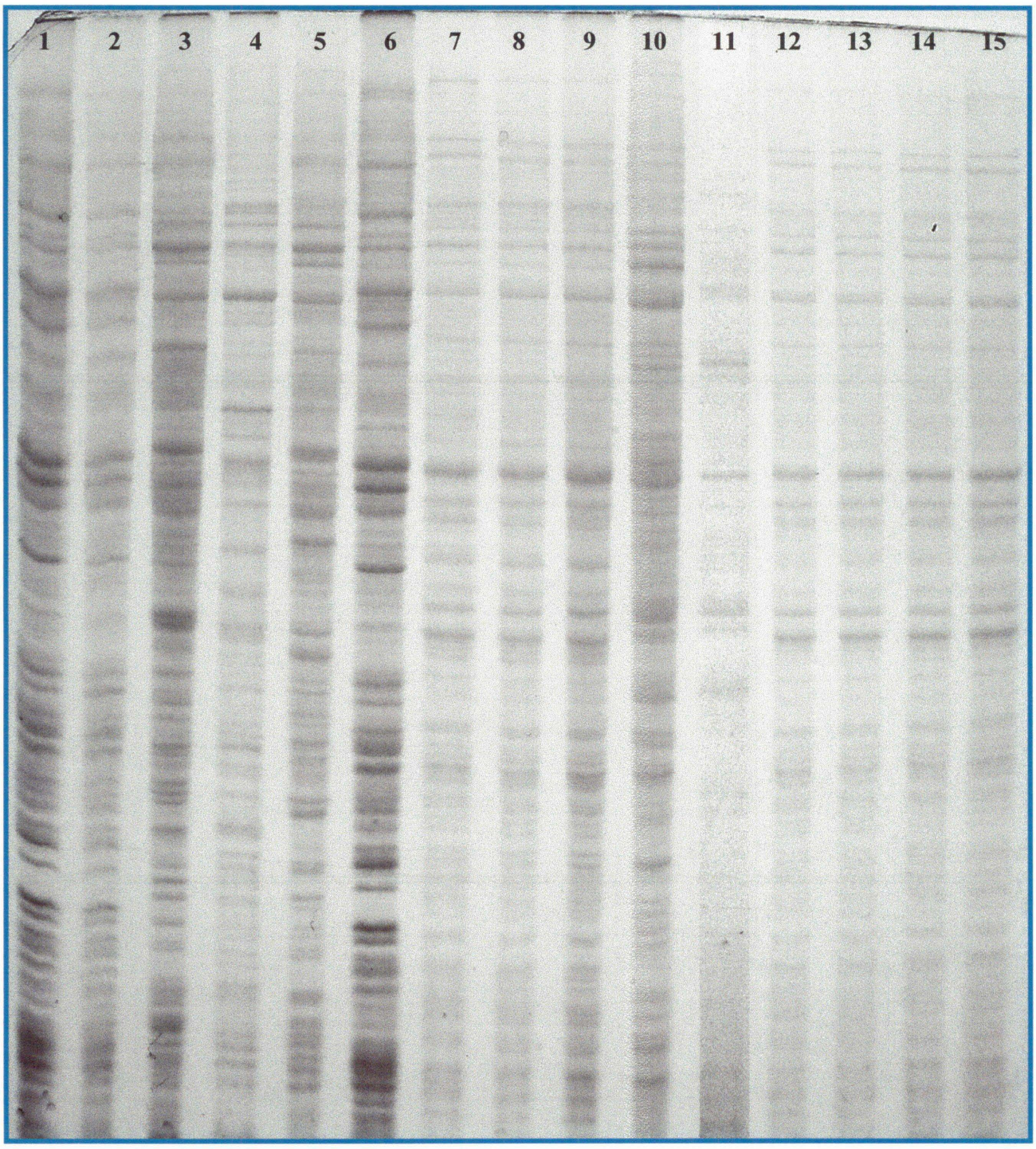

Figure 3.7. 1D-SDS-PAGE of whole cell proteins from various Antarctic yeasts
Lanes 1 through 4 - *Cr. gilvescens* [110c, 1028a, 1026b, 124g]
Lanes 5 & 6 - strains similar to *Cr. gilvescens* [189d, 189e]
Lanes 7 through 9 - *Cr. watticus* [41b, 22c]
Lane 10 - *Cr. antarcticus* [CBS 7687]
Lane 11 - *Rhodotorula sp.* [1028e]
Lane 12 - *R. mucilaginosa* [1103a]
Lane 13 - *R. laryngis* [1266b]
Lane 14 - *R. zsoltii* [CBS ?]
Lane 15 - *Mrakia gelida* [CBS 5272]

Cryptococcus nyarrowii

Isolates UNE64a, UNE73a, UNE116b, UNE125a, UNE157a, UNE172a, UNE180e and UNE182c were isolated from soil and snow samples collected in 1997. Molecular analysis indicated all these strains to be conspecific and identical to *Cryptococcus nyarrowii* CBS 8804^T. Phylogenetic analysis placed *Cr. nyarrowii* in a clade with *Cr. watticus*, also isolated from the Vestfold Hills, Antarctica (Guffogg *et al.*, 2004), three Korean strains, KCTC 17061, KCTC 17062 and KCTC 17063 isolated from tundra soils, *Cryptococcus species* CBS 7712 isolated from Antarctic soil (Golubev, 1977), *Cryptococcus species* CBS 8016 isolated from water, Sweden and *Holtermannia corniformis* CBS 6979 (Kobayasi, 1937). Colony appearance on YEP agar plates are presented in Figure 3.8.

UNE64a was used as the representative strain of *Cr. nyarrowii*. Growth in YEP broth after 3 days at 15°C, cells are ovoidal and occur singly or in pairs. Budding is polar. Aerobic growth results in pink convex circular colonies with an entire margin. Colonies are viscous in consistency. No sexual state was observed from mixed or pure cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. Growth on Yeast Nitrogen Base agar and cornmeal agar is slower than on YEP or malt agar. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, glycerol, inulin, *N*-acetyl-D-glucosamine, D-mannitol, ribitol, erythritol, and; negative or weak reaction for α-methyl-D-glucoside, D-arabinose, D-glucosamine, D-xylose, D-glucoronate, citrate, melibiose, raffinose, D-glucitol, D-ribose, inositol, hexadecane, sucrose, lactose, salicin, succinate, trehalose, L-sorbose, maltose, cellobiose, melezitose, L-arabinose, L-rhamnose, methanol, ethanol, galactitol, D-gluconate, DL-lactate. Assimilation of soluble starch is weak. Assimilation of nitrate is positive. Growth in vitamin-free medium and 10% NaCl 5% glucose is negative. Growth is positive in biotin-free and thiamine-free media and 50% (w/w) glucose-yeast extract agar. Gelatin liquefication and urease reactions are negative. Diazonium Blue B reaction is positive. Starch formation is negative. Growth at 25°C is weak, no growth at 30°C.

Protein profiles [Fig 3.6] for *Cr. nyarrowii* strains UNE64a, UNE73a, UNE116b, UNE125a, UNE157a, UNE172a, UNE180e and UNE182c were obtained by 1D-SDS-PAGE and comparative protein banding patterns indicated these strains to have identical 1D-protein profiles, with similar protein patterns to the closely phylogenetically related *Cr. watticus*. Fatty

predominant fatty acid present in the majority of strains with only trace amounts of linolenic ($C_{18:3}$). Strains UNE64a and UNE116b were predominantly rich in the monounsaturated oleic acid ($C_{18:1}$). *Cr. nyarrowii* was found to contain predominately $CoQH_2$-10 and CoQ-10 in the ratio 88% to 12% respectively [see Table 6.2].

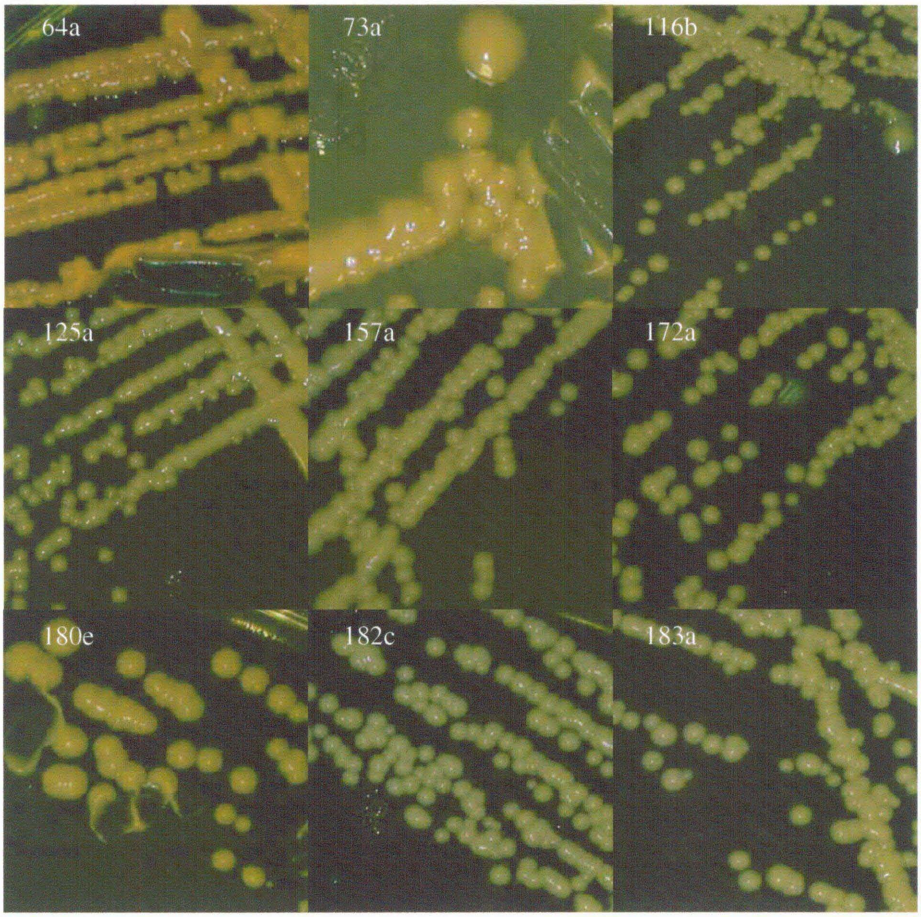

Figure 3.8. Colony photographs of various strains of *Cryptococcus nyarrowii*

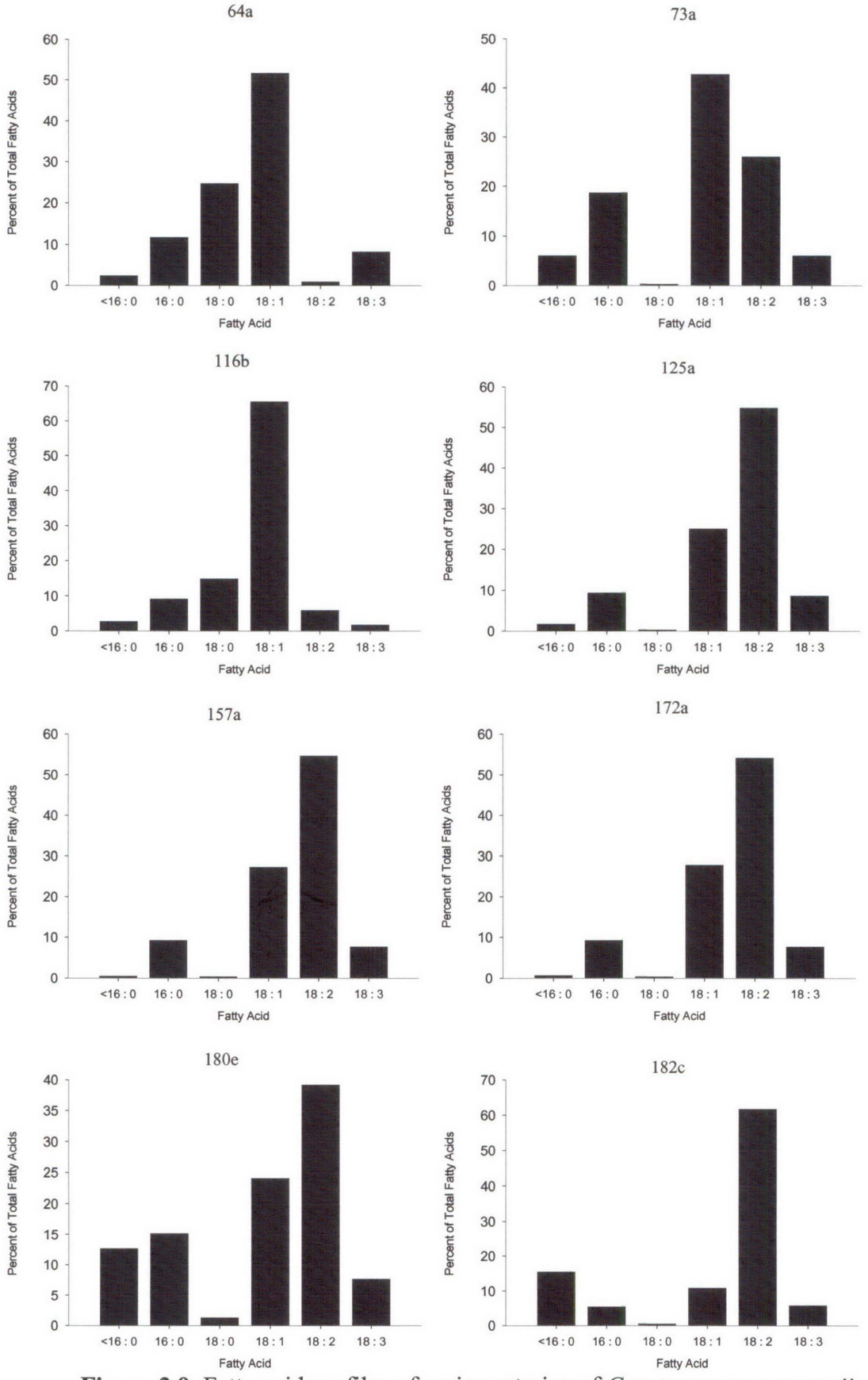

Figure 3.9. Fatty acid profiles of various strains of *Cryptococcus nyarrowii*

Cryptococcus gilvescens & Cr. gastricus

Isolates UNE110c, UNE124g, UNE189e and UNE189d were isolated from soil and snow samples collected in 1997, while isolates UNE1026b and UNE1028a came from a 1994 collection. Molecular analysis identified these strains as *Cryptococcus gilvescens*. UNE189e and UNE189d differed in 3 base pair (bp) substitutions in the D1/D2 region of the large ribosomal rDNA. Phylogenetic analysis placed *Cr. gilvescens* in the Filobasidiales clade [Fig 3.10]. There are four clusters in the Filobasidiales clade with the only teleomorphic genus being *Filobasidium*. One cluster consists of a number of Antarctic species including *Cr. antarcticus, Cr. friedmannii* and *Cr. vishniacii* (Fell *et al.*, 2000). *Cr. gilvescens* is found in the fourth cluster with *Cr. gastricus*. Molecular analysis of UNE25b indicated that it was identical in the D1/D2 region to *Cr. gastricus* (results not shown). Colony appearance on YEP agar plates of Cr. gilvescens is presented in Figure 3.13.

UNE110c is the representative strain. Growth in YEP broth after 3 days at 15°C, cells are round and occur singly or in pairs. Budding is polar. Aerobic growth results in cream to pink convex circular colonies with an entire margin. Colonies are viscous in consistency. No sexual state was observed from mixed or pure cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. Growth on Yeast Nitrogen Base agar and cornmeal agar is slower than on YEP or malt agar. Assimilation of carbon compounds is as follows: positive reaction for glucose, sucrose, trehalose, maltose, L-sorbose, inulin, D-xylose, L-arabinose, *N*-acetyl-D-glucosamine, citrate, and D-glucoronate; negative or weak reaction for galactose, D-arabinose, D-glucosamine, L-rhamnose, glycerol, erythritol, ribitol, D-glucitol, D-mannitol, melibiose, hexadecane, raffinose, cellobiose, salicin, melezitose, lactose, inositol, methanol, ethanol, galactitol, succinate, D-gluconate, D-ribose, DL-lactate, α-methyl-D-glucoside,. Assimilation of soluble starch is positive. Assimilation of nitrate is positive. Growth in vitamin-free medium, 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose is negative. Growth is positive in both biotin-free and thiamine-free media. Gelatin liquefication and urease reactions are negative. Diazonium Blue B reaction is positive. Starch formation is negative. Growth at 25°C is weak, no growth at 30°C.

Protein profiles (*Cr. gilvescens* strains Fig 3.6; *Cr. gastricus* not shown) for strains UNE110c, UNE124g, UNE1026b, UNE1028e, UNE189d and UNE189e were obtained by 1D-

SDS-PAGE and comparative protein banding patterns indicated the first four strains to be conspecific, with the latter two differing in a number of protein band positions from each other as well as from the *Cr. gilvescens* strains. The phylogenetic analysis showed clear separation in the 26S rDNA domain D1/D2 and the ITS1-5S-ITS2 region [Fig 3.10]. Fatty acid analysis [Fig 3.12] revealed oleic acid ($C_{18:1}$) to be the predominant fatty acid present with smaller amounts of the polyunsaturated fatty acids linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) in UNE110c, UNE124g and UNE25b and trace amounts in UNE189e gave a unique profile with almost equal amounts of the unsaturated fatty acids $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ and with 25% as the saturated fatty acid, palmitic acid ($C_{16:0}$). The predominant coenzyme Q system was identified to be CoQ_{10} [see Table 6.2].

This is the first report of *Cr. gilvescens* isolated from the Antarctic region although it is noteworthy that it was originally found in soil and decomposing plants in the Taimyr tundra in 1987 (Chernov and Bab'eva, 1988). These authors described *Cr. gilvescens* as a new species on the basis of the type of coenzyme Q system (CoQ_9) differing from *Cr. magnus, Cr. laurentii* and *Cr. flavus* (CoQ_{10}) as well as the presence of yellow pigmentation and variations in certain physiological properties. By contrast, all of the strains isolated in this study have a coenzyme Q system of CoQ_{10} and the majority exhibited pink pigmentation.

Cr. gastricus was originally isolated from gastric lavages in tuberculous patients in 1958, although the authors do mention also isolating the strain from New Zealand soil (Reiersöl and di Menna, 1958).

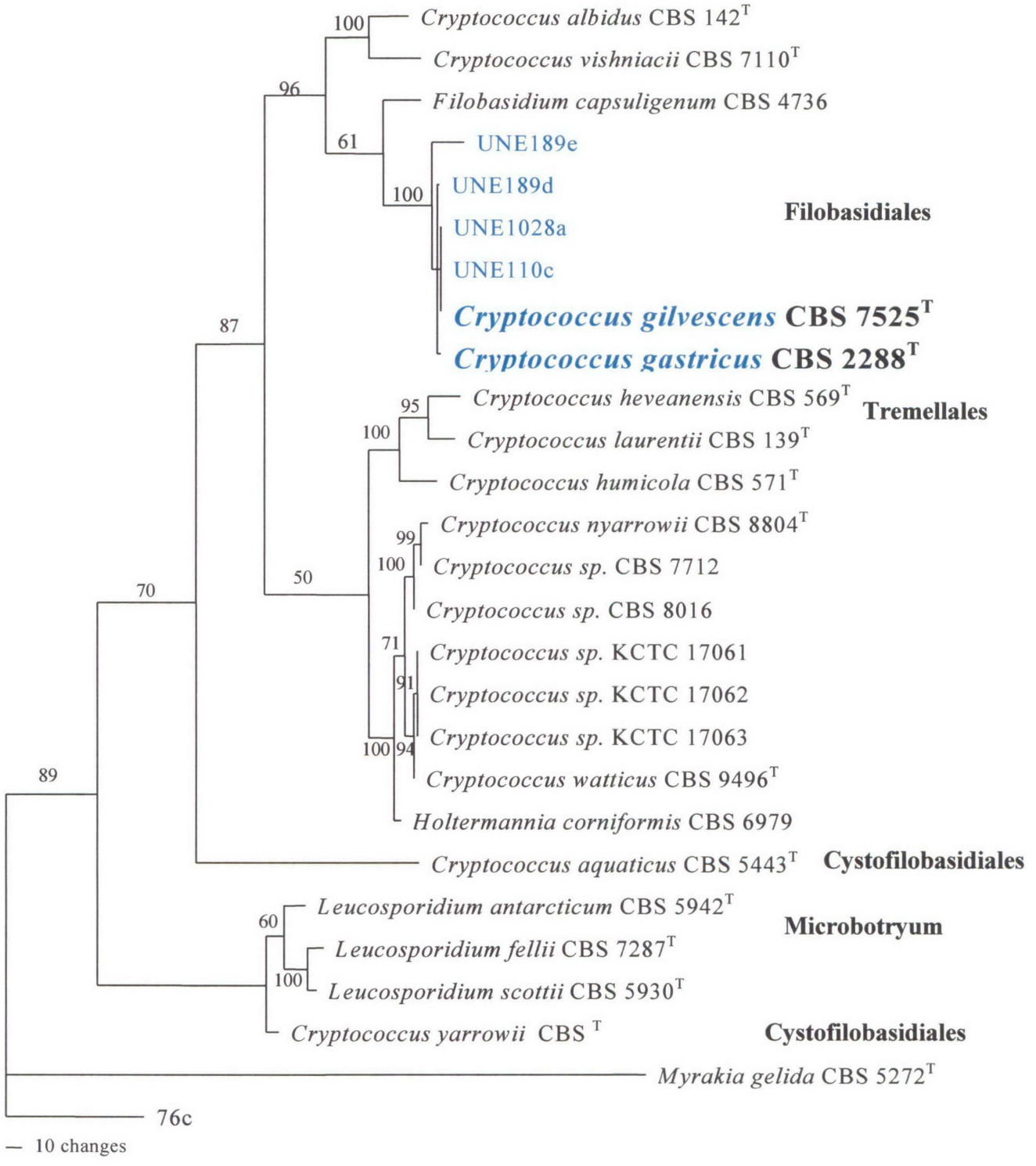

Figure 3.10. Phylogenetic tree showing placement of *Cryptococcus gilvescens* and *C. gastricus* among related basidiomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Mrakia gelida* representing the Cystofilobasidiales is the designated out-group species in this analysis. Labelled clades have representative species only.

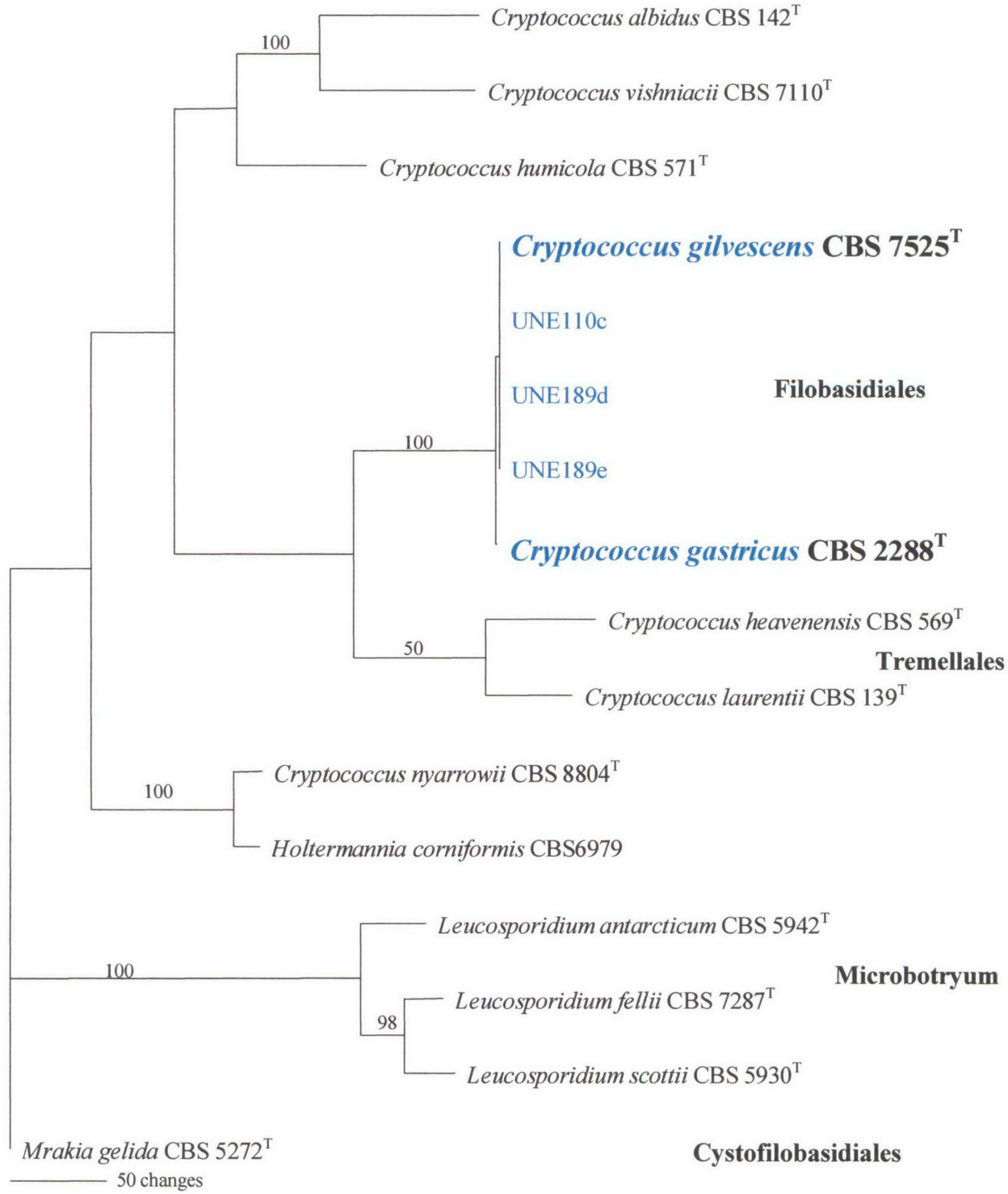

Figure 3.11. Phylogenetic tree showing placement of *Cryptococcus gilvescens* and *C. gastricus* among related basidiomycetous yeasts derived from maximum parsimony analysis of the ITS1-5S-ITS2 region. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Mrakia gelida* representing the Cystofilobasidiales is the designated out-group species in this analysis. Labelled clades have representative species only.

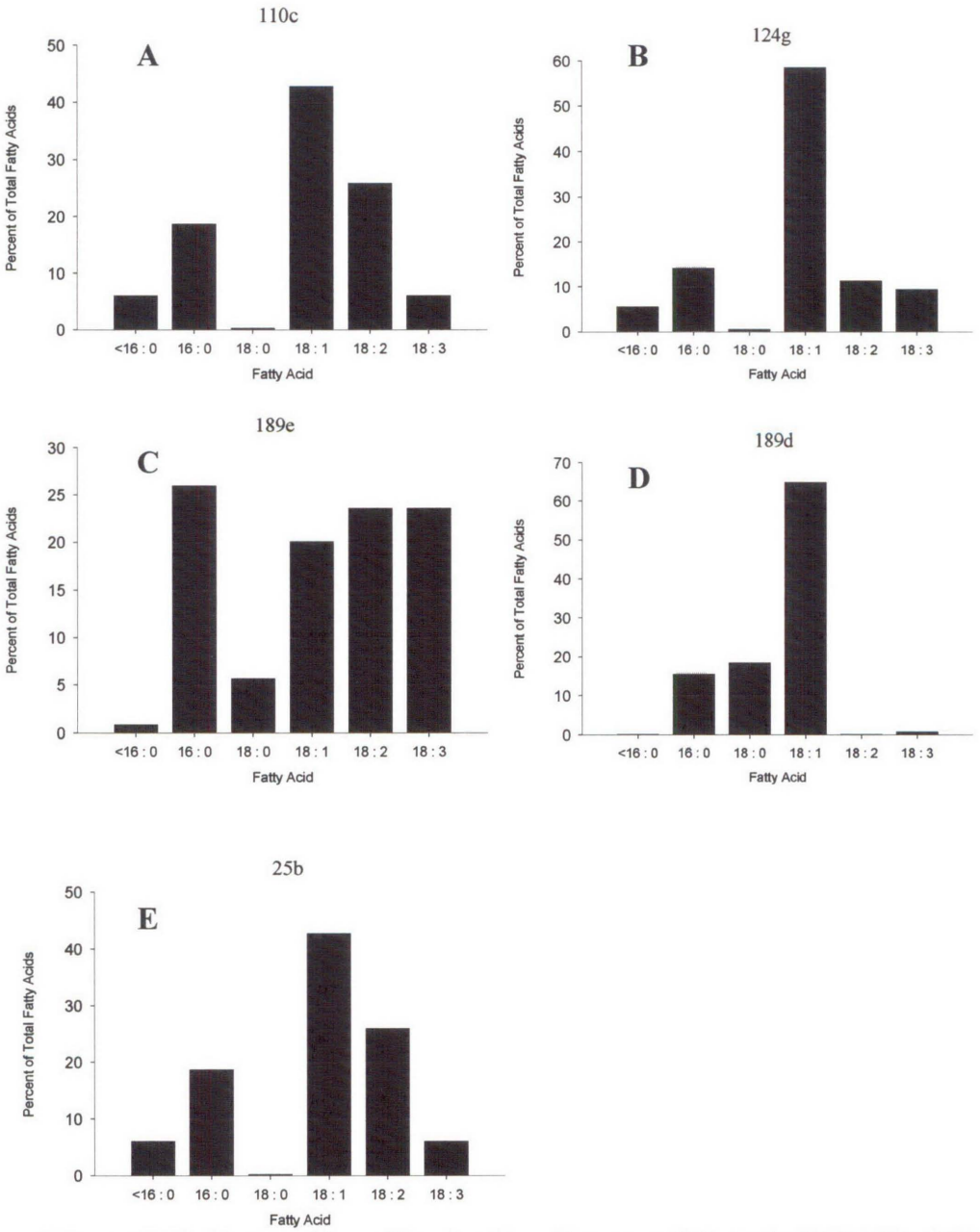

Figure 3.12. Fatty acid profiles for *Cr. gilvescens* [A] strain UNE110c [B] strain UNE124g [C] strain UNE189e [D] UNE189d and *Cr. gastricus* [E] UNE25b

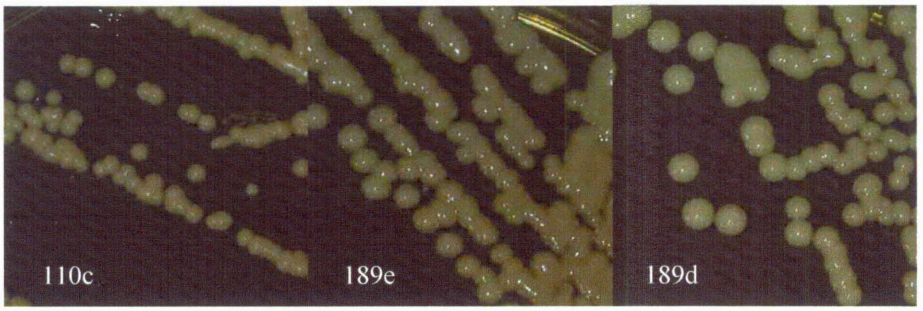

Figure 3.13. Colony formation of *Cr. gilvescens* variants after 14 days at 6°C on YEP agar

Cryptococcus victoriae

Molecular analysis revealed strains UNE76c, UNE98a, UNE151b and UNE190b to be identical in domain D1/D2 of the 26S rDNA to *Cryptococcus victoriae*. All strains were isolated from soil and snow samples collected in 1997. Phylogenetic analysis placed *Cr. victoriae* in the Tremellales clade in a cluster with *Cr. dimennae* [Figs 3.16 and 3.17]. *Cr. victoriae* was originally isolated from Southern Victoria Land, Antarctica from samples of soil, mosses and lichens (Montes *et al.*, 1999). More recently, *Cr. victoriae* was isolated from the Vestfold Hills region of the Australian Davis Base, Antarctica from soil samples (Thomas-Hall *et al.*, 2002). The isolates from this study originated from a variety of sources including soil, lichen and an algal mat located on top of ice. Colony appearance on YEP agar is presented in Figure 3.15.

UNE98a is the representative strain. Growth in YEP broth after 3 days at 15°C, cells are ovoidal and occur singly or in pairs. Budding is polar. Aerobic growth results in pink convex circular colonies with an entire margin. Colonies are butyrous in consistency. No sexual state was observed from mixed or pure cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. Growth on Yeast Nitrogen Base agar and cornmeal agar is slower than on YEP or malt agar. Assimilation of carbon compounds is as follows: positive reaction for glucose, sucrose, trehalose, maltose, L-sorbose, inulin, D-xylose, L-arabinose, *N*-acetyl-D-glucosamine, citrate, and D-glucoronate; negative or weak reaction for galactose, D-arabinose, D-glucosamine, L-rhamnose, glycerol, erythritol, ribitol, D-glucitol, D-mannitol, melibiose, hexadecane, raffinose, cellobiose, salicin, melezitose, lactose, inositol, methanol, ethanol, galactitol, succinate, D-gluconate, D-ribose, DL-lactate, α-methyl-D-glucoside,. Assimilation of soluble starch is positive. Assimilation of nitrate is positive. Growth in vitamin-free medium, 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose is negative. Growth is positive in both biotin-free and thiamine-free media. Gelatin liquefication and urease reactions are negative. Diazonium Blue B reaction is positive. Starch formation is negative. Growth at 25°C is weak, no growth at 30°C.

Protein profiles for strains UNE76c, UNE98a, UNE151b and UNE190b were obtained by 1D-SDS-PAGE and comparative protein banding patterns indicate the latter three strains to have identical 1D-protein banding patterns to *Cr. victoriae* CBS 8685^T with UNE76c differing

in a number of protein banding positions [Fig 3.6]. Fatty acid analysis (Fig 3.14) revealed oleic acid ($C_{18:1}$) to be the predominant fatty acid present in all strains examined with significant amounts of the polyunsaturated fatty acid linoleic ($C_{18:2}$) and trace amounts of linolenic ($C_{18:3}$). *Cr. victoriae* was found to contain predominately $CoQH_2$-10 and CoQ-10 in the ratio 61% to 39% respectively [see Table 6.2].

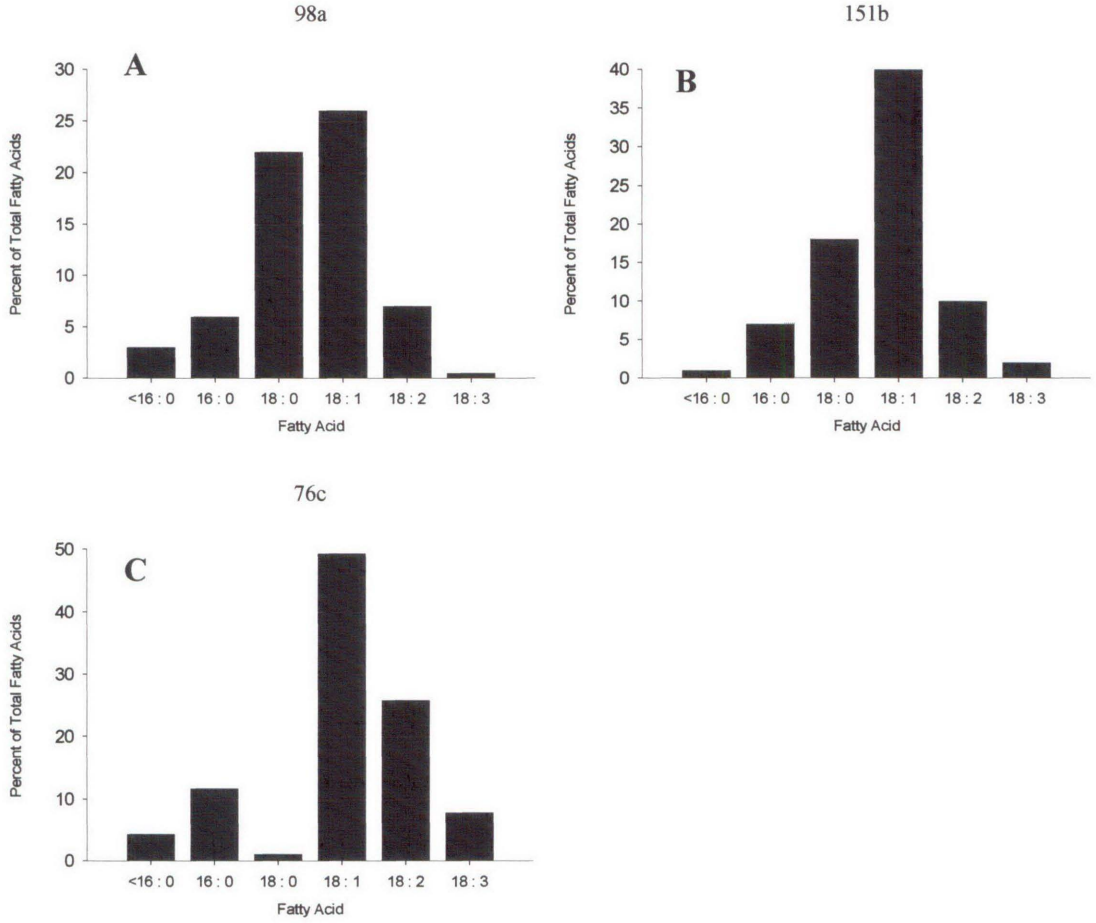

Figure 3.14. Fatty acid profiles for *Cryptococcus victoriae* strains [A] UNE98a [B] UNE151b and [C] UNE76c

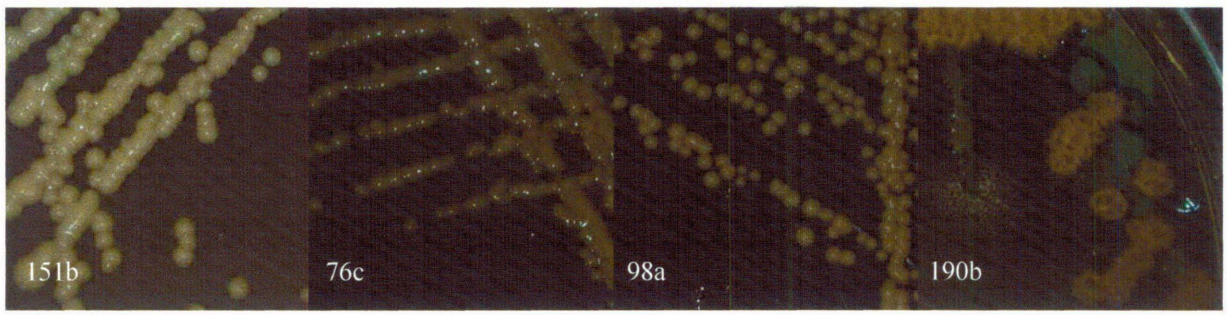

Figure 3.15. Colony formation of *Cr. victoriae* variants after 14 days at 6°C on YEP agar

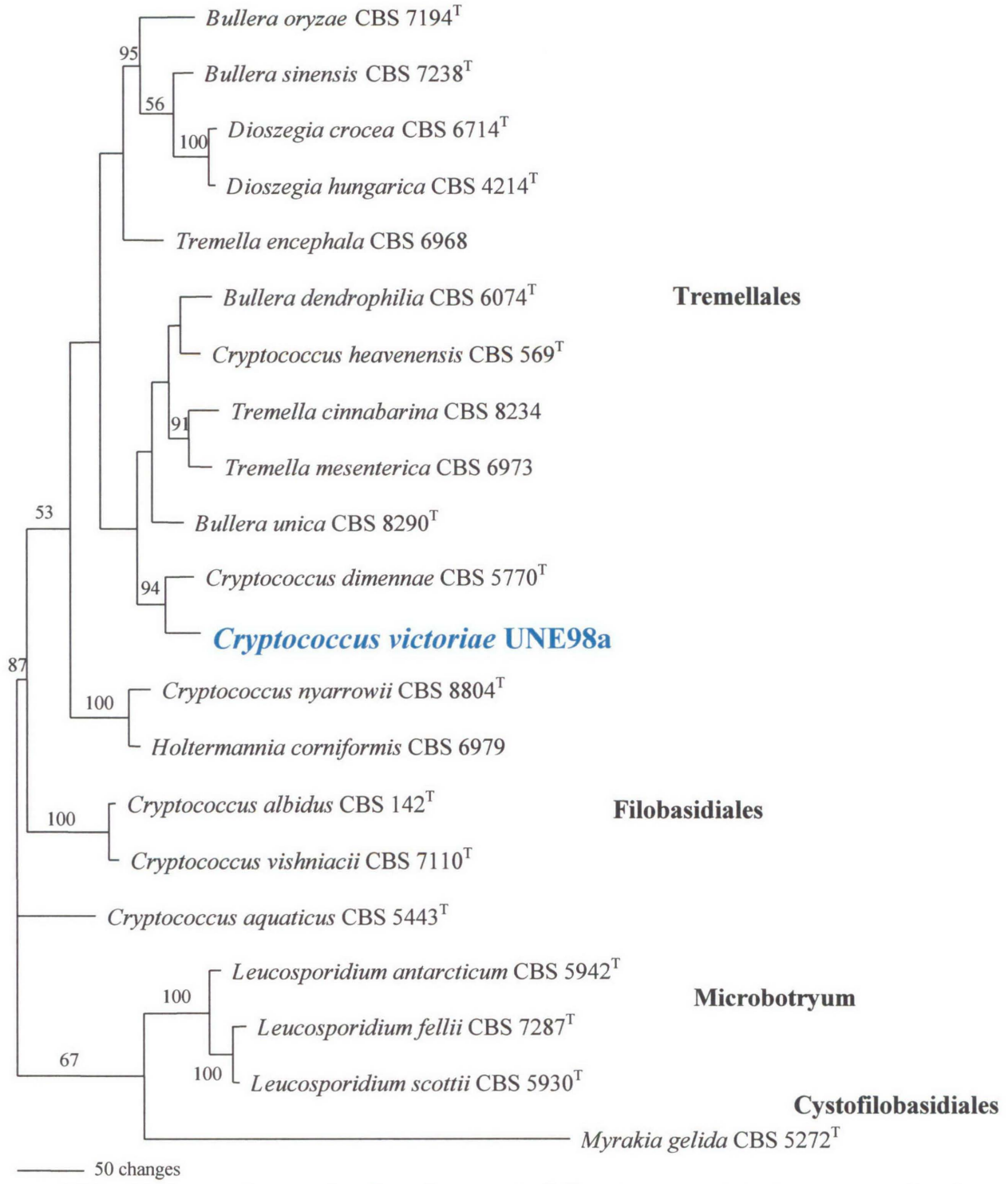

Figure 3.16. Phylogenetic tree showing placement of *Cryptococcus victoriae* among related basidiomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Cryptococcus aquaticus* and *Mrakia gelida* representing the Cystofilobasidiales are the designated out-group species in this analysis. Labelled clades have representative species only.

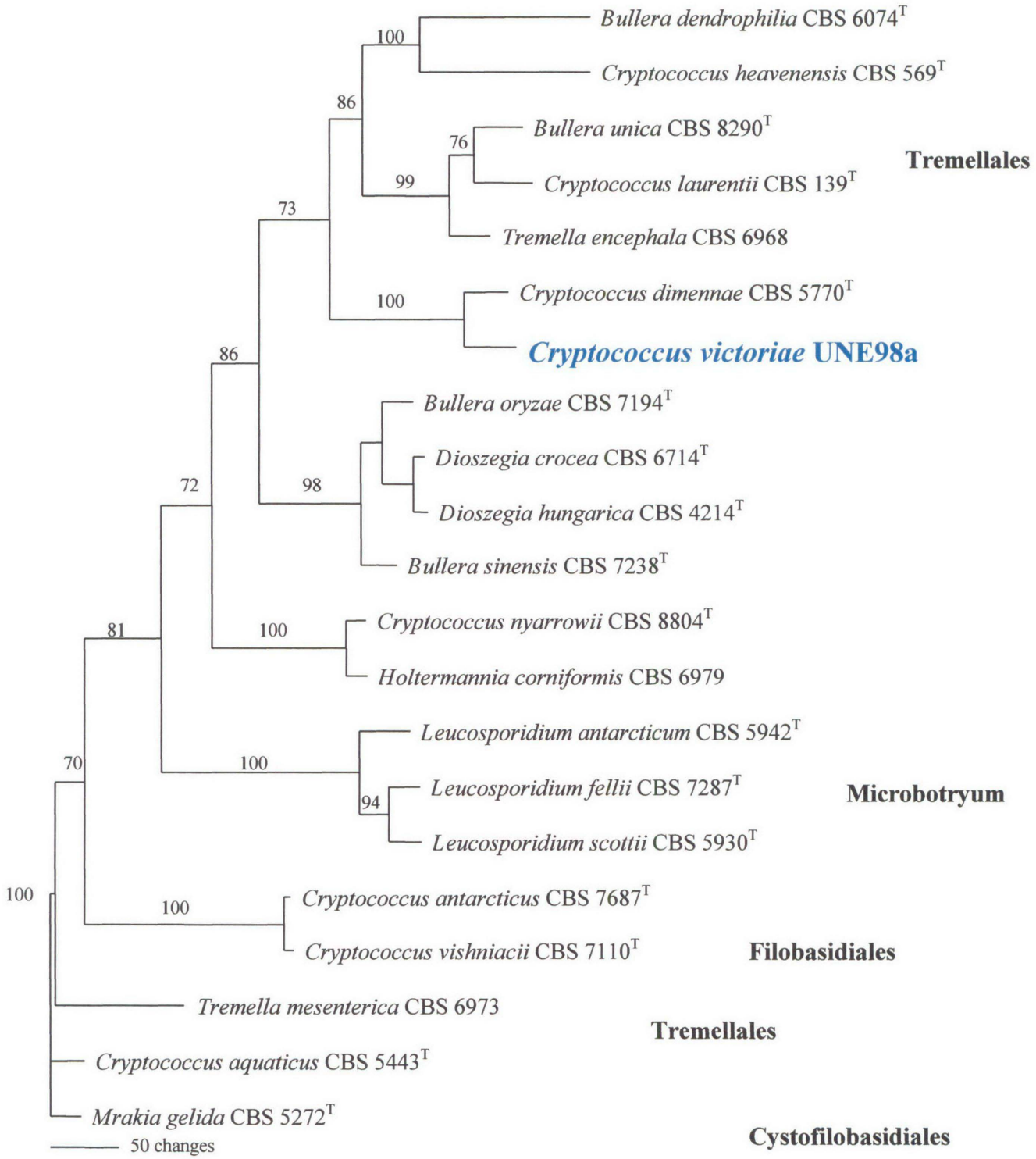

Figure 3.17. Phylogenetic tree showing placement of *Cryptococcus victoriae* among related basidiomycetous yeasts derived from maximum parsimony analysis of the ITS1-5S-ITS2 region. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Mrakia gelida* representing the Cystofilobasidiales is the designated out-group species in this analysis. Labelled clades have representative species only.

Rhodotorula species

Rhodotorula are commonly characterised by pink/red colonies and budding cells. *Rhodotorula*, along with *Sporobolomyces* and *Tilletiopsis*, are anamorphic genera and are assigned to the Urediniomycetes (Fell *et al.*, 2000). Comprising of four major clades, species of *Rhodotorula* are represented in the Microbotryum, Sporidiobolus and Erythrobasidium clades, but are not found in the Agaricostilbum clade. Although carotenoid pigments are not used for taxonomy purposes, the Sporidiobolus clade can be differentiated on the basis of this feature (Fell *et al.*, 2000). Three species of *Rhodotorula, R. glutinis, R. mucilaginosa* and *R. minuta* have been reported as pathogenetic to humans (Diekema *et al.*, 2005). Sequence analyses of the D1/D2 [Fig 3.18] and ITS [Fig 3.19] of the 26S ribosomal DNA identified UNE1130a as *R. mucilaginosa,* UNE1266b as *R. laryngis* and UNE17b as *R. minuta.*

Rhodotorula mucilaginosa

Physiological analyses of *R. mucilaginosa* UNE1130a confirming the phylogenetic results included the inability to assimilate inositol, positive reactions to urease and Diazonium Blue B. Starch formation is negative. No sexual state was observed from cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. *R. mucilaginosa* had a greater percentage of total fatty acids as unsaturated. Zlatanov *et al.* (Zlatanov *et al.*, 2001) reported a similar profile in a *R. mucilaginosa* strain isolated from Livingston Island, Antarctica using the same growth temperature as this study. This result confirmed earlier reports by Watson (Watson, 1980) and Watson & Arthur (Watson and Arthur, 1976) in which it was reported that psychrophilic organisms had a predominance for C_{18} unsaturated fatty acids. Optimal growth temperature studies indicated *R. mucilaginosa* to grow across a wide temperature spectrum, while maximum growth occurred between 15 and 20°C, adequate growth occurred up to 37°C with scant growth at 50°C. *R. mucilaginosa* was found to contain predominately $CoQH_2$-8 and CoQ-8 in the ratio 64% to 36% respectively [see Table 6.2]. A comprehensive study on stress tolerance in *R. mucilaginosa* UNE1130a is presented in Chapter 6.

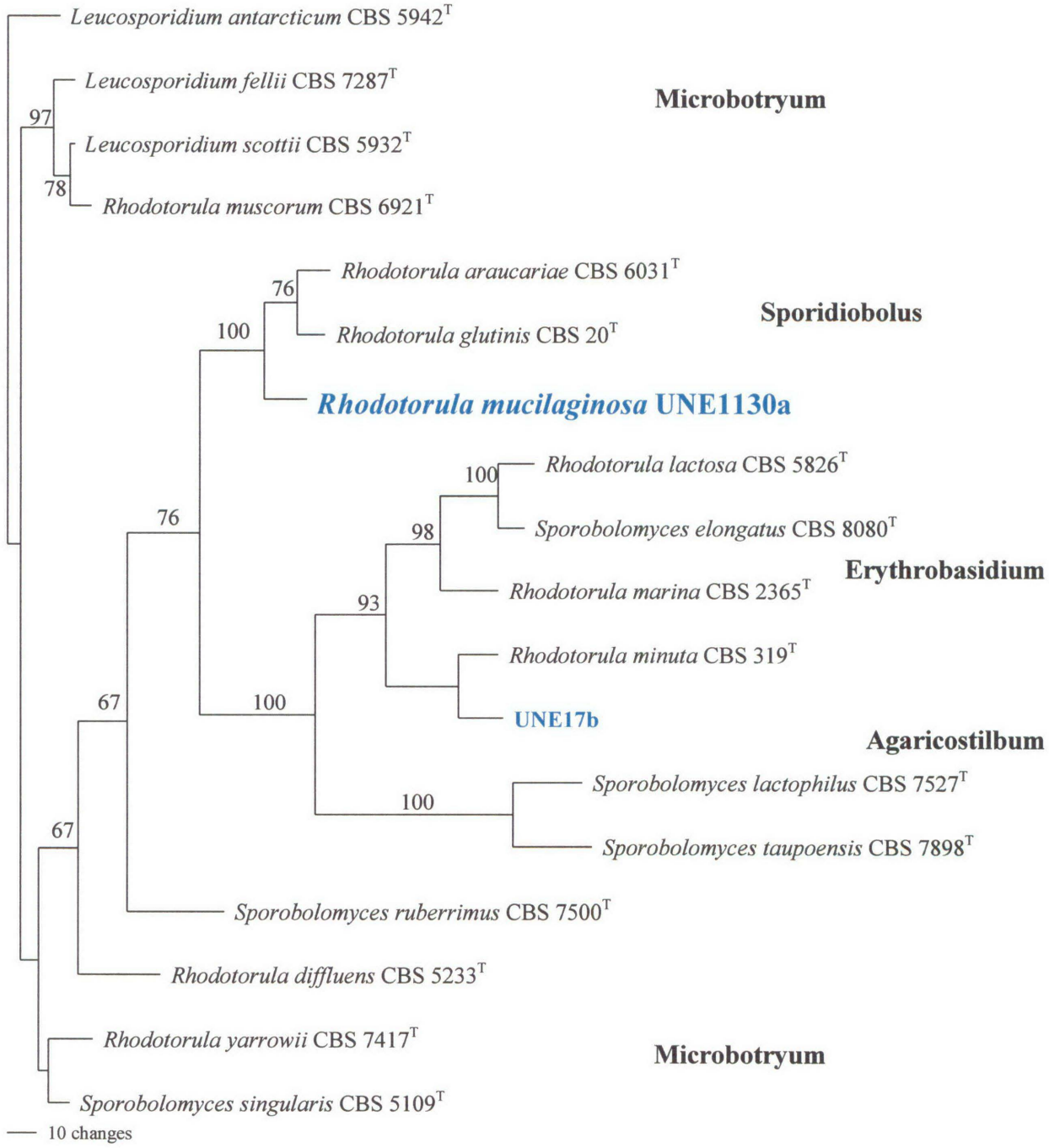

Figure 3.18. Phylogenetic tree showing placement of *Rhodotorula mucilaginosa* among related basidiomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. Labelled clades have representative species only.

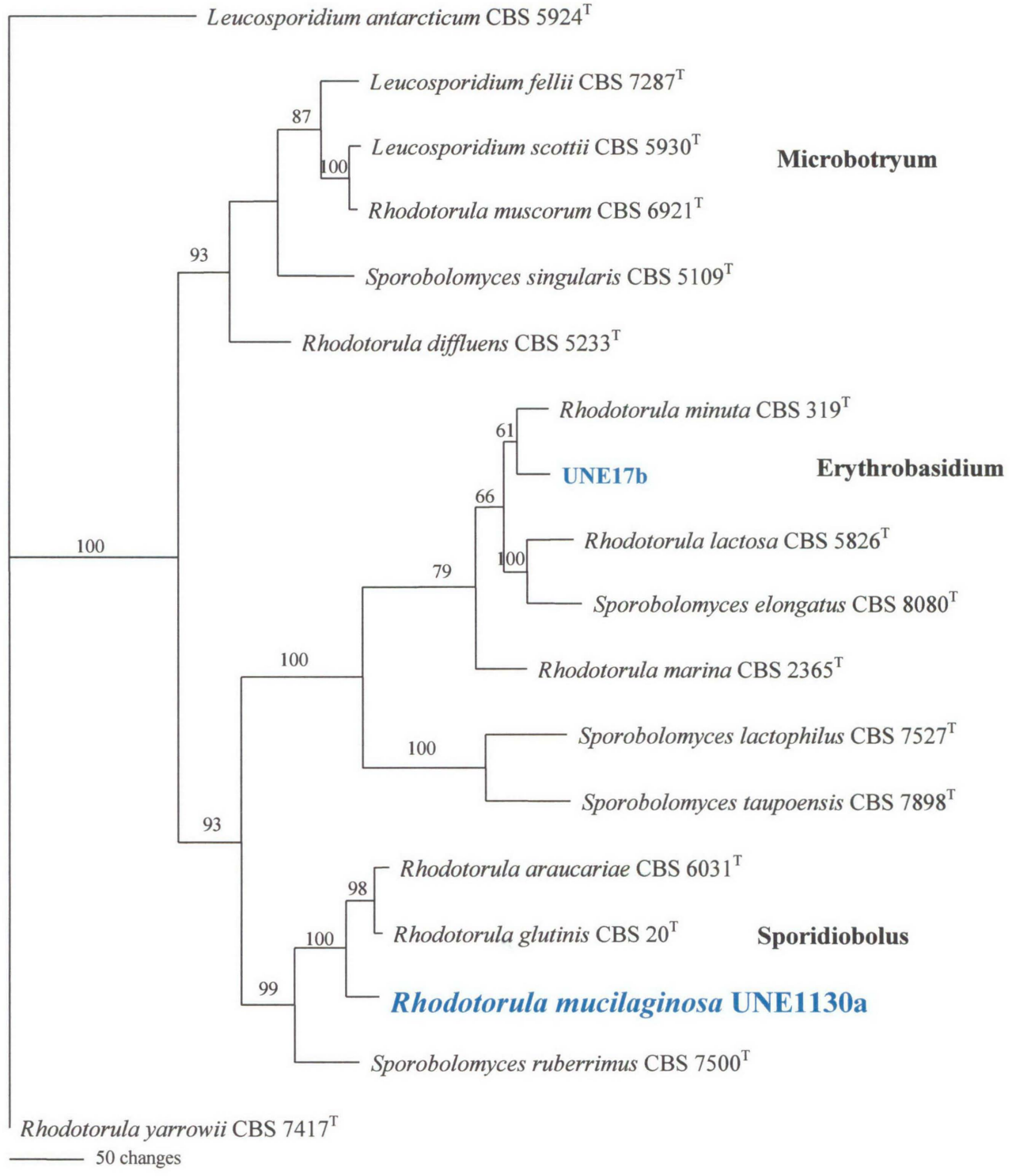

Figure 3.19. Phylogenetic tree showing placement of *Rhodotorula mucilaginosa* among related basidiomycetous yeasts derived from maximum parsimony analysis of the ITS1-5S-ITS2 region. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. Labelled clades have representative species only.