CHAPTER 4

The Ascomycetes

Introduction

The ascomycetes are the largest phylum of fungi, accounting for approximately 80% of all described fungi. The majority of pathogenetic and opportunistic species belong to the ascomycetes such as the yeast *Candida albicans*. Although *C. albicans* is the most common cause of candidiasis, other species such as *C. parapsilosis, C. tropicalis, C. guilliermondii* and *C. krusei* can cause candidiasis, occasionally with fatal outcomes. The clinical manifestations of a *Candida* infection are either local or systemic. Local infections include oral candidiasis (thrush), cutaneous candidiasis (nappy rash) and vulvovaginitis (Braunwald *et al.*, 2001). The most notable ascomycetous yeast is *Saccharomyces cerevisiae*, otherwise known as baker's/brewer's yeast. *S. cerevisiae* is the predominant yeast used by the wine industry although the uses of other 'wild-type' yeast strains are beginning to be utilized. The ascomycetes are divided into three lineages: The Hemiascomycetes, the Euascomycetes and the Archiascomycetes (Kurtzman and Robnett, 1998). As with the basidiomycetes, these classes are based on sequence analysis of the D1/D2 region of the 26S rDNA. Sexual reproduction in the ascomycetes is characterised by the presence of the ascus. The ascus is bulbous in shape, hence the term *sac fungi* is commonly used. Ascospores are produced within the asci. The arrangement of the asci varies from species to species as does the number of ascospores within them, however, the function of the asci is the same: to release haploid ascospores that are resilient to harsh environmental conditions. Due to the large number of species of ascomycetes, only the ascomycetous yeasts will be discussed in this communication.

Archiascomycetes

The "Archiascomycetes" consist of four orders, namely: The Schizosaccharomycetales comprising of the genera *Schizosaccharomyces*; the Taphrinales, which include the genera *Taphrina*; the Protomycetales consisting of genera *Protomyces* and *Saitoella*; and the

Pneumocystidales including the genera *Pneumocystis*. The "Archiascomycetes" are a diverse group including parasitic and pathogenetic fungi such as *Taphrina* that consist of a parasitic mycelial stage on plant hosts, and *Pneumocystis carinii* which is the infective agent of a pneumonia affecting immunocompromised individuals. This class was recently formed in 1993 by Nishida and Sugiyama based on sequence analyse of the rDNA (Kurtzman, 1998a).

Euascomycetes

The Euascomycetes are a sister group to the Hemiascomycetes consisting of the filamentous fungi and comprises over 90% of ascomycetes. The majority of the Euascomycetes form asci upon a fruiting body with the exception of the yeast clade (Kurtzman, 1998a). Yeast genera found in the Euascomycetes are *Endomyces* and *Oosporidium.*

Hemiascomycetes

The Hemiascomycetes include the budding yeasts and yeast-like genera. Comprising the order Saccharomycetales these species do not form asci upon fruiting bodies as do the Euascomycetes (Kurtzman and Robnett, 1998). The Hemiascomycetes consist of ten families within the order Saccharomycetales; Ascoideaceae, Cephaloascaceae, Dipodascaceae, Endomycetaceae, Eremotheciaceae, Lipomycetaceae, Metschnikowiaceae, Saccharomycetaceae, Saccharomycopsidaceae and Candidaceae. These yeasts are capable of replication either by budding or fission. Asci are either singular or in chains and are morphologically similar to the vegetative cell. The ascospores come in various shapes from the hat-shape asci of the species *Saturnispora* to the cylindrical asci belonging to *Dipodascopsis*.

The genus *Candida* is polyphyletic throughout the Saccharomycetales and is represented by 163 species with teleomorphs in such genera as *Pichia* and *Debaryomyces*. There are many clinically relevant forms of *Candida* including *C. albicans*. Although part of the normal body flora, they can become opportunistic in immunocompromised hosts.

Figure 4.1. Classification of the Ascomycetes

ASCOMYCETE [subdivision]
"ARCHIASCOMYCETE" [class]
Schizosaccharomycetales [order]
Schizosaccharomycetaceae [family]
Schizosaccharomyces
Taphrinales [order]
Taphrinaceae [family]
Taphrina
Lalaria
Protomycetales [order]
Protomycetaceae [family]
Protomyces
Saitoella
Pneumocystidales [order]
Pneumocystidaceae [family]
Pneumocystis
EUASCOMYCETES [class]
Endomyces
Oosporidium
HEMIASCOMYCETES [class]
Saccharomycetales [order]
Ascoideaceae [family]
Ascoidea
Cephaloascaceae [family]
Cephaloascus
Dipodascaceae [family]
Dipodascus
Galactomyces
Sporopachydermis
Stephanoascus
Wickerhamiella
Yarrowia
Zygoascus
Endomycetaceae [family]
Endomyces
Helicogonium
Myriogonium
Phialoascus
Trichomonascus
Eremotheciaceae [family]
Eremothecium
Coccidiascus

Lipomycetaceae [family]
- *Babjevia*
- *Dipodascopsis*
- *Lipomyces*
- *Zygozyma*

Metschnikowiaceae [family]
- *Clavispora*
- *Metschnikowia*

Saccharomycetaceae [family]
- *Ariozyma*
- *Citeromyces*
- *Cyniclomyces*
- *Debaryomyces*
- *Dekkera*
- *Issatchenkia*
- *Kluyveromyces*
- *Lodderomyces*
- *Pachysolen*
- *Pichia*
- *Saccharomyces*
- *Saturnispora*
- *Torulaspora*
- *Williopsis*
- *Zygosaccharomyces*

Saccharomycodaceae [family]
- *Hanseniaspora*
- *Nadsonia*
- *Saccharomycodes*
- *Wickerhamia*

Saccharomycopsidaceae [family]
- *Ambrosiozyma*
- *Saccharomycopsis*

Candidaceae [family]
- *Aciculoconidium*
- *Arxula*
- *Blastobotrys*
- *Botryozyma*
- *Brettanomyces*
- *Candida*
- *Geotrichum*
- *Kloeckera*
- *Myxozyma*
- *Schizoblastosporion*
- *Sympodiomyces*
- *Trigonopsis*

Table 4.1. Ascomycetous yeasts and reference species examined in the D1/D2 rDNA regions

Species	26S GenBank accession no.	CBS no.
Candida albicans	U45776	562^T
Candida atlantica	U45799	5263^T
Candida berthetii	U62298	5452^T
Candida boleticola	U45777	6420^T
Candida catenulate	U45714	565^T
Candida dendrica	U62301	6151^T
Candida dubliniensis	U57685	7987^T
Candida ergastensis	U45746	6248^T
Candida fragi	U71071	7702^T
Candida friedrichii	U45781	4114^T
Candida lodderae	U45755	1924^T
Candida maltosa	U45745	5611^T
Candida membranifaciens	U45792	1952^T
Candida mesenterica	U45720	602^T
Candida montana	U62305	8057^T
Candida norvegica	U62299	4239^T
Candida parapsilosis	U45754	604^T
Candida psychrophila	U45813	5956^T
Candida quercitrusa	U45831	4412^T
Candida rugosa	U45727	613^T
Candida savonica	U62307	6563^T
Candida schatavii	U45795	6452^T
Candida shetatae var. *shetatae*	U45761	5813^T
Candida stellimalicola	U84234	7853^T
Candida suecica	U45732	5724^T
Candida tropicalis	U45749	94^T
Ceratocystis fimbriata	U94917	146.53^T
Debaryomyces castelii	U45841	2923^T
Debaryomyces (Pichia) etchellsii	U45809	2011^T
Debaryomyces hansenii var. *hansenii*	U45808	767^T
Debaryomyces nepalensis	U45839	5921^T
Debaryomyces occidentalis var. occidentalis	U45804	819^T
Debaryomyces pseudopolymorphus	U45845	2008^T
Debaryomyces (Wingea) robertsiae	U45805	2934^T
Debaryomyces yamadae	U45837	7035^T
Endomyces scopularum	Y40092	131.86^T
Filobasidiella neoformans	U94941	882^T
Oosporidium margaritiferum	U40090	2531^T
Pichia amethionina var. *amethionina*	U75424	6940^T
Pichia caribaea	U75426	7692^T
Pichia dryadoides	U75422	6154^T

Pichia populi	U75427	8094^T
Pichia quercuum	U75416	2283^T
Pichia (Yamadazyma) segobiensis	U45742	6857^T
Pichia (Yamadazyma) stipites	U45741	5773^T
Schizosaccharomyces pombe	U40085	356^T
Williopsis californica	U75957	252^T
Williopsis (Komagataea) pratensis	U75964	7079^T

Candida species

The genus *Candida* includes over 150 anamorphic species with teleomorphs in as many as 13 genera. Of these, 20 species of *Candida* have now been linked to human infections (Guarro *et al.*, 1999). Species of *Candida* are found throughout the Hemiascomycetes in various clades such as *Saccharomyces, Pichia, Debaryomyces* and *Metschnikowia* (Kurtzman and Robnett, 1998) [Fig 4.1]. Characteristics of *Candida* species include asexual reproduction by either multilateral or bipolar budding; cells are either spheroidal, subglobose to ellipsoidal, ogival, or cylindroidal to elongate; pseudomycelium is often present; cultures are slow growing; Diazonium Blue B reaction is negative (Meyer *et al.*, 1998). 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 3 *Candida* species. 1D-SDS-PAGE was used to rapidly identify identical or closely related strains [Fig 4.7]. Following this initial screening process, further categorizations were made utilising morphological and physiological characteristics such as: biochemical assimilations, fatty acid analysis and coenzyme Q analysis.

Candida parapsilosis

Isolates UNE142a and UNE1217a were isolated from soil samples collected in 1997 and 1994 respectively. UNE142a was chosen as the type strain. Sequence analysis identified these strains to be *Candida parapsilosis*. Commonly associated with humans, this strain has also been isolated from various plant and soil samples (Meyer *et al.*, 1998).

Growth in YEP broth after 3 days at 15°C, cells are ovoidal and occur singly or in pairs [Fig 4.2]. Budding is polar. Aerobic cells are pink in colour and viscous in consistency with glossy colonies with a convex elevation and entire margin. 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. Growth on nitrogen base and cornmeal agar is weak. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, L-sorbose, sucrose, maltose, raffinose, D-xylose, L-arabinose, ethanol, erythritol, D-mannitol, α-methyl-D-glucoside, salicin, D-gluconate, and D-glucoronate; negative reaction for cellobiose, citrate trehalose, melibiose, melezitose, lactose, D-ribose, D-arabinose, L-rhamnose, methanol, glycerol, ribitol, galactitol, D-glucitol, *N*-acetyl-D-glucosamine, DL-lactate, succinate, inositol and hexadecane. Assimilation of soluble starch is negative. Assimilation of nitrate is negative. Growth in vitamin-free and biotin-free media is negative. Growth in thiamine-free media is negative. Growth is very weak on 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose. Gelatine liquefication is negative. Urease and Diazonium Blue B reaction is negative. Starch formation is negative. Growth at 20°C is positive, very weak at 25°C. The strain was isolated from soil, Mossell Lake, Vestfold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.

Phylogenetic analysis [Fig 4.5] placed *C. parapsilosis* in the Saccharomycetaceae family along with other pathogenetic *Candida* species such as *C. albicans* and *C. tropicalis*. All strains utilised in the phylogenetic analyses are listed in Table 4.1. Protein profiles for *C. parapsilosis* strains UNE142a and UNE1217a were obtained by 1D-SDS-PAGE and comparative protein banding patterns indicated these strains to have different 1D-protein profiles [Fig 4.7] even though the sequencing data (D1/D2 region) indicated the two strains were conspecific. Fatty acid analysis [Fig 4.3] revealed linolenic acid ($C_{18:3}$) to be the predominant fatty acid present (32%) as well as significant amounts of linoleic acid ($C_{18:2}$). *C. parapsilosis* was found to contain predominately $CoQH_2$-9 and CoQ-9 in the ratio 95% to 5% respectively [see Table 6.2].

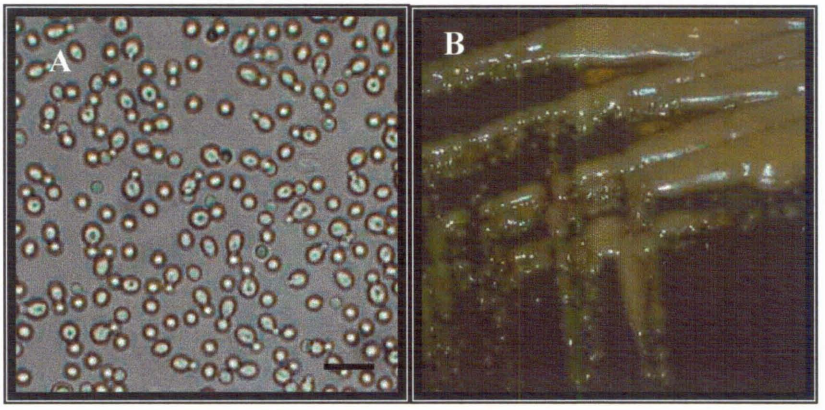

Figure 4.2. *Candida parapsilosis* after 3 days at 15°C in YEP broth showing budding cells [A]. Bar = 10 µm. [B] Colony formation of *C. parapsilosis* after 14 days at 6°C on YEP

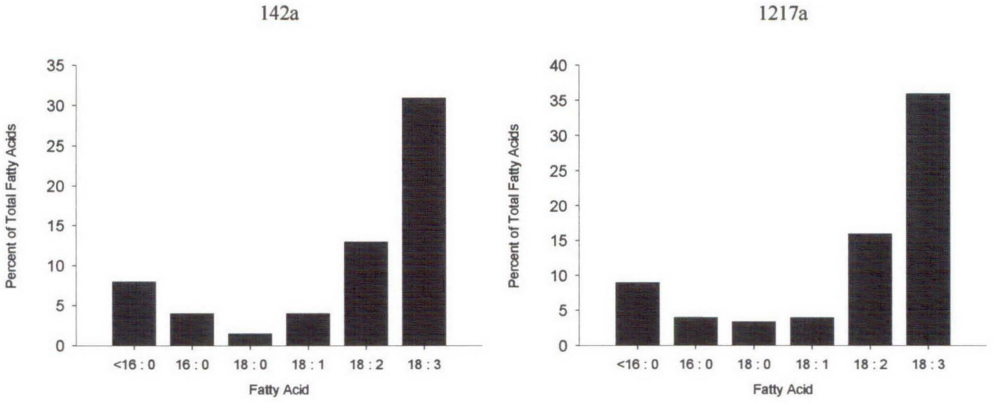

Figure 4.3. Fatty acid profiles for *Candida parapsilosis* [A] strain UNE142a [B] strain UNE1217a

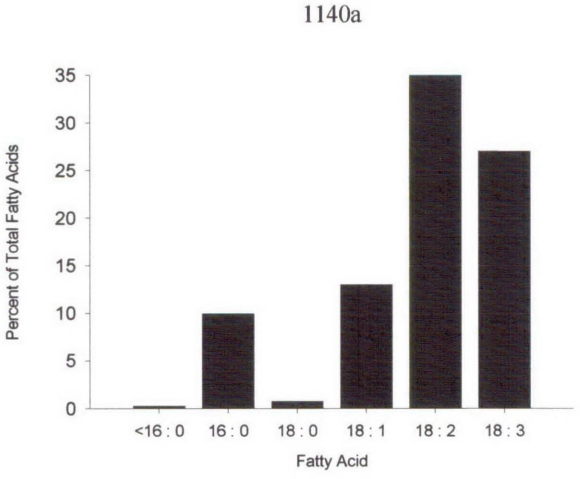

Figure 4.4. Fatty acid profile of *Candida norvegica* strain UNE1140a

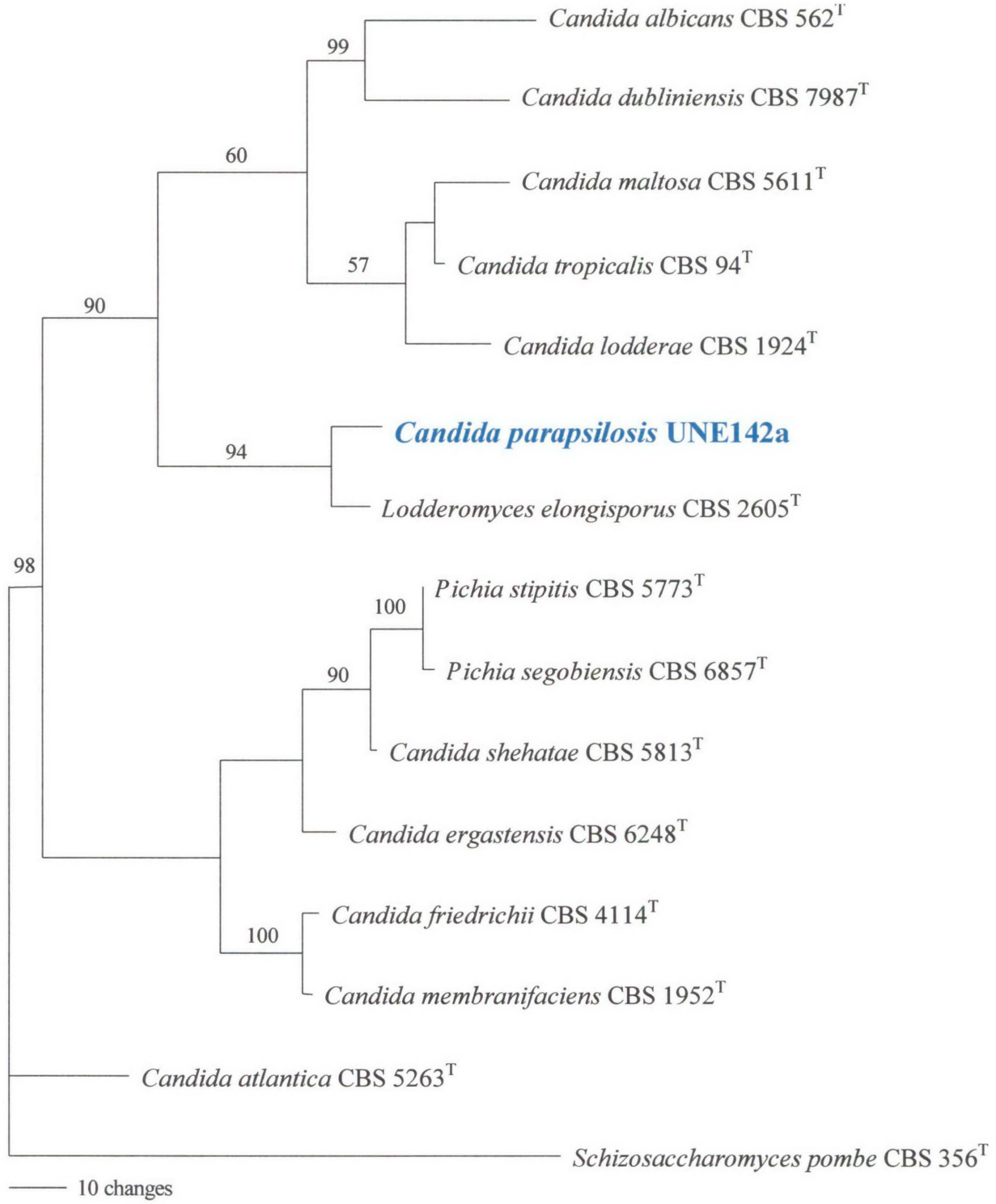

Figure 4.5. Phylogenetic tree showing placement of *Candida parapsilosis* among related ascomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Schizosaccharomyces pombe* is the designated out-group species in this analysis.

Candida norvegica

Isolate UNE1140a was isolated from soil samples collected in 1994. Sequence analysis identified this strain to be *Candida norvegica*. This strain has previously been isolated from seawater, fruit and miso (Meyer *et al.*, 1998).

Growth in YEP broth after 3 days at 15°C, cells are globose and occur singly or in pairs. Aerobic cells are white in colour and butyrous in consistency with smooth and shiny colonies with a convex elevation and entire margin. 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. Growth on nitrogen base and cornmeal agar is weak. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, cellobiose, inulin, L-rhamnose, ethanol, glycerol, D-mannitol, salicin, succinate, citrate, and D-glucoronate; negative reaction for L-sorbose, sucrose, maltose, trehalose, lactose, melibiose, raffinose, melezitose, D-xylose, L-arabinose, D-ribose, D-arabinose, *N*-acetyl-D-glucosamine, methanol, erythritol, ribitol, galactitol, D-glucitol, α-methyl-D-glucoside, D-gluconate, DL-lactate, inositol and hexadecane. Assimilation of soluble starch is negative. Assimilation of nitrate is positive. Growth in vitamin-free and biotin-free media is negative. Growth in thiamine-free media is negative. Growth is negative on 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose. Gelatine liquefication is negative. Urease and Diazonium Blue B reaction is negative. Starch formation is negative. Growth at 20°C is positive, very weak at 25°C. The strain was isolated from soil, Mossell Lake, Vestfold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.

Phylogenetic analysis placed *C. norvegica* in the *Pichia anomala* clade along with most *Williopsis* species [Fig 4.6]. All strains utilised in the phylogenetic analyses are listed in Table 4.1. Protein profiles [Fig 4.7] for *C. norvegica* strain UNE1140a was obtained by 1D-SDS-PAGE. Fatty acid analysis revealed the polyunsaturated linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) to be the predominant fatty acids present (35% and 27% respectively) [Fig 4.4]. *C. norvegica* was found to contain predominately $CoQH_2$-8 and CoQ-8 in the ratio 90% to 10% respectively [see Table 6.2].

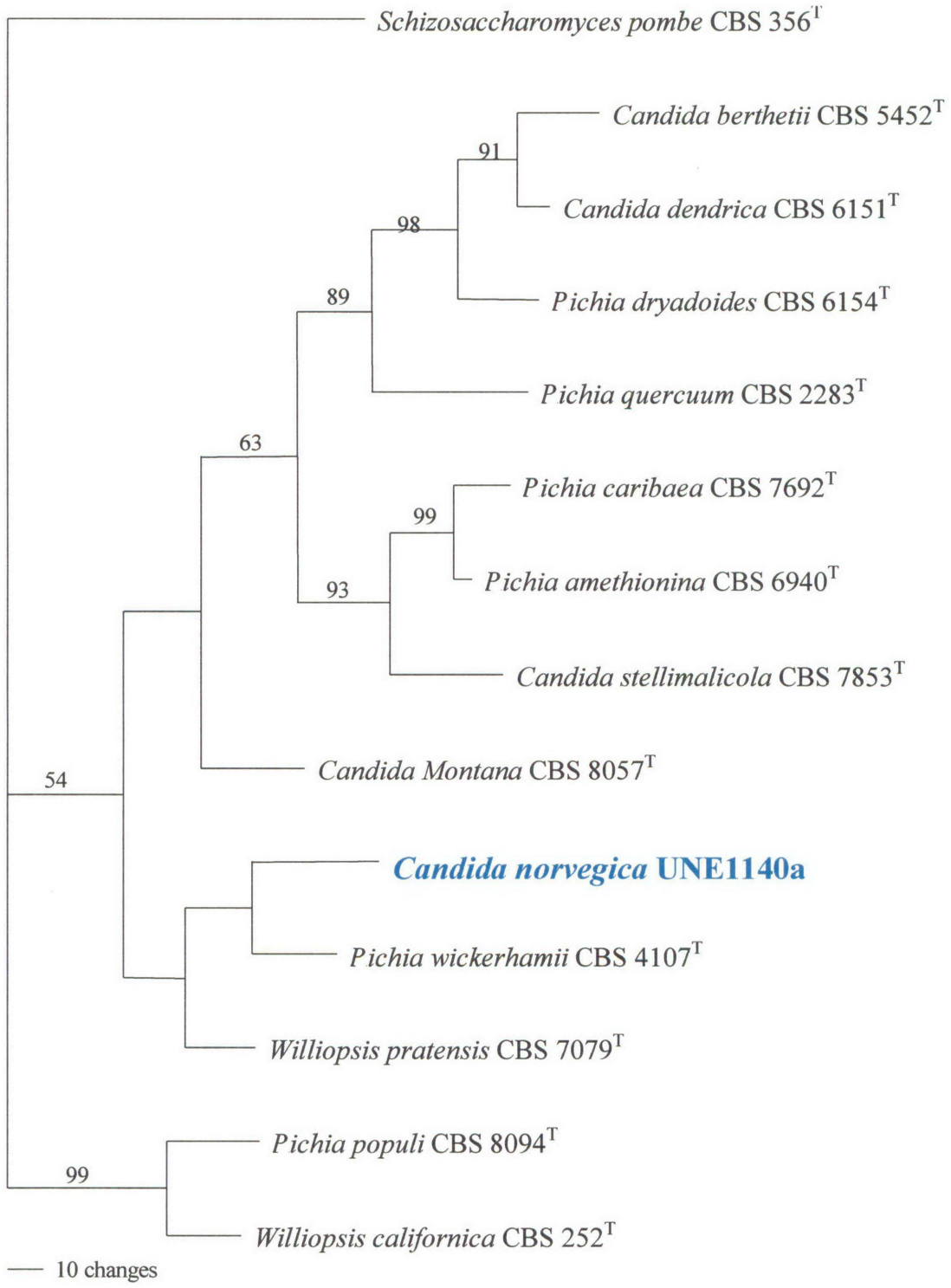

Figure 4.6. Phylogenetic tree showing placement of *Candida norvegica* among related ascomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Schizosaccharomyces pombe* is the designated out-group species in this analysis.

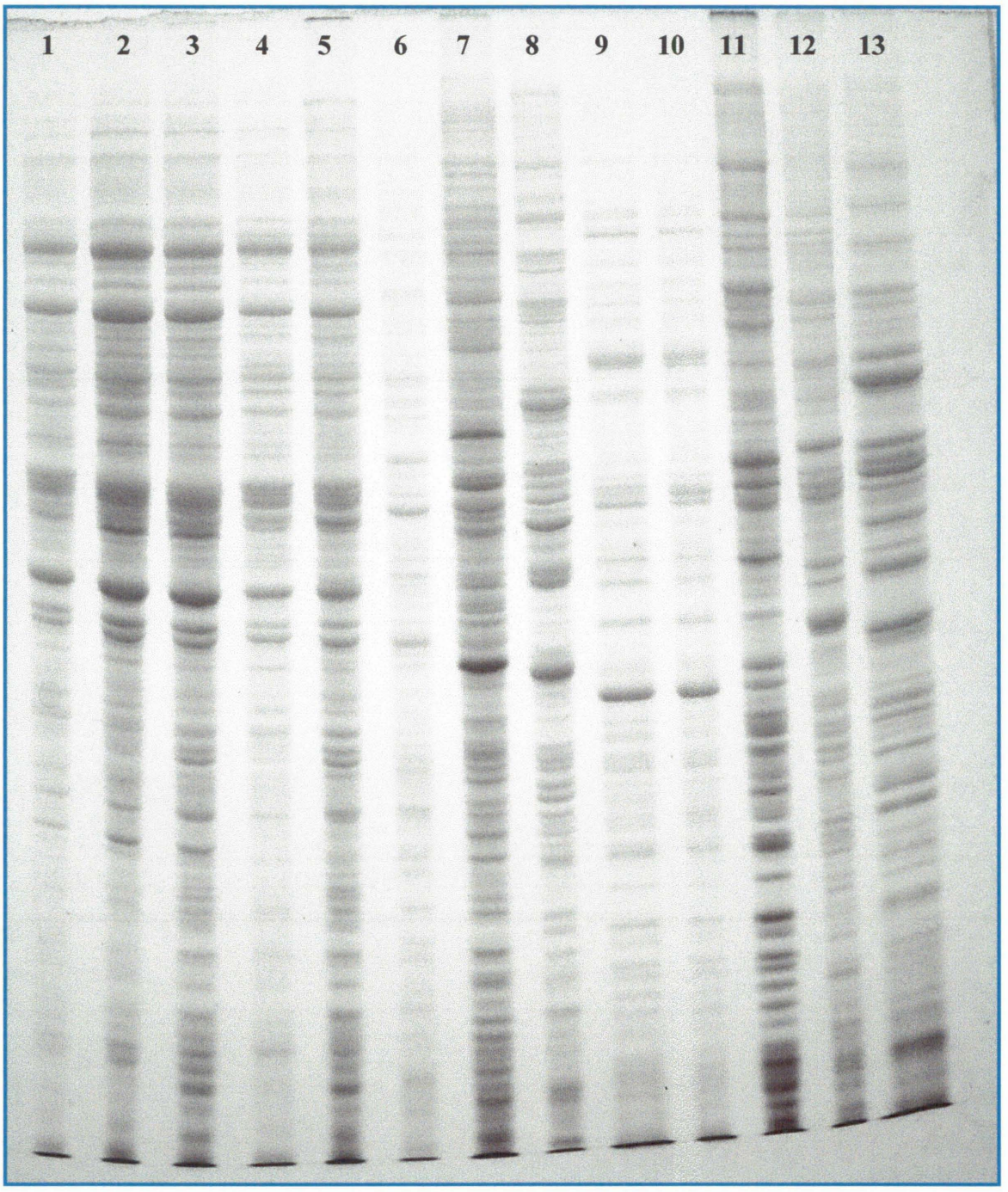

Figure 4.7. 1D-SDS-PAGE of whole cell proteins from various Ascomycetous yeasts

Lane 1 through 5 - Unknown Ascomycete yeast [98e, 116c, 1007a, 1033a, 1152c]
Lanes 6 & 7 - *C. parapsilosis* [1217a, 142a]
Lane 8 - *C. norvegica* [1140a]
Lanes 9 & 10 - *Debaryomyces hansenii* [1151a, 176a]
Lane 11 - *C. antarcticum* [CBS ?]
Lane 12 - *Pichia membranifaciens* [CBS 1952]
Lane 13 - *Saccharomyces cerevisiae* [K7]

Debaryomyces hansenii

Isolates UNE176a and UNE1151a were isolated from soil samples collected in 1997 and 1994 respectively. UNE176a was chosen as the type strain. Sequence analysis identified these strains to be *Debaryomyces hansenii*. Commonly associated with processed meats and cheeses, *D. hansenii* has also been isolated from human and fresh food sources (Meyer *et al.*, 1998) and is frequently isolated from seawater (Lachance and Starmer, 1998).

Growth in YEP broth after 3 days at 15°C, cells are ovoidal and occur singly, in pairs or chains [Fig 4.9]. Budding is polar. Cells grown aerobically are white in colour and viscous in consistency with glossy colonies with a convex elevation and entire margin. 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. Growth on nitrogen base and cornmeal agar is weak. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, raffinose, melezitose, inulin, D-xylose, L-arabinose, L-rhamnose, D-glucosamine, *N*-acetyl- ethanol, glycerol, ribitol, D-mannitol, α-methyl-D-glucoside, salicin, succinate, citrate and hexadecane; negative reaction for melibiose, D-arabinose, D-ribose, methanol, erythritol, galactitol, D-glucitol, D-gluconate, DL-lactate, inositol and D-glucoronate. Assimilation of soluble starch is negative. Assimilation of nitrate is negative. Growth in vitamin-free, biotin-free and thiamine-free media is positive. Growth is very weak on 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose. Gelatine liquefication is negative. Urease and Diazonium Blue B reaction is negative. Starch formation is negative. Growth at 20°C is positive, very weak at 25°C. The strain was isolated from soil, Lichen Valley, Vestfold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.

Phylogenetic analysis placed *D. hansenii* amongst other *Debaryomyces* species [Fig 4.10]. The *Debaryomyces* species separated into four clades, it was noteworthy that *D. hansenii* is in the same clade as *Candida psychrophila* which originates from Antarctica. All strains utilised in the phylogenetic analyses are listed in Table 4.1. Protein profiles [Fig 4.7] for *D. hansenii* strains UNE176a and UNE1151a were obtained by 1D-SDS-PAGE and comparative protein banding patterns indicated these strains to have identical 1D-protein profiles. On the other hand, the fatty acid profiles of the two strains were different in that UNE176a was rich in $C_{18:0}$ with similar amounts of $C_{18:1}$ and $C_{18:2}$ in comparison to UNE1152a

which was rich in $C_{18:2}$ as well as significant amounts of $C_{18:3}$ [Fig 4.8]. *D. hansenii* was found to contain predominately $CoQH_2$-9 and CoQ-9 in the ratio 94% to 6% respectively [see Table 6.2].

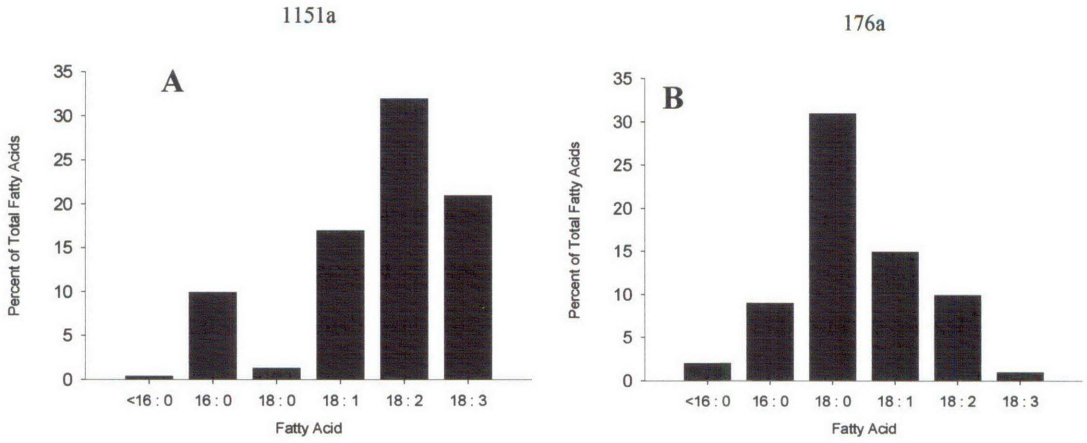

Figure 4.8. Fatty acid profiles for *Debaryomyces hansenii* [A] strain UNE1151a; [B] strain UNE176a

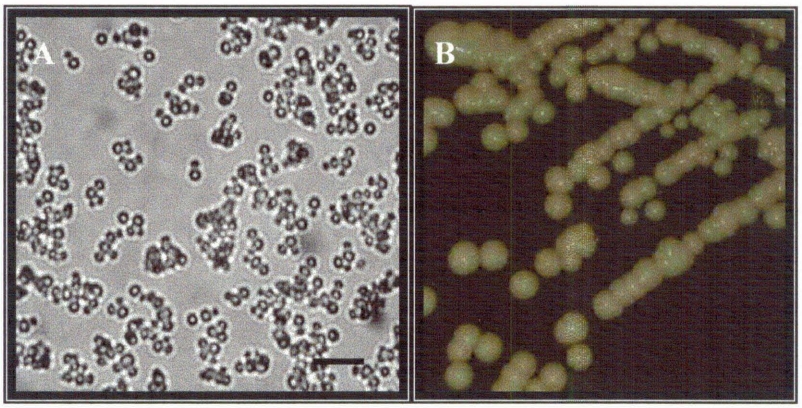

Figure 4.9. *Debaryomyces hansenii* after 3 days at 15°C in YEP broth showing budding cells [A]. Bar = 10 μm. [B] Colony formation of *D. hansenii* after 14 d at 6°C on YEP

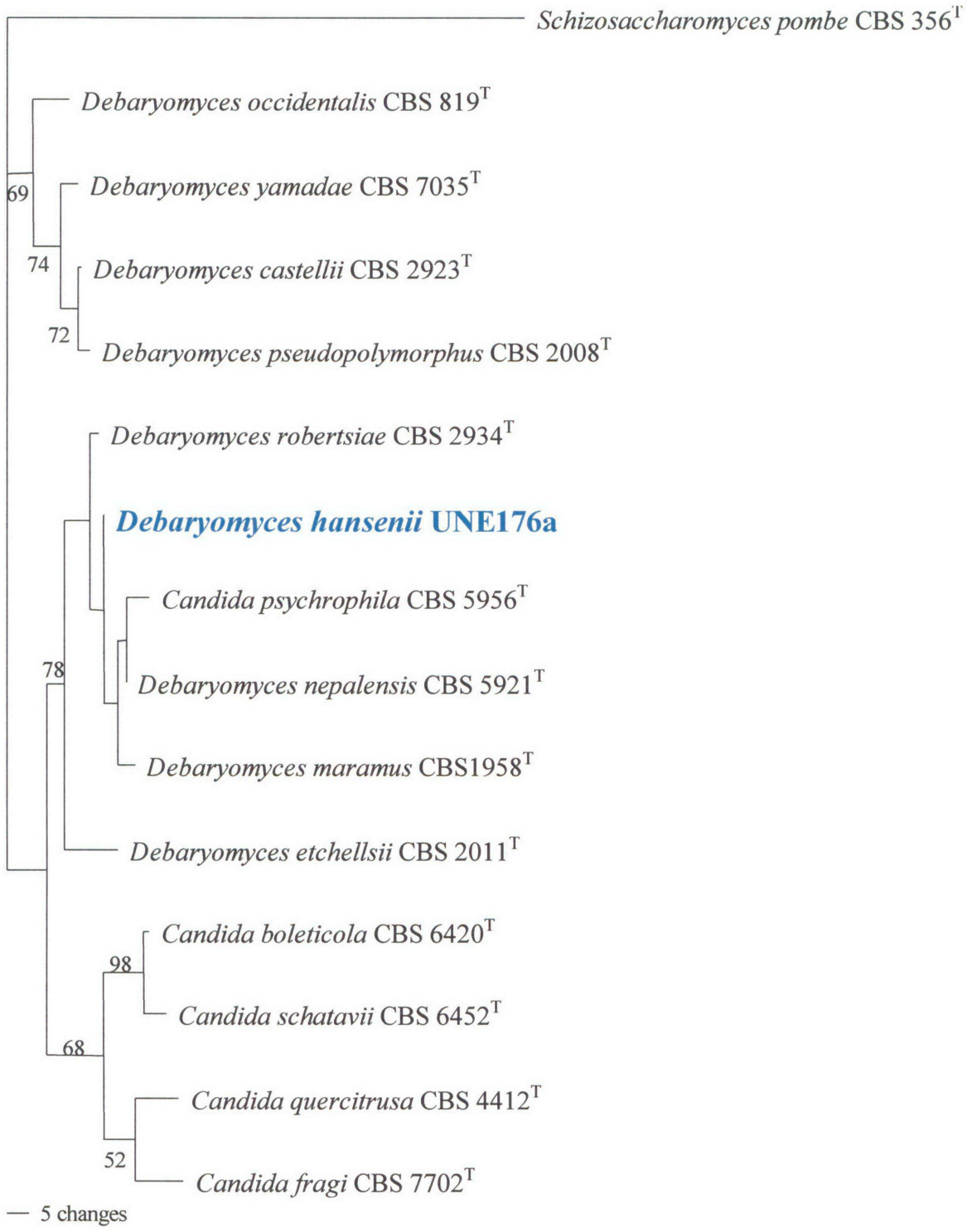

Figure 4.10. Phylogenetic tree showing placement of *Debaryomyces hansenii* among related ascomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. *Schizosaccharomyces pombe* is the designated out-group species in this analysis.

UNE116c

Isolates UNE98e and UNE116a were isolated from soil and lichen samples collected in 1997, while isolates UNE1007a, UNE1033a and UNE1152c were from soil samples collected in 1994. UNE116c was chosen as the type strain. Sequence analysis identified these strains to be unique.

Growth in YEP broth after 3 days at 15°C, cells are elongate and occur singly or in pairs [Fig 4.12]. Budding is polar. Cells grown aerobically are orange, dry and friable in consistency with punctiform colonies with a pulinate elevation and irregular margin. 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. Growth on nitrogen base and cornmeal agar is weak. Assimilation of carbon compounds is as follows: positive reaction for glucose, galactose, sucrose, maltose, cellobiose, trehalose, melibiose, melezitose, D-xylose, L-arabinose, D-ribose, L-rhamnose, *N*-acetyl-D-glucosamine, ribitol salicin, D-gluconate, citrate and D-glucoronate; negative reaction for L-sorbose, lactose, raffinose, D-arabinose, methanol, ethanol, glycerol, erythritol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, DL-lactate, succinate, inositol and hexadecane. Assimilation of soluble starch is weak. Assimilation of nitrate is positive. Growth in vitamin-free and biotin-free media is negative. Growth in thiamine-free media is positive. Growth is negative on 50% (w/w) glucose-yeast extract agar and 10% NaCl 5% glucose. Gelatine liquefication is negative. Urease and Diazonium Blue B reaction is negative. Starch formation is negative. Growth at 20°C is positive, negative at 25°C. The strain was isolated from soil and lichen, Moss Cirque, Vestfold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.

Sequence analysis of the D1/D2 region of the large ribosomal subunit (26S) of the proposed new species was phylogenetically analysed in a database containing all currently recognised ascomycetous and basidiomycetous yeasts. This analysis placed the unidentified psychrophilic strain in the Archiascomycete clade [Fig 4.13] with 14 nucleotide changes from *Leuconeurospora pulcherrima* and 38 nucleotide changes from *Oosporidium margaritiferum* thus indicating this to be a new species. This strain has since been sent to the Centraalbureau voor Schimmelcultures (CBS), Delft/Baarn, The Netherlands, where Dr. de Hoog is currently carrying out further analysis utilizing 18S sequencing. Preliminary sequencing results indicate

that UNE116c is 99.8% similar with a black, Aureobasidium-like strain. This is highly unusual due to UNE116c being orange in colour and not producing any black meristematic cells at any stage. All strains employed in the final rDNA analysis are listed in Table 4.1. No ascospores were observed. Fatty acid analysis [Fig 4.11] of UNE116c revealed oleic acid ($C_{18:1}$) to be the predominant fatty acid present with significant levels of the polyunsaturated fatty acids linoleic ($C_{18:2}$) and trace amounts of linolenic ($C_{18:3}$). Fatty acid analysis [Fig 4.11] of strain UNE1152c showed that the polyunsaturated fatty acids $C_{18:2}$ and $C_{18:3}$ made up approximately 60% of the total fatty acids present. Strain UNE116c was found to contain predominately $CoQH_2$-9 and CoQ-9 in the ratio 63% to 37% respectively together with smaller but significant amounts of $CoQH_2$-8/CoQ-8 (88%:12%) and $CoQH_2$-10/CoQ-10 (95%:5%) [see Table 6.2 and Fig 6.17].

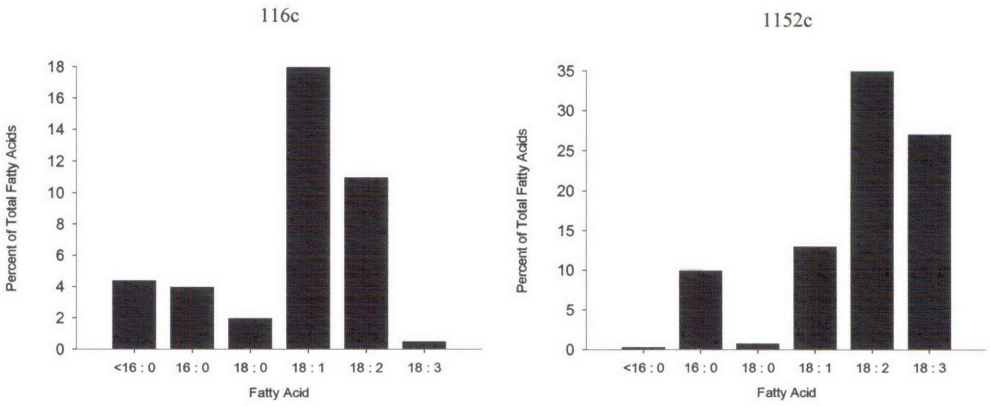

Figure 4.11. Fatty acid profiles for [A] strain UNE116c [B] strain UNE1152c

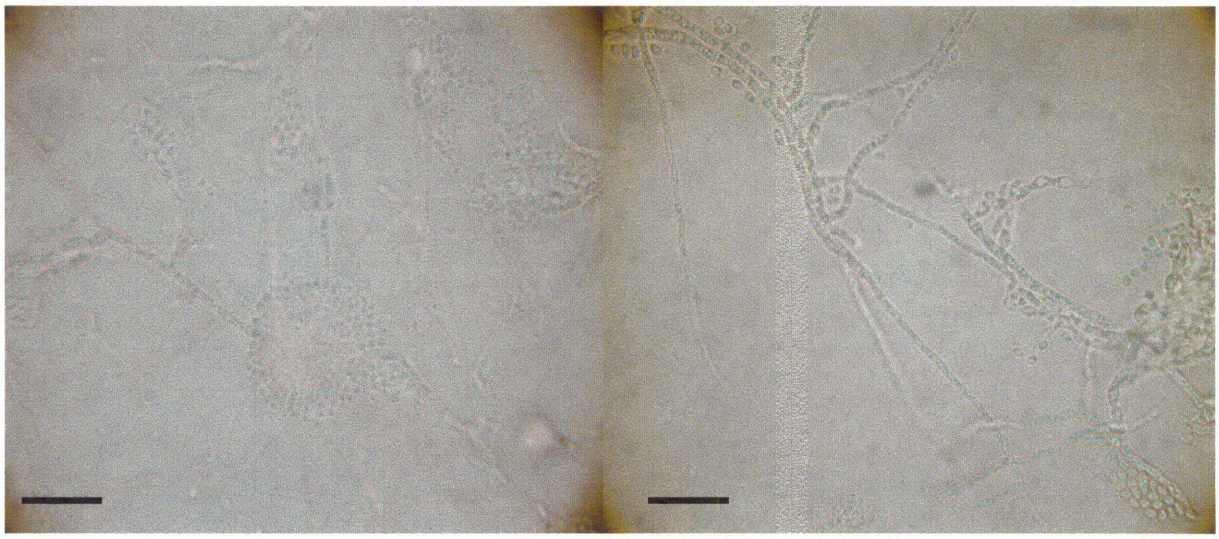

Figure 4.12. UNE116c after 3 d at 15°C in YEP broth demonstrating budding cells and pseudohyphae. Bar = 10 μm

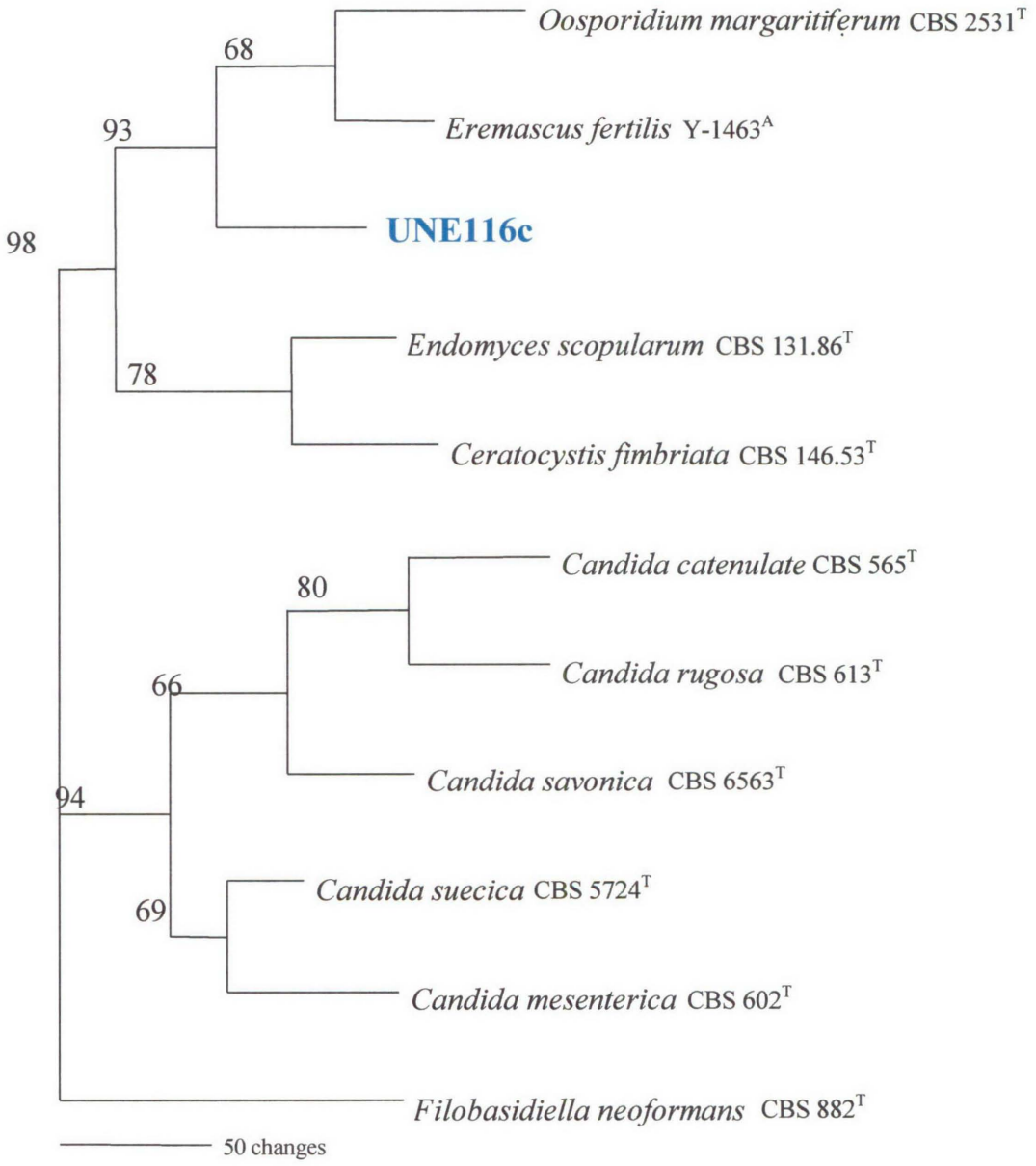

Figure 4.13. Phylogenetic tree showing placement of UNE116c among related ascomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications are displayed. *Filobasidiella neoformans* is the designated out-group species in this analysis. Sequence data of *Eremascus fertilis* is from a NRRL (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA) strain Y-1463^{A}.

CHAPTER 5

The Biodiversity of Antarctic Yeasts

Introduction

Biological diversity, or biodiversity, is the variability that exists among living organisms and ecosystems and includes diversity at the genetic, species and ecosystem levels. Microbial diversity is, quite simply, the measure of the number of different species of microorganisms within a given habitat. Research studies involving microbial diversity have increased dramatically over the past few decades as the profound significance of biodiversity for ecological processes have become apparent. Biodiversity is a measure of community productivity and is also a gauge of community stability. The majority of biodiversity studies (until the 1960s) were initially concerned with plant and animal communities, when the implications of biodiversity were explored in relation to the composition and function of microbial populations (Morris *et al.*, 2002). The total biomass of microorganisms is estimated to be 25 times the total biomass of all animal species. They are responsible for many fundamental ecological processes such as nutrient cycling and degradation of both naturally occurring and man-made compounds. Therefore, a deeper understanding of the biodiversity of microorganisms and the ways in which they interact with each other and their environment is essential to understanding, and protecting the world's ecosystems.

Diversity within the microbial community vastly exceeds that of the animal and plant communities, with 85 - 90% of the latter species already described. Studies have shown microorganisms to occupy a wide range of environmental niches, yet estimates are that only 1 or 2% of the microbial population has been elucidated. Attempts to estimate microbial numbers is problematic, species of well described macroorganisms, such as mammals, total just over 4000, yet a single soil sample can contain more than 4000 different bacterial genomes representing a similar number of different bacterial species (Torsvik *et al.*, 1990).

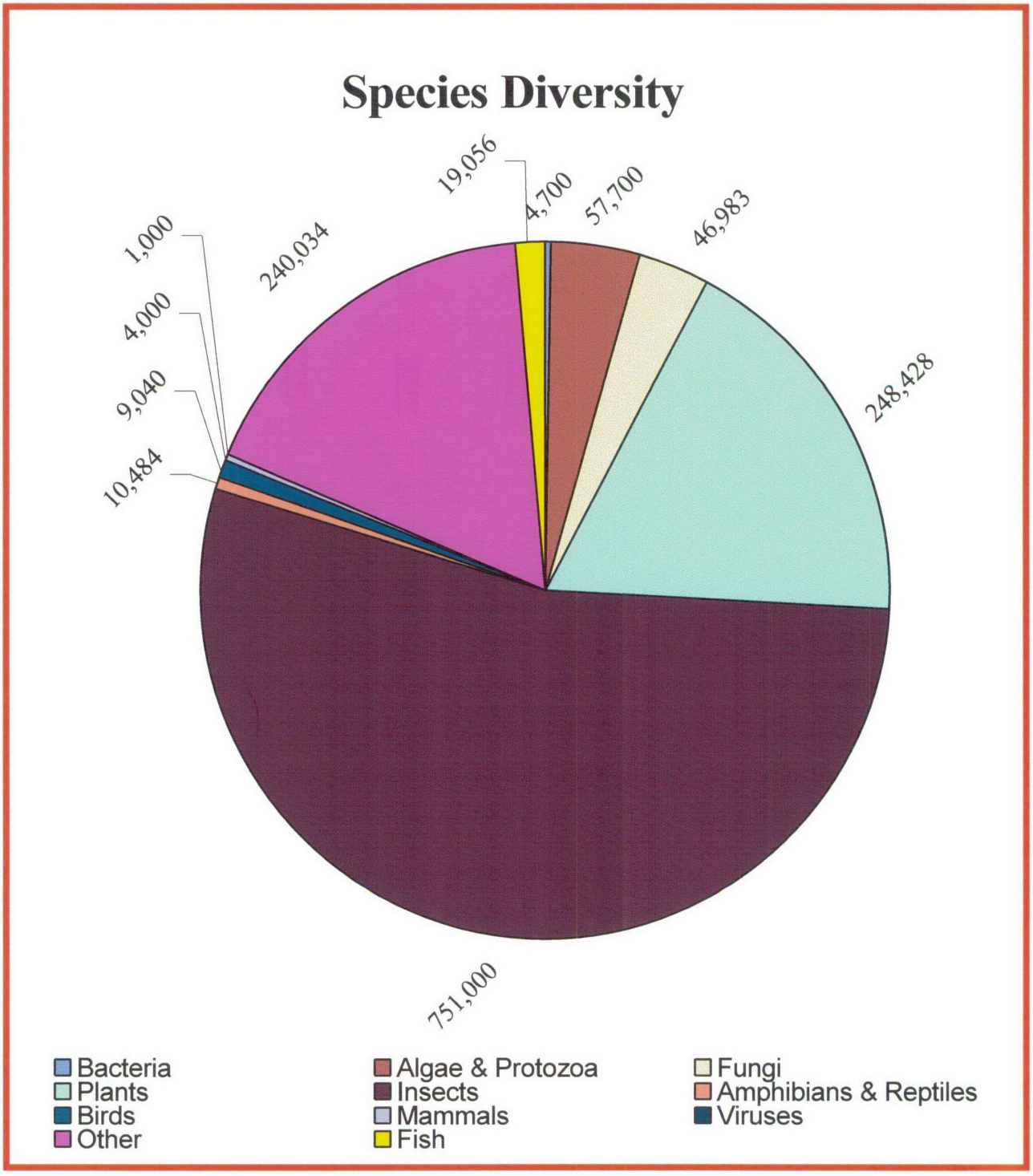

Figure 5.1. Diversity of known species. Adapted from McNeely *et al.*, (1990)

The disparity between identification of microbial communities has been partly attributed to difficulties in culturing microbes from the environment and the nature of the analysis required to classify and identify them. Traditionally, culturing methodology has been used, however, this method only represents a small fraction of organisms present in any given sample. This selectivity means that only microbes that are able to be cultured on a particular medium or media are isolated and identified [Fig 5.2 demonstrates culturable microorganisms]. Molecular techniques have greatly altered this picture. Specifically labelled oligonucleotides can be used to probe samples identifying a particular group of microbes. The Polymerase Chain Reaction (PCR) can be applied to determine the presence of specific organisms or genes. These methods, together with phylogenetic analysis and appropriate computer software, provide a more reliable and rapid method to determine biological diversity. More recently, the development of cultivation-independent techniques such as metagenomics has provided a new momentum to the field of biodiversity. Metagenomics is the analysis of microbial community genome sequence data recovered directly from the environment (Tringe *et al.*, 2005). cDNA libraries are constructed from environmental samples, such as soil, using the same methods as cloning genomic DNA from individual organisms, that is, fragmentation of the genomic soil DNA using restriction enzyme digestion, insertion of DNA fragments into appropriate vectors followed by transformation of the recombinant vector into a host. In theory it appears quite straightforward, however, the enormous numbers involved has proven to be onerous. Molecular techniques have since been used to screen libraries with sequenced based methods (Daniel, 2005). The combination of these methodologies has resulted in a number of pharmaceutical and biotech companies developing large-scale screening methods searching for novel enzymes, bioactive molecules and secondary metabolites (Lorenz and Eck, 2005). As the extent of microbial diversity becomes unearthed, it can be assumed that species numbers for microorganisms will far outweigh those of macroscopic organisms.

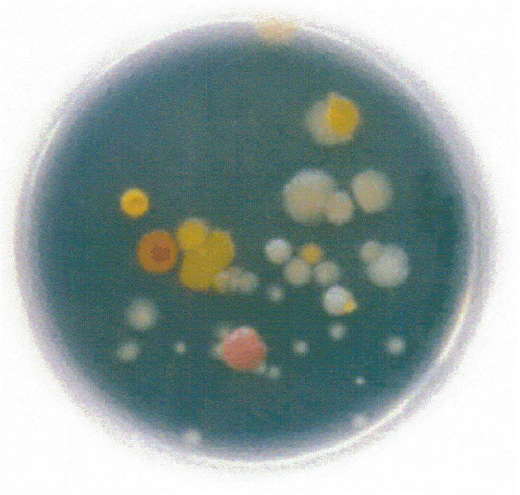

Figure 5.2. Colony formation of a variety of species on an agar plate

The Antarctic niche

The extreme low temperatures that prevail in the Antarctic limit the diversity of plants and animals in this geographically isolated area. Vegetation is limited, there are no trees or shrubs. The only animals (other than humans) are birds that can fly in and animals that can gain access from the sea such as penguins and seals. This isolation is in contrast to the Arctic where the land mass borders other regions, allowing access during the warmer summer months of the year. The lowest temperature on Earth was recorded in Antarctica in 1983 at -89.2°C. Taking into account the prevailing winds (measured at that time to be 320 km/hr) the wind-chill factor further lowers these temperatures, making survival, never mind growth, extremely difficult (British Antarctic Survey, 2004). Compounding the temperature extremes, the interior of Antarctica is classified as a desert with an annual precipitation of 50 mm. Blizzards occur regularly. Precipitation is much heavier along the coastal regions amounting to more than 200 mm yr^{-1} (British Antarctic Survey, 2004). Less than 2% of Antarctica is free from ice. Broadly speaking, the continent consists of two geological zones divided by the Transantarctic Mountains stretching from the tip of the Antarctic Peninsula to Cape Adare, a distance of 4800 km. East Antarctica (also known as Greater Antarctica as well as Continental Antarctica) is thought to be one stable plate with West Antarctica (Lesser Antarctica) consisting of numerous smaller, unstable plates. The South Pole, the axis of the Earth's rotation, is located on the polar plateau of East Antarctica.

Investigators began exploration of the Antarctic in the early 1800's and since the International Geophysical Year of 1957, many nations have established scientific stations (Fig 5.3). In 1961, the Antarctic Treaty suspended all territorial claims and states that the entire continent be dedicated to peaceful scientific investigation (see Chapter 1), and the Protocol on Environmental Protection, signed in Madrid in 1991, imposed a fifty-year moratorium on extracting oil and mining minerals. Australia has three bases in Antarctica; Davis, Mawson and Casey all of which are located along the Eastern coastline [Fig 5.3]. Davis Base is located in the Vestfold Hills, one of the few regions in Antarctica relatively ice-free. Covering an area of 420 km^2, this region offers a myriad of lakes, both fresh water as a result of glacial run-off in summer, and many marine-derived salt lakes.

With respect to bacteria, the lakes of this region have revealed a substantial number of novel bacterial isolates. The many lakes, including Ekho, Deep, Organic, Ace, Burton and Pendant Lakes have all been a source of bacterial diversity (Bowman and McMeekin, 1996). These lakes offer a wide scope of environmental niches, for example, Ekho Lake is hypersaline and, unlike other lakes, is relatively warm due to its depth and ice-covering. As a result, this lake has a wide range of microbial diversity. In contrast, the salinity levels of Deep Lake prevent it from freezing thereby losing heat to the atmosphere resulting in exceedingly cold and unwelcoming water temperatures, consequently only an Archaean (*Halorubrum lacusprofoundl*) and an alga have ever been isolated from these waters (Bowman and McMeekin, 1996).

Researchers have used the permanent ice sheets of Antarctica to study anabiosis at low temperatures (Abyzov, 1993). Microorganisms, spores, pollen and other living materials are blown in from all parts of the world to become embedded in the ice sheets, thus offering a detailed climate record. Many research stations are located along the coastal regions of Antarctica and, as such, the majority of microorganisms have been isolated from these areas. The discovery of Lake Vostok in 1996 by Russian and British scientists represents one of the most oligotrophic environments on Earth, as well as being one of the least accessible ecosystems on the continent. Lake Vostok (77°S, 105°E) is a large lake (10,000 km^2), located beneath approximately 4 km of glacial ice within the East Antarctic Precambrian craton (Kapitsa *et al.*, 1996). Ice cores from above Lake Vostok have revealed a continuous paleoclimatic record over the past 400,000 years. A diverse range of microorganisms has been recovered from these ice cores including algae, diatoms, bacteria, fungi, yeasts and actinomycetes. These organisms have been demonstrated to be viable at depths as deep as 2400 m (Abyzov, 1993). As for the lake itself, no data is available as yet as drilling was terminated approximately 120 m above the ice-water interface to prevent contamination of the underlying lake by drilling fluid. It has been postulated that some forms of microorganisms exist in Lake Vostok water and sediment. Culturable strains of bacteria have been isolated from 1 million year old Arctic permafrost soil. These isolates were capable of growth at -4.5°C and therefore growth at Lake Vostok with a temperature of -3.2°C should not be a limiting factor (Tiedje, 1998). The unique nature of this environment, permanently cold, dark and under extreme pressure, offers the opportunity to examine a microbial community isolated from the rest of the biosphere for perhaps a million years or more. Such a novel ecosystem as

this sub- glacial lake, may contain unique biota with data possibly contributing to our understanding of the evolution of microorganisms. Antarctica is the world's largest natural laboratory for the investigation of how organisms adapt and respond to extreme conditions, as well as how ecosystems and communities are structured and how these communities respond to environmental change. This geographically isolated continent offers scientists the opportunity to study microbial diversity in respect to climate change, the functioning of terrestrial and lake ecosystems and the interrelationships that exist between them. Understanding these interactions will assist in the conservation of this pristine ecosystem and provide advanced warning of impending changes in Antarctica.

Significance of microbial diversity

Microbes are responsible for practically all environmental processes and are, therefore, of prime importance in the functioning of the ecosystem. Processes such as photosynthesis, nutrient cycling, mineralisation, pest control and detoxification of chemicals are all vital for a healthy environment. The understanding of such processes and the interactions between organisms and their environment are paramount to the management and sustainability of microbial diversity in such delicate ecosystems as the Antarctic. Microorganisms constitute the largest biomass in an ecosystem, yet comprehensive biodiversity studies are severely lacking. To understand and manage microbial ecology in both natural and disturbed or threatened ecosystems, a fundamental understanding of the processes that govern microbial diversity patterns must first be established. It is crucial that baseline patterns are established so that future conservation strategies can be facilitated.

The improved techniques previously mentioned have helped to identify and classify many microbes, including yeasts, over the past decade. This information is the foundation of identifying yeast biodiversity by allowing baseline information to be established. Yeast communities can be assessed and monitored following fluctuations in environmental conditions. Soil organisms are one of the most sensitive environmental biomarkers and are therefore invaluable in the study of contaminated or disturbed ecosystems. Monitoring of microbial activity can thus provide advanced evidence of discreet changes prior to those changes being detectable as measured by organic matter levels.

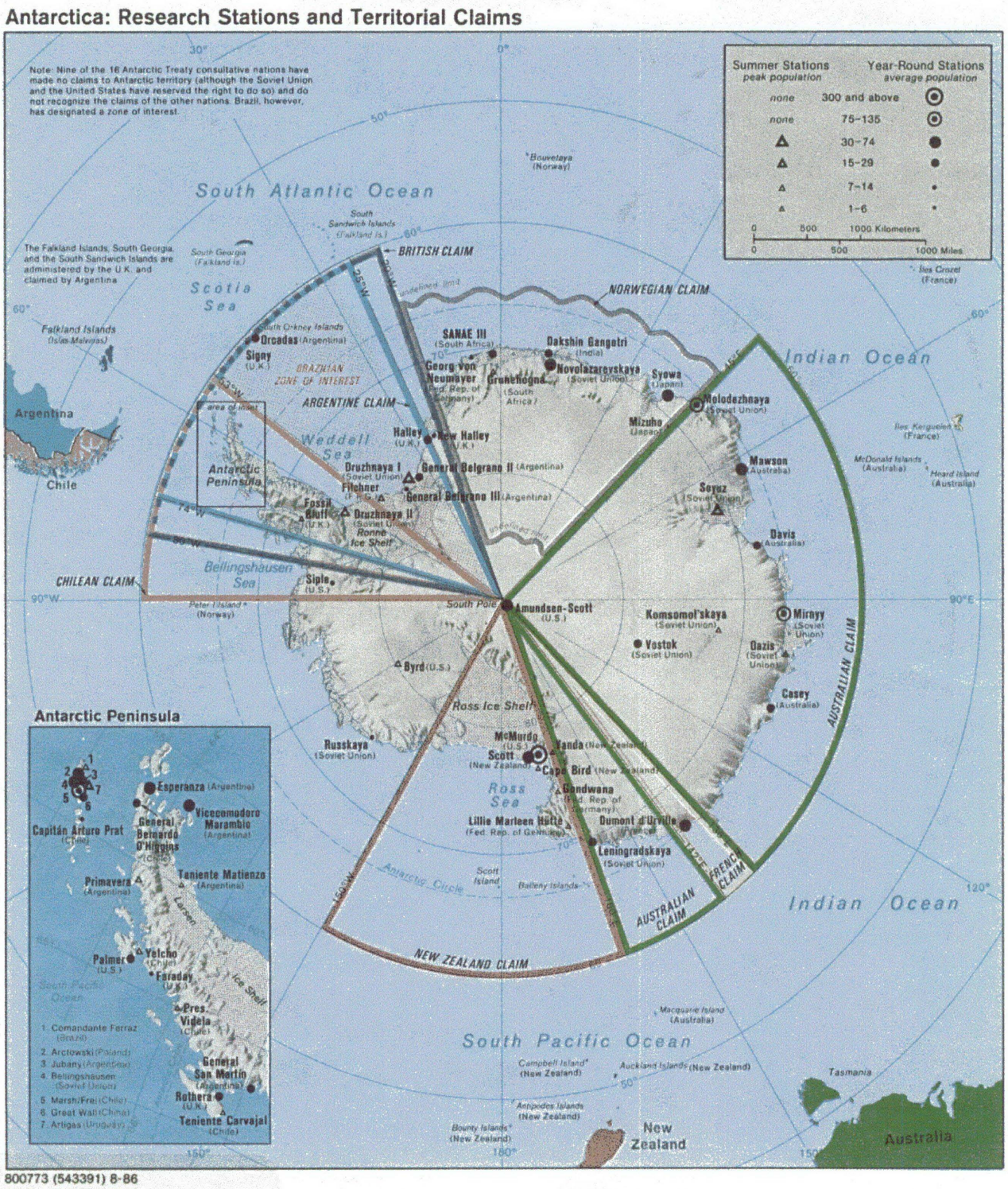

Figure 5.3. Map of Antarctica illustrating international territorial claims
http://www.1uptravel.com/worldmaps/antarctica5.html

Murray *et al.* (1998) report on the seasonal and spatial variability of psychrophilic bacteria in the surrounding oceans of Antarctica indicating significant changes in species composition. Psychrophilic organisms, being sensitive to alterations of temperature, could be dramatically affected by global temperature changes. For most species of micoorganisms, environmental or external temperature ranges for viability are very narrow and the same may be said of pH and oxidation states. Over the past few decades there has been a relative explosion in the isolation and identification of novel bacterial species isolated from various Antarctic environments. In particular, the last 5 years has seen numerous publications indicating that this environment is a rich source of prokaryotes (Bowman, 2000; Bowman *et al.*, 2000; McCammon and Bowman, 2000; Mevs *et al.*, 2000; Bowman and Nichols, 2002; Bozal *et al.*, 2002; Collins *et al.*, 2002). The increase in novel taxa being described from Antarctica is not only a reflection of an increased interest in the Antarctic as a source of novel isolates, but also the result of an improvement in the accuracy and reliability of modern methods. Traditionally, microbial enrichment techniques were used to isolated microbes. These methods, however, only produced a small subset of organisms representing only a fraction of those found in the environment. There are several reasons why only such a small fraction of organisms are recovered with this methodology: firstly, natural environments are not homogenous like an enrichment culture. Culture conditions may well be designed to mimic a natural growing environment, but can only represent a portion of the environment. Secondly, while culture conditions are created to isolate a specific organism, many others are selectively lost during subsequent rounds of isolation. And thirdly, species loss may occur when organisms live in symbiotic relationships.

Numerous mycological studies have led to the isolation of a number of fungal species from several regions of Antarctica (Onofri and Tosi, 1992; Onofri *et al.*, 1999; Onofri *et al.*, 2000; Tosi *et al.*, 2002). Commonly associated with species of moss, fungal isolates have also been found in association with lichens and organic substrates (Onofri *et al.*, 2000). A survey of fungal diversity on the Windmill Islands along the Eastern coast identified a total of 1228 isolates representing 22 genera with a significant increase in diversity in disturbed areas (Azmi and Seppelt, 1998). Among the filamentous fungi frequently isolated from Continental Antarctica include species belonging to genera of *Chrysosporium*, *Cladosporium, Geomyces, Mortierella, Mycelia, Penicillium*, *Phoma* and *Thelebolus* (Azmi and Seppelt, 1998; Tosi *et al.*, 2002).

Biodiversity as a bioresource

Biodiversity is not only important for its intrinsic value, but also in its importance as a major biotechnological resource. Microorganisms are the most abundant of all living organisms possessing the ability to circumvent an array of environmental conditions. A wonderful example of a significant biological advancement was the exploitation of *Thermus aquaticus*, a bacterium found in thermal springs in the USA (Brock, 1985). Isolation of the thermostable enzyme, *Taq* polymerase, revolutionized PCR technology by eliminating the need to replace enzymes after each hot/cold cycle due to denaturation processes.

Metagenomics has the potential to make a substantial impact on industrial applications. The immense microbial diversity demonstrated by Torsvik *et al.* (1990) where a single soil sample contained in the order of 10^4 different bacterial species, equates to more than a million open reading frames, many of which encode putative enzymes (Lorenz and Eck, 2005). The search for novel enzymes and biocatalysts has given rise to a new wave in biotechnology: white biotechnology (Lorenz and Eck, 2005). The term has been adopted to denote all industrial biotechnological processes that are not covered by medical (red) or plant (green) biotechnology. White biotechnology encompasses such products as vitamins, biofuels, bioplastics and enzymes in detergents, dairy and bakery industries. Long-term applications of white biotechnology are focussed on replacing fossil fuels with renewable resources, replacing conventional processes with bioprocesses as well as generating new bioproducts, for example, nutraceuticals, performance chemicals and bioactives (Lorenz and Eck, 2005). Industrial enzymes are a multibillion dollar industry. Global estimates for enzyme sales in 2003 were US$2.3 billion with detergents earning the highest profit followed by food applications, agriculture, textiles and chemicals (Lorenz and Eck, 2005).

The application of biodiversity to waste management and environmental clean-up programs through bioremediation is growing rapidly. Microorganisms, such as bacteria and yeasts, are being examined for their ability to break down substrates such as phenol (Margesin *et al.*, 2005), *n*-alkanes, triglycerides (Fickers *et al.*, 2005), benzene, toluene (Nicholson and Fathepure, 2005; Shim *et al.*, 2005) and crude oil components (Medina-Bellver *et al.*, 2005). A major factor that will determine future bioremediation will be the discovery of microorganisms that possess the ability to convert waste compounds that are at present non-biodegradable.

Yeasts in the Antarctic

DiMenna in 1960 reported on the isolation of ninety-five yeasts from various samples taken around the McMurdo Valley region in 1957, 1958 and 1959 (Di Menna, 1960). The majority of isolates were identified as *Cryptococcus albidus, Cr. laurentii* and *Rhodotorula minuta*. Strains of *Candida scottii* (*Leucosporidium scottii* Fell *et al.*), *R. mucilaginosa, R. glutinis, Sporobolomyces odorus, Cr. diffluens,* and *Cr. luteolus* were also isolated in smaller numbers from the same samples (Di Menna, 1960). In 1964, Sinclair and Stokes (Sinclair and Stokes, 1964) reported isolating sixty-seven psychrophilic organisms from soil, snow and ice samples from Antarctica, although the exact location was not reported. Three samples turned out to be psychrophilic yeasts (one *Torulopsis* and two *Candida* species) with a further twenty-three psychrotolerant yeast species. Unfortunately the latter isolates were not studied further and no identifications were made. Three new species; *Candida nivalis, C. gelida* and *C. frigida* were described by DiMenna in 1966 (Di Menna, 1966), again isolated from soil samples originating from the Ross Sea region. In 1969, Goto *et al.* (1969) isolated thirty-one yeast strains belonging to twelve species from a variety of sample types including soil, algae, moss and penguin dung taken from both South Victoria Land and Ross Island. The authors stated that many of the isolates belonged to the Cryptococcaceae family but classified the majority of strains as either *Candida* or *Rhodotorula*. Eight strains were identified for the first time as being new Antarctic yeasts: *Sporobolomyces antarcticus, Torulopsis psychrophila, Candida diffluens, C. humicola, C. australis (C. sake), Trichosporon cutaneum* var. *antarcticum, Rhodotorula glutinis* var. *rufusa* and *R. Rubra* var. *miersensis*. The three *Candida* strains have all since been reassigned to *Cryptococcus*. Established strains isolated included *C. scottii* (*Leucosporidium scottii* Fell *et al.*), *Cr. albidus*, *R. rubra* (*R. mucilaginosa* (A. Jörgensen) F.C. Harrison) and *R. texensis* (*R. minuta* (Saito) F.C. Harrison). Goto noted in his methodology that almost three times as many yeast strains were able to be isolated at 10°C as compared with 25°C regardless of salt concentration, thereby identifying temperature as a critical determinant. In 1969, Fell *et al.* (1969) proposed a new genus; *Leucosporidium*, incorporating *Candida* strains *C. scottii, C. frigida, C. gelida, C. nivalis, Torulopsis capsuligenus* as well as novel strains *L. antarcticum* and *L. stokesii* [*L. stokesii* was tentatively described as *Candida* by Sinclair & Stokes (Sinclair and Stokes, 1964)]. *Cr. vishniacii* was isolated in 1973 from the arid highlands of the Ross Desert, and has been found to dominate this region due to it's minimal nutritional requirements (Fell and Statzell-Tallman, 1998a).

Yeasts have also been isolated from Antarctic marine waters. A number of species were reported by Fell (1976) from various locations surrounding the continent. *Torulopsis austromarina* (*Candida sake*) was isolated for the first time from two geographically distinct regions, Indo-Pacific and the Indian Ocean sector of the Antarctic (Fell and Hunter, 1974). Along with *C. sake*, numerous yeast species have been found to inhabit Antarctic ocean waters, species representing *Candida, Cryptococcus, Debaryomyces, Leucosporodium, Rhodotorula, Sporobolomyces* and *Torulopsis* were isolated from several explorations to oceanic regions (Fell, 1976).

Abyzov *et al.* (Abyzov, 1993) isolated six strains of two viable yeast species [*Cryptococcus albidus* (Saito) and *Rhodotorula glutinis* (Fresenius)] dating between 700 and 3,250 years old from ice cores taken from above Lake Vostok. Electron microscopy revealed ultrastructural changes, such as smaller mitochondria, when compared to strains from temperate climates, thus demonstrating the ability of these yeasts to adapt to their conditions and survive (Abyzov, 1993). Other Russian investigators (Soina *et al.*, 2000) have also reported findings of yeasts in permafrost dated at 10,000 years. Yeasts belonging to the genera *Cryptoccoccus, Rhodotorula* and *Sporobolomyces* were among a myriad of microorganisms found including bacteria, fungi and algae.

The Victoria Land surrounding the Ross Sea has been the site from which the majority of yeasts have been isolated. McMurdo station (USA) is Antarctica's largest research station. The soils of the McMurdo Dry Valleys are saline, coarse and periglacially active, with organic carbon content and moisture very low (Campbell and Claridge, 1987). In close proximity to McMurdo is Scott Base, the New Zealand research station. *Cr. friedmannii, Cr. socialis* and *Cr. consortionis* were isolated from this region in 1985 (Vishniac, 1985a, 1985b). *Cr. antarcticus* and *Cr. albidosimilis* followed in 1992 (Vishniac and Kurtzman, 1992) along with *Cr. victoriae* (Montes *et al.*, 1999). *Cr. adeliensis* and *Cr. antarcticus* var. *circumpolaris* were both isolated in 2000 and 2002 respectively (Scorzetti *et al.*, 2000; Vishniac and Onofri, 2002).

Yeast biodiversity in the Vestfold Hills

In the austral summer of 1997/1998, 196 soil and snow samples were collected from the Vestfold Hills and stored at the CRC for Antarctic Research at the University of Tasmania, Hobart. Samples were appropriately labelled with the site of origin, site description, dated and

stored at -80°C. From these samples, over 500 yeasts were isolated with 37 isolates chosen for in-depth investigations (Thomas-Hall, personal communication). This resulted in 12 new species including *Cr. nyarrowii, Cr. statzellii*, three variant strains of *Cr. victoriae* and a further 18 sub-species that were identified (Thomas-Hall and Watson, 2001; Thomas-Hall *et al.*, 2002). Of the yeasts isolated, only half have been fully characterised to date. The majority of the yeasts described were found to belong to the Basidiomycota with only a small number of Ascomycetous yeasts (*Candida psychrophila*, and three conspecific isolates belonging to the genera *Chalara*). Within the Basidiomycota, the Hymenomycetes were the best represented with species in three of the four clades.

Although the Antarctic yeasts were by and large uniformly distributed throughout the Hymenomycetes, they clustered together indicating that they evolved from a common ancestor. A number of species were found to belong to the Uredinomycetes with two of the four clades represented. Species included *Leucosporidium antarcticum* and six novel species; *Leucosporidium watsonii*, *Rhodotorula jonesiana*, *R. guffoggiae, R. tangerine, R. partizanica* and *R. zenithia* (Thomas-Hall, 2005).

The yeasts described in this study follow the trends of the study by Thomas-Hall (2005) with the majority of the yeast isolates being basidiomycetous (>70%). Of these, the majority belonged to the genus *Cryptococcus*. Four isolates were identified as *Cr. victoriae* and were found in soil samples taken from Moss Cirque and Lichen Valley, a lichen sample from Mossell Lake and an algal mat from Lake Fletcher. Montes *et al.* (Montes *et al.*, 1999) who first described *Cr. victoriae,* isolated this species from samples of moss, lichens and soil in Southern Victoria Land. The regions with the highest levels of biodiversity were found to be Lichen Valley and Moss Cirque (located approximately 1 km north of Lichen Lake). Species such as *Cr. nyarrowii, Cr. victoriae* and *Cr. gilvescens* were all represented in samples of soil and/or lichen. Areas such as Marine Plain, Lichen Valley, Mossell Lake and Ekho Lake have been designated as protected regions. Marine Plain has yielded a number of vertebrate fossil fauna dating back to the time of Gondwana, has a relative abundance of mosses and lichens and during the Austral summer is home to a number of species of birds, penguins and seals. It is, therefore, not surprising that this area yielded a number of yeast isolates including *Cr. gilvescens* and *Cr. gastricus*. While this appears to be the first report of these isolates from Antarctica, *Cr. gilvescens* is one of the dominant yeast species in the Tundra soils of Russia as

well as the wetland soils of Alaska, and is well documented as being psychrotolerant (Chernov and Bab'eva, 1988; Poliakova *et al.*, 2001). However, although *Cr. gastricus* has been isolated from soil in New Zealand on a number of occasions, it has also been isolated from a tuberculosis patient in Norway (Fell and Statzell-Tallman, 1998a). This leaves some doubts as to whether this is an indigenous yeast species capable of withstanding the harsh Antarctic conditions, or if it is in fact a cosmopolitan species introduced into the area. *Cr. gastricus* is not a true psychrophile as it is capable of growth at 25°C but neither this strain nor the type strain is capable of growth at 30°C (Fell and Statzell-Tallman, 1998a). A possible explanation may be that the strain isolated from the tuberculosis patient is in fact a sub-species of *Cr. gastricus* which is adapted to growth at warmer temperatures.

Cr. nyarrowii was originally isolated from two samples taken from Lichen Valley, one soil and the other snow petrel carnage (Thomas-Hall and Watson, 2001). In this study, eight isolates were identified as *Cr. nyarrowii*. Three isolates were also from Lichen Valley in soil and moss samples as well as one sample from petrel carnage. Two of the isolates were from Moss Cirque, and were from samples of lichen and/or soil. The sixth isolate came from Marine Plain soil, located further south from the other isolates. The geographic diversity of this species, coupled with the fact that it has only ever been isolated from Antarctica indicates that this may be a truly indigenous species. Similar conclusions may be drawn in the case of *Cr. watticus*. Three samples, two from the Watts Lake region (one soil, one shell/stromatolite) and a sample from Larsemann Hills (sample type not available) yielded a novel *Cryptococcus* species described in this study as *Cr. watticus* (Guffogg *et al.*, 2004). The diverse localities, in addition to the molecular data, also suggest this to be an indigenous species.

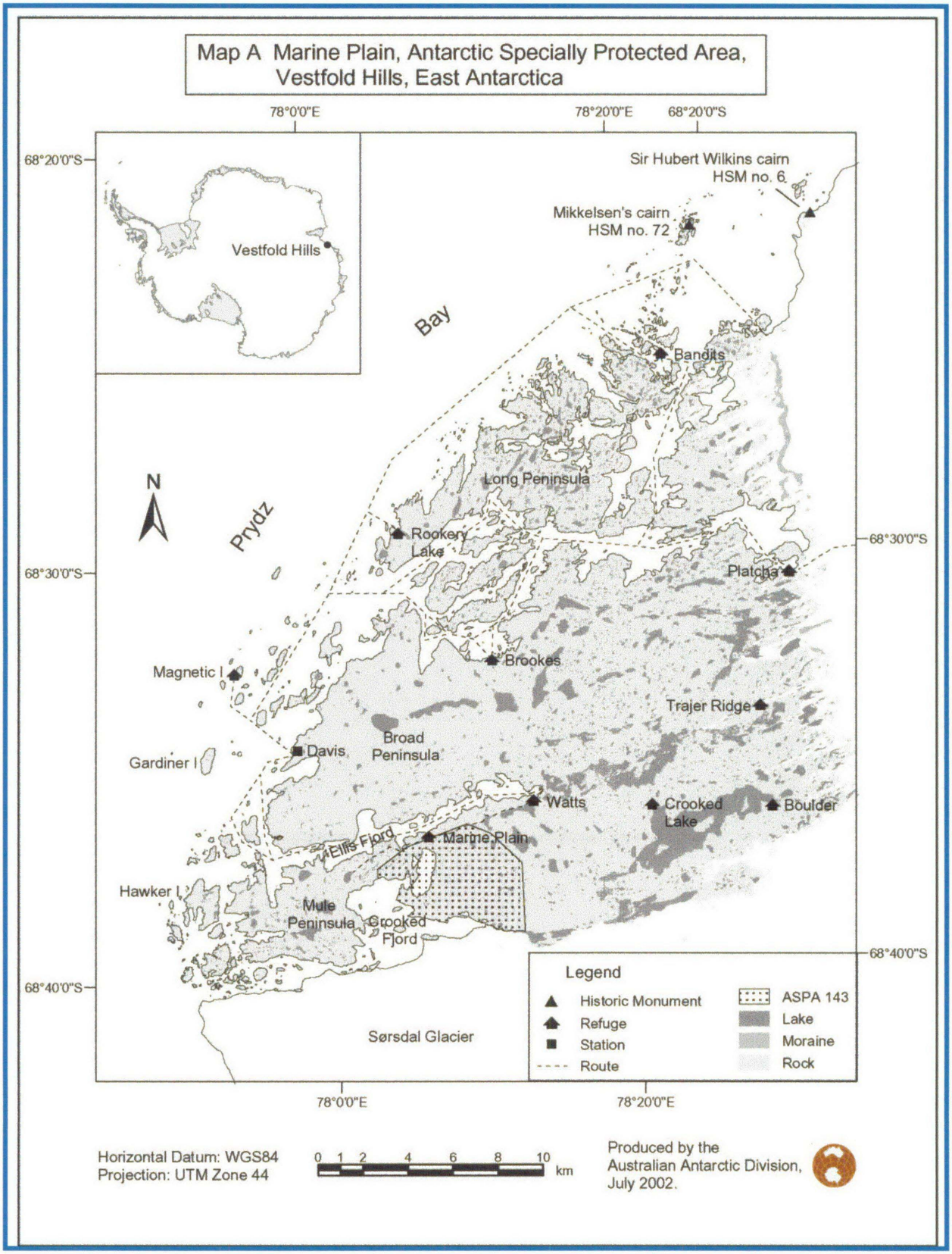

Figure 5.4. Map of Vestfold Hills, Davis Base, East Antarctica showing key areas where yeasts were isolated such as, Marine Plain and Watts Lake.
http://aadc-maps.aad.gov.au

Three *Rhodotorula* species were isolated from a range of soil samples. *R. mucilaginosa* from Apple Hut, *R. minuta* from Watts Lake and *R. laryngis* from Turners Island. *R. mucilaginosa* has been previously isolated from various regions in Antarctica including the McMurdo Valley in 1960 (Di Menna, 1960) and again in South Victoria Land in 1969 (Goto *et al.*, 1969). More recently, *R. mucilaginosa* was isolated from a soil sample from Livingston Island off the Antarctic Peninsula (Zlatanov *et al.*, 2001). *R. mucilaginosa* has been found world-wide in terrestrial, marine and aquatic habitats. This species has frequently been isolated from human sources and is quite possibly the only *Rhodotorula* species to cause human infections. *R. mucilaginosa* has a long list of synonyms (59 in *The Yeasts: A Taxonomic Study* 4th ed. 1998) and, until recently, was known as *R. rubra* (Fell and Statzell-Tallman, 1998b). The strain isolated in this study is capable of surviving at temperatures up to 50°C with an optimum growth temperature of 25°C. This strain also grew well at low temperatures at least down to 6°C. The ability of this yeast to grow across such a wide spectrum of temperatures, as well as its UV resistance (see Chapter 6) suggests an enhanced capacity to adapt to a number of environmental conditions. Likewise, *R. minuta* is prevalent across a wide variety of substrates including, air, clinical, terrestrial, plants, animals, fresh and marine water as well as food sources (Fell and Statzell-Tallman, 1998b). First isolated in Antarctica in 1969 (Goto *et al.*, 1969) and described as *R. texensis*, this species also has a number of synonyms (Fell and Statzell-Tallman, 1998b). The widespread occurrence of *Rhodotorula spp.*, indicate that while this species has the ability to occupy a plethora of niches, *R. minuta* and *R. mucilaginosa* are not indigenous species of Antarctica.

Of the ascomycetous yeasts isolated, only one species was isolated repeatedly from various locations. Still to be classified, UNE116c (designated representative strain) was found in soil and lichen samples from Mossell Lake, Moss Cirque, Wilkes Station and Davis Station. Phylogenetic analysis of this species in the D1/D2 region of the large ribosomal subunit placed this strain in the Archiascomycete clade with 14 nucleotide changes from *Leuconeurospora pulcherrima.* Although morphologically yeast-like, preliminary sequencing of the 18S region indicate that UNE116c is 99.8% similar to a black, Aureobasidium-like fungal strain (de Hoog, personnel communication). Other ascomycetous yeasts isolated include, *Debaryomyces hansenii* (Lichen Valley, Wilkes Station), *Candida parapsilosis (C. sake)* (Mossell Lake, Turner Island) and *C. norvegica* (Davis Base). All but one of these isolates, (the *D. hansenii* strain from Turner Island) were isolated from samples associated with human impact such as

rotting food cans. These samples are likely to be contaminated with species that are not normally associated with cold climates.

Many of the yeasts that appear to be indigenous to the Antarctic [Table 5.1] are psychrophilic organisms. That is not capable of growth above 20-25°C. This is a common characteristic associated with Antarctic organisms, however, a number of these yeasts are psychrotolerant, and capable of growth at temperatures above 25°C. Are these yeasts contaminants? Are they psychrophiles that have adapted to surviving the warmer temperatures experienced in a particular habitat? The isolation of *Cr. watticus* from three separate sites in the Vestfold Hills region indicate that this species is a psychrotolerant indigenous species, however, very recent reports indicate that *Cr. watticus* has been isolated from another Antarctic site (exact location is unknown at present) as well as in Northern regions such as Alaska, Russian Far East and Iceland (Vishniac, 2005). *Cr. adeliensis* on the other hand, is also psychrotolerant but its isolation, to date, is restricted to that of Antarctica alone. A list of selected yeasts which have been isolated from Antarctica but which have also been isolated elsewhere is presented in Table 5.2.

Table 5.1. Indigenous Antarctic yeast species

Species	Year	Antarctic location
Candida psychrophila	1969	South Victoria Land
Cryptococcus adeliensis	2000	Terre Adelie
Cryptococcus albidosimilis	1992	South Victoria Land
Cryptococcus antarcticus	1992	South Victoria Land
Cryptococcus consortionis	1985	Ross Desert
Cryptococcus friedmannii	1985	Ross Desert
Cryptococcus niccombsii	2005	Vestfold Hills, Davis Base
Cryptococcus nyarrowii	2001	Vestfold Hills, Davis Base
Cryptococcus socialis	1985	Ross Desert
Cryptococcus statzelliae	2002	Vestfold Hills, Davis Base
Cryptococcus vishniacii	1979	South Victoria Land
Cryptococcus watticus	2004	Vestfold Hills, Davis Base
Leucosporidium antarcticum	1969	Antarctic Peninsula; Vestfold Hills, Davis Base
Leucosporidium watsonii	2005	Vestfold Hills, Davis Base
Mrakia blollop	2005	Vestfold Hills, Davis Base
Mrakia robertii	2005	Vestfold Hills, Davis Base
Rhodotorula guffoggiae	2005	Vestfold Hills, Davis Base
Rhodotorula jonesiana	2005	Vestfold Hills, Davis Base
Rhodotorula partizanica	2005	Vestfold Hills, Davis Base
Rhodotorula redmena	2005	Vestfold Hills, Davis Base
Rhodotorula tangerina	2005	Vestfold Hills, Davis Base
Rhodotorula zenithia	2005	Vestfold Hills, Davis Base

Table 5.2. Antarctic yeast species found in other locations

Species	Substrate of original isolation	Year	Antarctic location
Debaryomyces hansenii	Cheese, sausage, sake, human	1985	Vestfold Hills
Candida sake	Sake-moto, Japan	1935	Indian Ocean & Ross Sea, Antarctica
Candida norvegica	Washing machine	1958	Vestfold Hills
Candida parapsilosis	Sprue, Puerto Rico	1932	Vestfold Hills
Cryptococcus albidus	Air, Tokyo	1922	McMurdo Valley
Cryptococcus gastricus	Soil, NewZealand	1958	Vestfold Hills
Cryptococcus gilvescens	Humus, Russia	1988	Vestfold Hills
Cryptococcus laurentii	Palm wine, Congo	1920	McMurdo Valley
Cryptococcus luteolus	Air, Tokyo	1922	McMurdo Valley
Leucosporidium scottii	Soil and frozen beef, Australia	1936	Scott Base
Mrakia frigida	Soil, Antarctica	1966	Scott Base, Mawson, Vestfold Hills
Mrakia gelida	Soil, Antarctica	1966	Scott Base, Mawson, Vestfold Hills
Rhodotorula glutinis	Air, Germany	1912	Lake Vostok
Rhodotorula minuta	Air, Tokyo	1928	Vestfold Hills,
Rhodotorula mucilaginosa	Air, humans, Tokyo	1928	Vestfold Hills, Antarctic Peninsula

Environmental impact on yeast biodiversity

Response to environmental changes occurs individually at the species level. Environmental factors such as temperature, nutrient availability, moisture and UV radiation effect the taxa composition and abundance of soil mycota. Changes in any of these factors may lead to a cascade of events such as an increase in UV radiation exposure leading to an increase in air temperature resulting in a decrease in moisture levels. Increases in air temperature occur slowly over time and are consistent with fluctuations that have occurred over geological time. Such gradual alterations are unlikely to have dramatic effects on soil biota. Depletion of stratospheric ozone on the other hand, has been occurring only in the past few decades. This has resulted in an increase in surface UVB radiation as well as an increase in atmospheric carbon dioxide (CO_2) concentrations. Bacteria in Arctic soils have been shown to be significantly effected by increased UVB and CO_2 levels (Johnson *et al.*, 2002), which in turn has affected the soil composition. Ratios of nitrogen (N), sulphur (S) and phosphorus (P) are indicative of the condition of the soil and are directly related to the organic carbon content of the soil. Organic carbon has both a direct and indirect effect on the supply of soil nutrients; firstly, organic carbon serves as a direct source of N, P and S through mineralisation by microorganisms present in the soil and secondly, organic carbon is required as an energy source for nitrogen-fixing bacteria.

In the Dry Valleys of McMurdo Sound, the food chain within the soil is very simplistic. Bacteria, yeast and nematodes are the only soil organisms found. It is therefore reasonable to assume that yeasts play a fundamental role in this ecosystem. Recent reports from McMurdo suggest that yeasts provide the nematodes with ergosterol, a lipid produced by yeast that is normally supplied by plants in more temperate climates (Kuenning, 2003), indicating that some yeasts may play a role other than as a saprophyte. In marine waters, the role of yeasts is unknown. Distribution of some yeasts are limited by their physiological properties such as *L. antarcticum* and *C. sake* found only in Antarctic marine waters as both species are psychrophilic (Fell, 1976). The distribution limitations of the psychrophilic yeasts would suggest that these species have a specific role in their given habitat.

The detrimental effects of UV radiation on the primary producers of the Antarctic marine ecosystem are well documented. Many unicellular marine organisms are highly sensitive to UV radiation. The delicate balance of ecosystems can be altered by the selection of

UV-resistant species thereby threatening the growth and survival of other more UV-sensitive microorganisms (Hader *et al.*, 2003). Ocean dwelling microbes are exposed to UVB radiation levels inversely proportional to the depths at which they reside. UVB penetrates to significant depths of 20 – 30 m at intensities that cause measurable biological damage (Smith *et al.*, 1992; Malloy *et al.*, 1997). Protective mechanisms such as synthesis of carotenoid compounds, production of mycosporine-like amino acids (MAAs) as well as biochemically associated antioxidant defences have been reported in several yeast genera such as *Rhodotorula, Sporobolomyces, Cryptococcus* and *Phaffia* (Buschges *et al.*, 1994; Libkind *et al.*, 2004b; Sommaruga *et al.*, 2004). Temperature is indisputably one of the foremost environmental factors affecting the growth and survival of any microorganism. The selective expression of a set of proteins collectively known as the *heat shock proteins* (hsps) constitutes the universal *heat shock response* found in every organism from bacteria to humans (Morimoto *et al.*, 1990; Watson, 1990). High evolutionary conservation of the genes involved in the heat shock response suggests that this function may be essential for survival during hostile conditions such as can be found in the Antarctic (Schlesinger, 1990; Trautinger, 2001). Psychrophilic yeasts have been shown to induce hsp synthesis when exposed to a mild heat shock of 25°C. Such exposure resulted in acquired thermotolerance for the psychrophilic yeasts *M. gelida, M. frigida* and *L. antarcticum* (Deegenaars and Watson, 1998a).

It has long been established that the fatty acid composition of yeasts varies with temperature of growth (Watson, 1987). The ratio of fatty acids within the membranes determines fluidity. The greater the ratio of unsaturated versus saturated fatty acids, the more fluid the membrane and vice versa. Isolates in this study in general had a greater percentage of total fatty acids as unsaturated. This result confirmed earlier reports by Watson (1980) and Watson & Aurthur (1976) in which it was reported that psychrophilic organisms had a predominance for C_{18} unsaturated fatty acids. No major differences or patterns in fatty acid composition emerged between the psychrophiles and psychrotolerant yeasts in this study. Oleic acid ($C_{18:1}$) predominated across both types, averaging 34% of the total fatty acids present, but in some cases higher than 60% (strains of *Cr. nyarrowii* and *Cr. gilvenscens*). The two omega fatty acids, linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) varied across all strains. Eleven of the thirteen psychrophiles produced quantities of $C_{18:2} > 25\%$. Six psychrophiles recorded levels of $C_{18:2} > 45\%$ (four strains of *Cr. nyarrowii* and one of *L. antarcticum*), with $C_{18:2}$ making up 61% of the total fatty acids present in the psychrophile strain UNE182c. A

majority (65%) of psychrotolerant yeasts recorded higher levels of $C_{18:1}$ compared with $C_{18:2}$, while the remaining yeasts had opposite profiles. Linolenic acid in general recorded <15% of total fatty acids, with the notable exceptions of *C. parapsilosis* (52%) and *Cr. gilvescens* (24%). The relatively high percentages of the omega-3 ($C_{18:3}$) and omega-6 ($C_{18:2}$) fatty acids in *C. parapsilosis* and *Cr. nyarrowii* respectively, indicate the potential of exploiting these strains for production of these commercially important omega fatty acids for use as dietary supplements. *R. mucilaginosa* had a greater percentage of total fatty acids as unsaturated. 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. The consistently high percentage of oleic acid compared with the other C_{18} fatty acids would indicate its significance as a vital membrane component in organisms from such a cold environment. Further investigation of membrane fatty acid composition is warranted as, in the present studies, strains were only examined at a constant growth temperature of 15°C. Differentiation of the lipid profiles between the true psychrophiles and the psychrotolerant yeasts may be more clearly achieved by growth of strains at different temperatures for example; 2°C, 15°C and 23°C. The latter temperature corresponding closely to the maximum growth temperature of psychrophiles. In addition, detailed examination of the membrane phospholipid classes, including fatty acid composition of individual phospholipids, is likely to shed new light on the role of membranes and temperature adaptation of Antarctic yeasts.

Conclusion

Comprehensive studies of the biodiversity of yeasts in Antarctica are severely lacking. Historically, this has been attributed to limitations associated with culturing methods as well as the logistic obstacles that need to be overcome when exercising any research in Antarctica. One important issue that is being addressed is one of contamination. The isolation of valid indigenous yeasts will be dependant upon the sampling techniques used. Over the past decade there has been a conscious effort to keep Antarctica as pristine as possible, for instance the designation of protected areas such as Marine Plain in the Vestfold Hills region. Samples need to be recorded with a high degree of accuracy including data on the exact location, substrate composition, UV-radiation levels, temperature, moisture levels and presence or/absence of flora and/or fauna.

The advent of sophisticated molecular techniques has resulted in an influx of yeasts being described as well as many taxonomic synonyms being identified. This data has expanded the current knowledge of yeast biodiversity in the Antarctic building the baseline data so that a more comprehensive picture of yeast biodiversity can be established. Culturing isolates will remain an important goal, as molecular data alone will not provide vital information concerning the characteristics and traits of these organisms.

The number of yeasts isolated from the Vestfold Hills thus far represents the greatest biodiversity data available for yeasts from Antarctica. The question, however, remains as to the indigenicity of those yeasts isolated from this habitat. One thing that is perfectly clear however, is the ability of these yeasts, as for example *R. mucilaginosa* (Chapter 6), to protect themselves from such harsh environmental conditions warrants further investigation. Yeast biodiversity in the Antarctic is of prime importance to answer the poignant question: why are they there?
