YEASTS FROM LESOTHO – THEIR CLASSIFICATION AND POSSIBLE APPLICATIONS

by

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Chapter 1

Introduction

1.1 Motivation

In view of the rapid decline of many natural habitats due to urban and industrial development, the need to search for new microorganisms, including yeasts, in these areas is pressing. Also, the increased awareness of the importance of documenting biodiversity allows one to be filled with optimism about the future of taxonomic research.

Yeasts are unicellular fungi and this enables them to occupy a wide variety of habitats, which include aquatic and terrestrial environments. A typical environment where yeasts are found is one that is moist and has abundant supply of simple, soluble nutrients such as sugars and amino acids (Lachance & Starmer, 1998). This nutritional requirement explains why they are common on leaf and fruit surfaces, on roots and in various types of food. After isolating yeasts, from a particular habitat, it is important that proper identification methods are used.

Yeast identification, which was previously based only on conventional identification methods, has undergone significant transformation over the last two decades due to the increase in basic biological knowledge as well as interest in the practical applications and biodiversity of this microbial group. Since conventional identification techniques are laborious and time-consuming, rapid yeast identification techniques using among others fatty acids profiles was proposed. These methods are well established and comprehensive databases are available, which can facilitate comparisons of strains to be identified (Kock *et al.*, 1985; Viljoen *et al.*, 1986; Cottrell & Kock,

1989; Miller *et al.*, 1989; Botha *et al.*, 1992; Botha & Kock, 1993). Molecular taxonomy methods are now also being used due to their speed and accuracy. These techniques have also found application in monitoring effectively the succession of active yeast species during wine production (Guillamón *et al.*, 1998; Esteve-Zarzoso *et al.*, 1999). Also, the analysis of restriction fragment length polymorphism of the ITS region allows for detection and quantification of different yeast species (Querrol & Ramon, 1996; Vasdinyei & Deak, 2003). The use of two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA allows for rapid and accurate species identification (Herzberg *et al*., 2002; Daniel & Meyer, 2003).

Lesotho can be considered an unexplored region in regard to yeasts. This country is ideally suited to harbour yeasts due to the region's relative high rainfall, rich forest and fauna. Also, it is one of the few countries in the world with all its land lying at an altitude in excess of 1000m thereby being exposed to increased UV radiation, which would subsequently increase diversity through mutations. Therefore, it is believed that yeasts from Lesotho should be more diverse than reported for areas in other parts of the world.

Consequently, the aim of this study was to survey the diversity of ascomycetous yeasts in some pristine moist habitats of Lesotho. In addition, attempts were made to isolate ascomycetous yeasts, which can utilize unusual complex substrates such as cyclohexanes, monoterpenes as well as halogenated compounds, which might be similar to intermediates in the degradation of chlorophenols. This is important since these compounds are

toxic environmental pollutants. Yeasts able to degrade them would be useful in biocatalysis, bioremediation, wastewater applications as well as high value compound-production from the detoxification of these compounds. Here conventional morphological and physiological tests, fatty acid profiles, restriction fragment length polymorphism as well as D1/D2 sequencing methods will be used to identify yeast isolates.

1.2 Lesotho

Lesotho is a mountainous developing country in Southern Africa completely surrounded by South Africa (Figure 1) and has a total area of 30, 355 km^2. It lies between latitude 28^0 35^1S and 30^0 40^1 S, and longitude 27^0 00^1E and 29^0 30^1E. The country rises from a plain at an altitude of about 1500m in the west, to mountains over 3350m in the east. This altitude suggests abundant supply of sub-lethal UV irradiation that can influence fungi (Stevens *et al*., 1997) and cause diversification through mutations.

The landscape of Lesotho is divided into two major units, lowland and highland, and these are related to the underlying geology. The lowlands, found primarily in the narrow western part of the country are composed of sedimentary formations and minor igneous intrusions while the highlands, comprising 75% of the country, consist of basaltic plateaus with maximum height at Thabana-Ntlenyane of 3841m (11,425ft). The soil is moist, humus-rich suggesting that it may be a good source for the presence of yeasts.

The climate in the lowlands is generally characterized by cold dry winters and summers with occasional rains. In the highlands, the winters from May to early September are colder with temperatures ranging from –7 to 15^{0}C and summer temperatures of 20 to 30^{0}C. Annual rainfall varies from 500mm to 1200mm with an average of 750mm. Consequently, less yeasts are expected to be present in winter and in the South of the country where the rainfall is less (Germond, 1997).

Figure 1. Map of Lesotho (landlocked by South Africa).

1.3 Definition of yeasts

Several definitions have been used to describe yeasts. According to Lodder (1970), yeasts were defined as unicellular fungi, which reproduce by budding or fission as their mode of asexual reproduction. This definition has a drawback because it regards only true unicellular fungi as yeasts and excludes many yeast species that are dimorphic and produce pseudohyphae and/or true hyphae in addition to unicellular growth. The overlap in morphological appearance has led to some authors regarding yeasts merely as fungi that produce unicellular growths (Flegel, 1977; Kendrick, 1987). In 1987, Oberwinkler placed the yeasts in a phylogenetic framework and regarded them as unicellular ontogenetic stadia of either ascomycetes or basidiomycetes (Van der Walt, 1987).

In summary, yeasts are unicellular fungi that can be classified into two phylogenetic groups i.e. teleomorphic and anamorphic ascomycetous or teleomorphic and anamorphic basidiomycetous yeasts that reproduce by budding or fission and that form their sexual states (i.e. asci), which are not enclosed in a fruiting body (Boekhout & Kurtzman, 1996; Kurtzman & Fell, 1998; Querol & Belloch, 2003). During sexual reproduction ascomycetous yeasts (i.e. yeasts of interest in this study) form asci, which contain varying numbers of ascospore shapes. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores (Van der Walt, 2000).

1.4 Ecology

Yeast ecology can be of economic importance due to the impact of human activity on nature. The potential role of yeasts in environmental ecology as agents of pollution, bioremediation or biological pest control as well as being central components of some industrial processes is well documented (Brakhage & Turner, 1995; Pöggeler, 2001). Yeasts are not randomly distributed throughout the biosphere and form communities of species defined by their habitat. The habitat is the actual place where an assemblage of yeasts lives and by the niches of its component species. The niche includes all physical chemical or biotic factors required for successful existence. The nature of yeasts generally limits them in the range of habitats they can occupy. The mineral, nutrient as well as organic source of carbon and energy differs between habitats and influence the type of yeasts present in that habitat (Phaff & Starmer, 1980; Phaff, 1986; Lachance & Starmer, 1998).

The unicellular nature of yeasts makes them better suited for deep liquid substrates or moist and uneven surfaces. Therefore, yeasts grow typically in moist environments where there is an abundant supply of simple, soluble nutrients such as sugars and amino acids. This explains why they are common on leaf and fruit surfaces, on roots and in various types of food. A few exceptions are able to degrade polymers, such as starch and cellulose that are used by many hyphal fungi. Yeasts are found in widely different aquatic and terrestrial sources, the atmosphere as well as certain restricted habitats. They may also be found associated with the body of certain animals since they act as intestinal commensals (Lachance & Starmer, 1998).

The type of nutrients reaching the soil determines the yeast microflora found while some yeasts are permanent residents in the soil. They include *Cryptococcus*, *Lipomyces lipofera*, *Lipomyces starkeyi*, *Lipomyces tetrasporus*, *Rhodotorula* species, *Schizoblastosporion starkeyi-henricci* and *Sporobolomyces* species, all of which have been repeatedly and exclusively isolated from soil (Spencer & Spencer, 1997; Lachance & Starmer, 1998).

The distribution of yeast species in various water sources is different and the numbers vary quite widely from a few cells/ml in unpolluted water to more than a million/ml in effluents. In polluted water the number of yeast increases proportionally to the degree of pollution (Hagler & Ahearn, 1987; Lachance & Starmer, 1998). Some yeasts such as red yeasts have been suggested as indices of pollution.

The abundance of *K. aestuarii* in crabs has been reported by Hagler's group (de Araujo *et al.*, 1995) probably explaining why they are scattered in the sea (Lachance & Starmer, 1998). Fresh water yeasts as well as those found in the sea probably also include those from other ecosystems, which have been washed into these water sources (Vincent, 1988). The species that are commonly found include *Candida*, *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* (Hagler & Ahearn, 1987).

Yeasts are found to participate in complex relationships with other organisms in terrestrial ecosystems. These interactions include, microbe-microbe interaction, commensalism, synergism, mutualism, competition, amensalism,

predation and parasitism (Phaff & Starmer, 1987; Atlas and Bartha 1993; Lachance & Starmer, 1998). The important outcomes of these interactions are whether or not they enhance or inhibit the growth of any particular species or strain (Fleet, 2003).

The cactus-yeast-Drosophila system was used as an example (Starmer *et al*., 1991) to show dispersal as a factor that is highly specific as it may involve insects that feed only on certain cactus species (Dobzhansky *et al.*, 1956; Starmer *et al.*, 1976; Lachance, 2003). The yeasts present in certain habitats must provide sufficient benefits such as food enrichment and detoxification to the vector in order to ensure further dispersal of the yeast community to a new environment. Furthermore, the compatibility of yeast species within a niche influences the species found in that habitat (Lachance & Starmer, 1998).

Yeast habitats are often rich in simple organic carbons, sometimes very high in moisture, acidic or occasionally alkaline. This diversity in habitat-types confirms that yeasts are able to grow over a broad range of growth conditions.

These features enable us also to predict their distribution. However, new yeast species isolated from the varying habitats are formed due to selection pressures exerted by the environment. The observed similarities and differences of yeasts found in a particular environment can play a vital role in observing evolution as it happens. Thus, the ecology of yeasts is believed to involve the effects of the physical environment on the yeast cells and the

interaction of the yeast species with the other microorganisms (Spencer & Spencer, 1997).

New yeast habitats may be discovered accidentally whereas in other cases the finding may be influenced by previous knowledge. However, once yeast populations have been located for study, their habitat should be identified in order to appreciate the whole community (Lachance & Starmer, 1998).

1.5 Isolation and maintenance

1.5.1 Isolation of yeasts

Yeasts, like other heterotrophic living organisms require carbon, nitrogen, phosphorus, trace elements and growth factors as sources of nutrition. Yeasts rarely occur in the absence of either molds or bacteria and since they do not occur naturally as pure cultures it is important to know which components to consider. Hence, selective techniques are often used for the recovery of yeasts, using media that permit the yeast to grow while suppressing molds and bacteria (Yarrow, 1998).

For isolation purposes, the direct streak plating technique is recommended. The preferred media is YM agar which is acidified to pH 3.7 using either hydrochloric acid or phosphoric acid. This acidification is preferred over the incorporation of antibiotics and fungistatic agents (Lachance & Starmer, 1998) that are used to suppress bacteria and molds respectively. Fungistatic agents are to be used with caution as some of these compounds may also inhibit certain yeasts (Yarrow, 1998).

Cultures are usually incubated between 20°C and 25°C since most yeasts are mesophilic, though for taxonomic studies, 25°C is preferred. Optimum temperatures for growth are higher for some yeasts and lower for others. The psychrophilic taxa require temperatures of between 4°C and 15°C as their optimum temperatures. Higher temperatures, in the range of 30-37°C, are often required for yeasts that are strictly associated with warm-blooded animals (Yarrow, 1998).

1.5.1.1 Isolation

The isolation of yeasts present in low numbers require enrichment using media and conditions that favour the growth of yeasts over other microorganisms (Wickerham, 1951). Usually a sample is inoculated into a liquid medium with a pH of 3.7 to 3.8. Air can be excluded from the culture to discourage the development of moulds although this method leads to the development of fermentative strains excluding the aerobic strains. Sterile pharmaceutical paraffin can be poured on the surface of the media to a depth of 1cm to exclude air (Yarrow, 1998).

1.5.2 Isolation of yeasts able to utilize non-carbohydrate carbon sources

The use and ability of some yeasts to either transform toxic compounds to valuable compounds or to detoxify these compounds has been well documented (Copley, 1998; Fetzner, 1998). It is against this background that yeasts from Lesotho were also isolated using enrichment culture techniques using unusual compou nds such as cyclohexane derivatives as well as chloro - compound derivatives as carbon sources.

The carbon sources usually utilized by microorganisms contain glucose or other carbohydrates and are used as carbon and energy source. Microbes with the ability to utilize non-carbohydrate carbon sources such as alkanes, branched alkanes (Demain *et al.*, 1998), low molecular weight aromatics and cyclic alkanes are important because these compounds are environmental pollutants (Van Beilen *et al.*, 1998; 2003). In addition, the degradation of cyclic alkanes by microbes is important in nature and in technological applications such as wastewater, waste gas treatment, bioremediation and biocatalysis (Sikkema *et al.*, 1995). In particular, cyclohexane is becoming increasingly important as an industrial solvent because it is relatively nontoxic compared to benzene, a known carcinogen, used previously as an industrial solvent (Uribe *et al*., 1990; Sikkema *et al.*, 1995).

Monoterpenes are branched chain C_{10} hydrocarbons widely distributed in nature. The most widespread terpene in the world is limonene, which is formed by over 300 plants (Colocousi *et al.*, 1996; Van der Werf & de Bont, 1998; King & Dickinson, 2000; 2003). The biotransformation of limonene by microorganisms with a view towards potential production of more valuable natural flavour compounds has been reported in bacteria and basidiomycetous yeasts (Van der Werf *et al.*, 1999). Remarkably, information regarding microbial metabolism of monoterpenes is scarce and thus far the degradation pathway for limonene has been established for only one microorganism (i.e. *Pseudomonas putida*). This degradation pathway was determined by biochemical studies (Van der Werf *et al.*, 1999).

The microbial degradation of monoterpenes is hampered by their toxicity. A few yeast strains, all belonging to the alkane-utilizing yeasts, can hydroxylate monoterpenes (Van Rensburg *et al.*, 1997), but we found no reference to ascomycetous yeast strains with the ability to utilize monoterpenes as the only carbon source. The only report on the isolation of yeasts able to grow on monoterpenes has been on basidiomycetous yeasts (Thanh *et al.*, 2004).

1.5.3 Maintenance of cultures

The best medium for maintaining yeast cultures requires the addition of glucose to the media as the only source of carbon. This is preferred since the risk of changes in growth and fermentative pattern, due to the selection of mutants is minimized (Scheda & Yarrow, 1966). However, an unstable strain's properties can change within a few days due to the above selection when grown on media containing malt extract. Consequently, YM agar slopes as well as yeast-glucose-peptone or malt agar are used to maintain yeast (Yarrow, 1998).

Numerous yeast strains may be stored at temperatures between 4^0C and 12^0C after sub-culturing for intervals of six to eight months. The frequency of subculturing differ among yeasts, with some such as *Arxiozyma* and *Malassezia* having to be subcultured every month because they are more sensitive to prolonged storage. The teleomorphic members of ascomycetous and basidiomycetous yeasts may lose the ability to sporulate on successive cultivation on laboratory media. The extents to which yeasts lose their ability

to sporulate differ among yeasts and may range from a few weeks to several years (Yarrow, 1998).

As a result, it is best to preserve important strains using appropriate techniques such as lyophilization (Kirsop & Kurtzman, 1988), L-drying (Mikata & Banno, 1989) and freezing in either liquid nitrogen or a mechanical freezer at temperatures between -60^0C and -135^0C (Yarrow, 1998). The method currently being mostly used is freezing in liquid nitrogen and is termed cryopreservation, which uses a cryoprotectant such as glycerol to ensure high rates of survival as well as genetic stability.

1.6 Taxonomy of the yeast

The principles of yeast taxonomy encompass identification, naming and placing organisms in their proper evolutionary framework. Historically, ascomycetes have been placed in two taxonomic classes or subclasses namely the Hemiascomycetes and the Euascomycetes. Hemiascomycetes are characterized by asci that are not enclosed in a fruiting body (ascocarp), whereas Euascomycetes usually form asci within or upon fruiting bodies.

1.6.1 Natural classification system

Natural classification systems are based on evolution, and have been the preferred method for systematics. This system addresses the species concept, and also shows the phylogeny or sequence of events that are involved in evolution. This includes the new species and interpretation of ascomycetous species, which no longer produce ascal stages. The species

concept recognizes that the different species have different phenotypic characters by which they will be recognized (Van der Walt, 2000). This approach has weaknesses highlighted by several authors (Phaff, 1981, 1989; Kurtzman *et al.*, 1983; Kurtzman & Phaff, 1987; Van der Walt, 1987, 2000). Also, it has been recognized that some characters are subject to intra-group variability, convergent or parallel evolution (Price *et al.*, 1978; Fuson *et al.*, 1980; Kurtzman, 1984).

Studies carried out by several independent laboratories have shown that there is a phylogenetic connection between the ascogenous and the anamorphic species despite the fact that the anamorphs do not produce asci but instead have characteristics that make them suitable for being assigned to the ascomycetes (Barns *et al.*, 1991; Hendriks *et al.*, 1991, 1992; Cai *et al.*, 1996; Suzuki *et al.*, 1999). The anamorphic species' inability to produce asci is believed to be due to either the absence of genes for ascospore formation or that the genes are present but not expressed or are silent. The anamorphs account for about half of the ascomycetous yeasts and are widely distributed. This implies that the anamorphs are as environmentally successful as their ascogenous types despite being completely asexual.

1.6.2 Biological species concept

Modern biologists have arrived at the biological species concept that is characterized by four distinct aspects. These are a reproductive unit, an ecological unit, a genetic unit and lastly an evolutionary entity. This was further enhanced by another dimension of the species through the

introduction of the so-called phylogenetic species concept that focused on the interpretation of the ribosomal nucleotide sequence analysis thereby excluding the phenotypic, genetic or ecological criteria. The two concepts namely the biological species concept and phylogenetic species concept have little in common. The biological species concept is not easily applied in practical systematics resulting in having to adhere to the type-based species and the extensive use of phenotypic differentiation (Kurtzman, 1987; Boekhout & Kurtzman, 1996). Also, by definition, the biological species concept excludes the asexual (anamorphic) yeast species. Barnett and his co-authors (2000) listed about 93 characters for identification purposes concentrating on just phenotypic characteristics.

To overcome the noted drawbacks from the two preceding concepts, yeast taxonomists had to consider analysis of the nuclear genome that had already been extensively used in prokaryotic systems. The base composition of the nuclear genome was believed to reflect the ancestral descent at molecular level reducing reliance on phenotypic characterization (Van der Walt, 2000).

1.6.3 Molecular taxonomy

The introduction of DNA studies provides a parameter for estimating evolutionary distances among taxa. Different taxonomic levels are resolved by different methods. The two features of the nuclear genome that were thought to be useful in recognition of species, involved accurate determination of the mean molar percentage guanine plus cytosine (mol% G + C) of the presumed homogenous nuclear DNA first adopted in the laboratory at H.

Phaff (Meyer & Phaff, 1969; Fuson *et al*., 1980; 1987). However, the mean mol% G+C does not reflect differences in base sequences. It is mainly exclusionary and therefore different species may have more or less coincidental G+C values. To overcome the limitation, another concept of nDNA homology of strains was considered whereby closely related species were denatured and the reassociation of the single stranded fragments of nDNA would determine how closely related the species were (Van der Walt, 2000). This method is still being extensively used and preferred although it is laborious and crude by modern perception (Daniel & Meyer, 2003). The initial finding of this method was promising and showed reassociation values in the order of 70 to 100% indicative of conspecificity. When the numbers of strains were increased, the reassociation values decreased to between 40 and 65%. Therefore, when new strains are to be described using this method, reassociating of strains remains problematic. This has led to genetic relatedness in terms of nDNA data being referred to as a sliding scale that requires calibration in terms of absence of genetic exchange. This is restricted to amphimictic species, the life cycles of which involve the fusion of haploid vegetative cells.

Molecular taxonomists have conceded that nDNA complimentarity had an important impact on yeast systematics but it does not allow for elucidation of relationships above the species level (Kurtzman, 1998; Vaughan-Martini, 2003). Therefore, nDNA reassociation data on their own serve no useful purpose in the demarcation of genera as seen from merging of the genera *Pichia* and *Hansenula* (Kurtzman, 1984). This merger was found to be

taxonomically incorrect since it ignored the ruling that the merging of genera be based on the comparison of the relevant type species (Van der Walt, 2000).

In the development of the species concept, the recognition of the phylogenetic species seemingly requires no recourse to phenotypic, sexual or ecological criteria. It is based exclusively on nucleotide sequence analysis of selected informative elements in the ribosomal genome (Kurtzman & Robnett, 1994; 1995). This means that the number and sequence of substituted nucleotides identify the species, and that the resolution of taxa does not go beyond sister species. The ribosomal base sequence analyses permits alignment of species providing a more readily accessible source of data which provides a basis from which phylogenies may be either inferred or hypothesized (Van der Walt, 2000; Daniel & Meyer, 2003).

The noted limitations of the proposed concepts led to consideration of genomic data, which included ribosomal markers that relied on base sequence analysis of the ribosomal genome. This is especially useful due to its stability and subsequent highly conserved status. Blanz and Unseld cautioned that ribosomal base sequence data should not be the key feature to be taken into account but that other numerous characters have to be considered when phylogenetic conclusions are to be drawn (Blanz & Unseld, 1987). In the last few years, the system of classification for yeasts, as well as for other fungi, has been revolutionized by molecular analysis of phylogenetic sequences. A higher percentage of these studies have been focused on

rRNA gene sequences, but comparisons of other molecules have given similar results (Petersen & Kurtzman, 1991; Kurtzman, 1994).

Initial studies undertaken were limited to the 18S and 26S rRNA. Preliminary results confirmed relationships between related species but it was clear that not all regions of the 18S and 26S subunits were equally useful (Hendriks *et al.*, 1991, 1992; Cai *et al.*, 1996). Regions that were found to resolve certain taxa in one failed to do so in others. The noted limitations of the rRNA base sequence led to consideration of aspects of rDNA together with randomly amplified polymorphic DNA (Vaughan-Martini & Kurtzman, 1985). However, mycologists had already found a range of domains that sufficiently resolve both species and genera (Van der Walt, 2000).

Kurtzman and Robnett carried out a comprehensive study in 1998 on all known ascomycetous yeasts. This study involving 760 strains, representing 500 species, was based on sequence analysis of approximately 600 bases of the D1/D2 domain of the 26S subunit. In addition, nine strains representing Euascomycetous yeasts were included to increase the scope of the investigation. The study confirmed that the ascomycetous yeasts are polyphyletic. However, the information obtained did not address the anticipated connection between the Saccharomycetales and Euascomycetes. The study did not show whether D1/D2 domain provides sufficient information for classification of all ascomycetous yeasts or whether an alternative investigation could be carried out on other regions including the ITS 1 and 2 regions of the 5.8S subunit together with the β-tubulin and histone genes.

The limitation of the method led to the conclusion that more gene sequences will have to be analyzed before most genera could be phylogenetically classified (Kurtzman & Robnett, 1998; Fell *et al.*, 2000; Yang *et al.*, 2001; Herzberg *et al.*, 2002).

1.6.4 Current classification system

The current classification of the ascomycetous yeasts, which is based on the biological species concept, comprises three classes, namely Archiascomycetes, Euascomycetes and Hemiascomycetes. Most of the ascomycetous yeasts are found in the orders Schizosaccharomycetales (fission yeasts) and the Saccharomycetales, which are in the classes Archiascomycetes and Hemiascomycetes respectively (Barnett *et al.*, 2000). The focus of the study was on the Hemiascomycetes, which comprises nine families, 51 genera and 432 species. These families have been demarcated on the basis of the 18S, 26S RNA sequence data as well as phenotypic characteristics.

Table 1. The nine families in the order Saccharomycetales, under the class Hemiascomycetes with some representative genera (Barnett *et al*., 2000).

Family & characteristic features	Genera (examples)	Distinctive features
Candidaceae Budding cells, pseudo-hyphae, septate hyphae and no sexual reproduction.	*Aciculoconidium*	Septate hyphae with chains of blastoconidia.
	Arxula	Septate hyphae and arthroconidia.
	Brettanomyces	Non-septate hyphae.
	Candida	
	Geotrichum	Arthroconidia and no budding cells.
	Kloeckera	Polar budding, lemon-shaped or oval cells.
	Myxozyma	Fluid to mucoid colonies
	Sympodiomyces	Conidia formed on conidiophores in sympodulae.
	Trigonopsis	Triangular cells, budding from corners, also oval cells with multilateral budding.

Dipodascaceae Budding cells, pseudohyphae, and septate hyphae.	*Dipodascus*	Asci with 2 to 4 smooth ascospores with a regular mucilaginous sheath.
	Galactomyces	No budding cells and each ascus has one spiny or warty ascospore.
	Yarrowia	Asci with 1 to 4 round, oval, walnut, hat or saturn shaped ascospores, usually formed after mating pairs of compatible strains.
Eremotheciaceae Budding cells, pseudo-hyphae or septate hyphae, asci with 4 to 16 needle-shaped ascospores.	*Eremothecium*	Budding cells, pseudo-hyphae or septate hyphae, asci with 4 to 16 needle-shaped ascospores.
Lipomycetaceae Budding cells with polysaccharide capsule, no hyphae	*Lipomyces*	Asci with 1 to 16 oval dark ascospores.
	Smithiozyma	Asci with 1 to 4 round ascospores.
Metschnikowiaceae Characterized by budding cells, sometimes pseudohyphae.	*Clavispora*	Asci with 1 to 4 conical or clavate ascospores.
	Metschnikowia	Ascus isclub-shaped with 1 or 2 needle-shaped ascospores.

Phaffomycetaceae Is characterized by budding cells, asci with 1 to 4 hat-shaped ascospores.	*Phaffomyces* *Starmera*	Occasionally pseudohyphae No hyphae
Saccharomycetaceae Budding cells, with 1 or 2 round ascospores.	*Arxiozyma* *Citeromyces* *Debaryomyces* *Dekkera* *Issatchenkia* *Kluyveromyces* *Pichia* *Saccharomyces* *Williopsis*	Asci persistent Also warty ascospores, heterothallic. Conjugation usually between bud cell and bud. Sometimes pseudo-hyphae 1 to 4 round ascospores 1 to 60 smooth, round or reniform ascospores. Variation in ascospore morphology from round, hat or Saturn shaped. Asci persistent and formed direct from diploid cell, has 1 to 4 ascospores. 1 to 4 saturn-shaped ascos pores

Saccharomycodaceae Is characterized by bipolar budding, lemon-shaped or oval cells, and asci with 1 to 4 spherical or hat-shaped ascospores.	*Hanseniaspora*	Rarely pseudo-hyphae, 1 to 4 spherical or hat-shaped ascospores.
	Nadsonia	1 or 2 rough, round ascospores.
Saccharomycopsidaceae Budding cells, plugged septal pores, asci arranged in lateral clusters containing 1 to 4 hat –shaped or saturn-shaped ascospores.	*Ambrosiozyma*	Pseudo and true hyphae
	Saccharomycopsis	Septate hyphae Variation in ascospore morphology ranging from reniform, round, or elliptical.

1.6.5 Identification

It is essential to ensure that a culture to be identified is pure. Conventional taxonomic procedures that analyze strain phenotypic characteristics, which include ecological origin, morphology, physiology, biochemical and sexual aspects are carried out from absolutely pure cultures and are clearly described (Kreger-van Rij, 1984; Van der Walt & Yarrow, 1984; Yarrow, 1998; Barnett *et al*., 2000).

1.6.5.1 Conventional identification

The criteria and tests for identification of yeasts involve firstly, observation of culture characteristics, which include colour, shape and texture of the colonies. The texture can either be mucoid, viscous, butyrous, friable or membranous. The production of extra-cellular polysaccharide material may

be observed by the resulting mucoid growth. There are distinctive colours such as yellow, orange and red that is peculiar to certain genera. This includes *Phaffia*, *Rhodosporidium* and *Sporidiobolus*. The colour produced by a majority of yeasts ranges from whitish through cream to buff (Yarrow, 1998).

This is followed by observation of asexual structures, which include shape and size of the vegetative cells. Then the mode of conidia formation is investigated and provides information, which aids in the identification of a strain. Budding starts by forming a small outgrowth at some point on the surface of the cell without the cell changing in size. The increase in size is seen in a newly formed bud, which eventually separates from the parent cell (mother). Holoblastic budding result from outgrowth of the entire cell wall of the parent cell, the bud separates from the narrow base leaving a scar through which no further budding occurs. This type of budding is characteristic of the *Saccharomycetales* and their anamorphic states while enteroblastic is characteristic of basidiomycetous yeasts and results in formation of a collaret due to recurrent formation and abscission of a succession of buds (Yarrow, 1998).

The position and the site of bud formation facilitate classification. This can either be monopolar budding which refers to buds arising at one pole of the cell whereas involvement of both poles result in a bipolar budding, which is characteristic of the apiculate yeasts (Yarrow, 1998).

Fission is the type of reproductive method whereby duplication of vegetative cells involves growth of the septum inwards from the cell wall dividing the long axis of the cell. The fission cells that are newly formed are termed arthroconidia (arthrospores). They elongate and the process is repeated. Recurrent fission by a cell results in transverse multiple scars or annelations that are characteristic of *Schizosaccharomyces* (Yarrow, 1998).

The vegetative cells have different shapes, which in turn can be used for identification. The noted shapes include globose, subglobose, ellipsoidal, ovoidal, cylindrical, botuliform, elongate, apiculate, lunate and triangular. The shape may reflect the type of reproduction and in some cases it is peculiar to particular genera or species. Examples include the lemon-shaped cells of the apiculate yeasts *Hanseniaspora* and *Wickerhamia*, the bottle-shaped cells of *Malassezia*, triangular cells of *Trigonopsis* and the lunate cells of *Metschnikowia lunata* and *Candida pellata* (Yarrow, 1998).

Sexual structures are investigated which include arrangement, cell wall ornamentations, number, shape and size of ascospores or basidiospores. The form of the asci can characterize a genus. In *Lipomyces* they appear as sac-like appendages whereas in *Metschnikowia* they are long and clavate. Ascospores liberated tend to aggregate in masses. They vary in number present in the asci, shape, size, ornamentation and colour. Asci with more than eight spores are usually characteristic of *Lipomyces*. There are various shapes of ascospores known. They include, globose, cylindrical, ellipsoidal,

clavate, crescentric, hat-shaped, saturnoid, needle-shaped and spindle-shaped (Yarrow, 1998).

Other types of spores formed that aid identification include endospores which are vegetative cells, formed within discreet cells and hyphae. They cannot be stained selectively. However, they can also be observed in old cultures on YM agar. Asexual endospores are observed in strains of the genera *Candida*, *Cryptococcus*, *Cystofilobasidium*, *Oosporidium* and *Trichosporon* whereas they are uncommon in other genera. Chlamydospores are thick-walled asexual spores rich in lipids. The asexual nature of the chlamydospore distinguishes it from the teliospore of the *Sporidiales* and *Ustilaginales*. Chlamydospores are characteristic of *Candida albicans* and *Metschnikowia* species. The formation of germ tubes by *Candida albicans* is a reliable and rapid feature used in medical laboratories for identification (Van der Walt & Yarrow, 1984; Yarrow, 1998).

Physiological properties can be used primarily to describe and identify yeast species and to a very minor extent genera. The tests most used for routine identification purposes include fermentation of and growth on carbon sources, growth on nitrogen sources, requirements for vitamins and growth at various temperatures and on media with high content of sugar or sodium chloride. The ability to ferment sugars is tested in Durham tubes containing 2% (w/v) solution of sugar with the exception of raffinose where 4% (w/v) is used. Raffinose is used at this concentration because some strains use only part of the molecule.

These tests are not standardized and are thus dependent on techniques employed. Therefore, methods employed for identification tests should be strictly adhered to, as well as having an authentic strain of the supposed species which can be used as a reference strain (Yarrow, 1998; Barnett *et al.*, 2000).

1.6.5.2 Fatty acids in yeast taxonomy

Currently, the identification of yeasts is based on conventional methods as already mentioned, which are time-consuming, laborious and not always accurate. This led to the investigation of other techniques that would address the shortcomings of conventional methods. Consequently, the use of fatty acid composition to distinguish species within genera (Kock *et al.*, 1985; Viljoen *et al.*, 1986) was investigated. These workers went further by ensuring that standardized conditions were taken into account that included nature of medium, growth conditions, extraction and analyses of fatty acids (Kock *et al.*, 1985; Viljoen *et al.*, 1986).

The use of standardized techniques led to a surge of studies being carried out and these studies showed that fatty acid composition was useful in distinguishing between species within a genus as seen in *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schwanniomyces* and taxa in the family Lipomycetaceae (Cottrell & Kock, 1989; Cottrell *et al.*, 1986; Miller *et al.*, 1989; Van der Westhuizen *et al.*, 1987; Viljoen *et al.*, 1986; Viljoen & Kock, 1989; Van der Walt, 1992). The revision of the genus *Nadsonia* also

included fatty acid composition in combination with other phenotypic characteristics such as ascospore morphology (Golubev *et al.*, 1989).

1.6.5.3 Restriction Fragment Length Polymorphism (RFLP)

Another method of delineating yeast strains is a DNA-based procedure based on variations in the DNA structure by detecting restriction fragment length polymorphisms (RFLPs). This involves amplification of a specific DNA region followed by cleavage into fragments by DNA restriction enzymes (Cutler *et al.*, 1988; Merz, 1990). These restriction enzymes (RE) are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4-6 bp (Merz, 1990). Sample DNA is digested with one or more RE and the resulting fragments are separated according to molecular size using gel electrophoresis (Magee *et al.*, 1992). Molecular size standards are used to estimate fragment size. Ethidium bromide staining is used to reveal the fragments under UV (260nm) light. The observed differences result from base substitutions, additions, deletions or sequence rearrangement within restriction enzyme recognition sequences or sites (Magee *et al.*, 1992). RFLP is most suited to studies at the intraspecific or among closely related taxa (Dlauchy *et al.*, 1999). Presence and absence of fragments resulting from changes in recognition sites are used to identify species or even populations of organisms from one another (Merz, 1990).

PCR-based techniques have gained popularity over the years because of the ease and speed with which they can be performed as well as the huge

database available as a result of sequencing studies (Vaughan-Martini, 2003), which allow comparison for rapid presumptive yeast strain identification. According to Deák *et al.* (2000), restriction analysis of the internal transcribed spacer (ITS) that includes the more conservative 5.8S rRNA gene (ITS-PCR) has been found the most useful, since it allows species identification and typing of isolates (Valente *et al.*, 1997; Guillamón *et al.*, 1998; Fernández *et al.*, 1999; Cadez *et al.*, 2002). This method has found amongst others, application in the rapid identification of yeasts involved in the spoilage of yoghurt (Caggia *et al.*, 2001) and in rapid identification of medically important yeasts (Trost *et al.*, 2004).

1.6.5.4 The Internal transcribed spacer region

According to literature (Egli & Henick-Kling, 2001; Petersen *et al.*, 2001; Van der Aa Kühle & Jespersen, 2003), phylogenetic analysis of closely related species is possible from the use of the region spanning the two intergenic transcribed spacers (ITS1 & ITS2) and the 5.8S ribosomal subunit. This region is located between the 18S and the 28S rRNA genes in yeasts. The ITS region is subdivided into the ITS 1 region which separates the conserved 18S and the 5.8S rRNA genes (James *et al.*, 1996, Guillamón *et al.*, 1998; Frutos *et al.*, 2004). The ITS 2 region is found between 5.8S and 28S rRNA genes. The ITS 1 and 2 have been shown to play a role in primary rRNA processing (Musters *et al.*, 1990).

The ITS regions are less conserved as a result of less evolutionary constraints and hence can be used to discriminate species within some

genera (Egli & Henick-Kling, 2001; Kurtzman, 2001). This region was reported useful for differentiation of species within *Saccharomyces* (Oda *et al.*, 1997; Fernandez-Espinar *et al*., 2000), rapid identification of yeasts (Guillamón *et al*., 1998), a rapid method for species identification and the differentiation of Flor yeasts (Fernandez-Espinar *et al.*, 2000) as well as rapid identification of yeasts involved in spoilage of yoghurt (Caggia *et al.*, 2001).

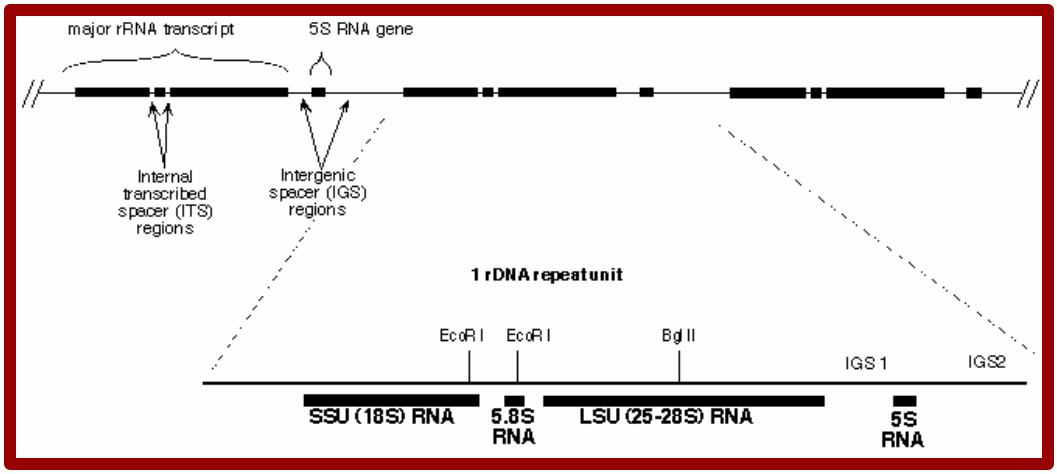

Figure 2. Schematic representation of the ITS region (Gargas & De Priest, 1996).

1.6.5.5 D1/D2 domain

This region refers to the variable domain of the large subunit (26S) ribosomal DNA or the complete small subunit and is approximately 600 bases in size. Conspecific strains are separated by less than 1% nucleotide substitution whereas biological species are separated by a greater than 1% nucleotide substitution. Kurtzman and Robnett have shown that most yeast species can be identified from sequence divergence in this region, which represents a

partial sequence of the 26S rDNA (Kurtzman & Robnett, 1998; Fell *et al.*, 2000).

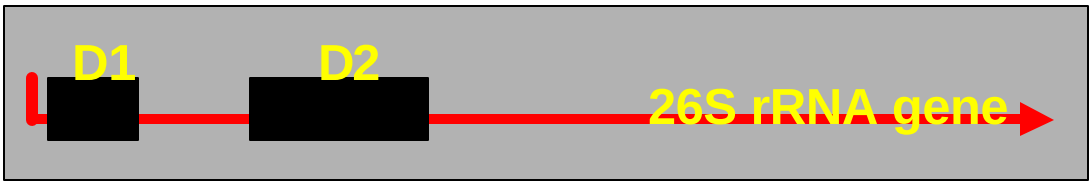

Figure 3. Schematic representation of the 26S rRNA gene and the location of the D1 and D2 hypervariable domains are illustrated.

The sequencing of the D1/D2 domain is increasingly being used to identify yeasts (Phaff *et al.*, 1999; Hong *et al.*, 2001; Scorzetti *et al.*, 2002) and according to Frutos *et al.* (2004) it is accepted universally as the main tool for yeast taxonomy. This method has enabled identification of new ascomycetous yeasts in the *Pichia anomala* clade previously not recognized as novel when conventional identification techniques were used (Kurtzman, 2000). Also, the observed large number of nucleotide substitutions in the D1/D2 domain among *Metschnikowia* species supports the idea that the genus is highly diverged and that there maybe a great number of species still to be detected (Giménez-Jurado *et al.*, 2003).

Databases of the D1/D2 sequences are now available for all currently recognized ascomycetous and basidiomycetous yeasts (Kurtzman & Robnett, 1995; 1997; 1998; Guffogg *et al.*, 2004). This extensive available database makes the task of species identification much easier (Kurtzman, 2001;

Starmer *et al.*, 2001; Wesselink *et al.*, 2002) and could serve as reliable and practical criteria for identification of most known yeasts (Abliz *et al.*, 2004).

1.7 Purpose of Study

With the above as background the purpose of this study became the following:

1. To isolate and identify yeasts from different habitats in different regions of Lesotho using: (**Chapter 2**)
    - Conventional identification techniques
    - Fatty acid profiles
    - Restriction fragment length polymorphism of the ITS 1 and 2 region
    - D1/D2 sequencing data.

2. To isolate and identify ascomycetous yeasts capable of utilizing derivatives of the cyclohexanes or chlorocompounds (**Chapter 3**).

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Chapter 2

Isolation and characterization of yeasts from the Lesotho highlands

2.1 Introduction

South African soils proved to be a rich source for the isolation of various yeasts (Jansen van Rensburg *et al.*, 1992; Mothibeli *et al.*, 1997). Examples include isolation of new yeasts such as *Arxula terrestris* (Van der Walt *et al.*, 1990a), *Candida mokoenaii* sp. nov. (Mokwena *et al*., 2000), *Debaryomyces polymorphus* var. *africanus* (Van der Walt *et al.*, 1994) *Debaryomyces udenii* (Van der Walt *et al.*, 1989a), *Kluyveromyces africanus* (Van der Walt, 1956a), *Kluyveromyces polysporus* (Van der Walt, 1956b), four new species in *Lipomyces* (Van der Walt *et al.*, 1999), *Lipomyces spencermartinsiae* (Van der Walt *et al*., 1997), *Myxozyma geophila* (Van der Walt *et al.*, 1987), *Myxozyma kluyveri* (Van der Walt *et al.*, 1989b), *Myxozyma lipomycoides* (Van der Walt *et al.*, 1987), *Myxozyma melibiosi* (Van der Walt *et al.*, 1981), *Myxozyma monticola* (Candidaceae) (Pretorius *et al*., 1993), *Myxozyma neglecta* sp. nov. (Candidaceae) (Spaaij *et al.*, 1998), *Myxozyma vanderwaltii* (Spaaij *et al.*, 1993), *Saccharomyces transvaalensis* (Van der Walt, 1956c), *Williopsis mucosa* (Kurtzman, 1991) and *Zygozyma smithiae* (Van der Walt *et al.*, 1990b) to name but a few.

On the other hand, the north-eastern region of Lesotho do not only border to an area in South Africa (Drakensberg mountains), where new species such as *Lipomyces mesembrius* (Van der Walt *et al.*, 1997) and *Myxozyma monticola* sp. nov. (Candidaceae) (Pretorius *et al.*, 1993), have been isolated, but has rich wet soils at high altitude posing the possibility of housing diverse populations of yeasts.

With this as background, it became the aim of this study to isolate and characterize ascomycetous yeasts from this area of Lesotho. The isolated yeasts were identified by conventional methods, whilst the lipomycetous yeasts were further characterized by molecular methods using restriction fragment length polymorphism (RFLP) and D1/D2 sequencing data analyses.

2.2 Materials and methods

2.2.1 Strains

Hundred and sixty-five yeast strains were isolated from soil collected from 10 north-eastern regions of Lesotho with each region comprising 10 sites each as well as from two types of traditional local brews, Hopose and Sesotho. These strains are held at the University of the Free State UNESCO-MIRCEN culture collection. The regions sampled are shown in Table 1.

2.2.2 Isolation

Soil samples obtained from 100 sites from the 10 regions were stored at room temperature and plated directly onto solid and liquid complex media such as YM, various selective media such as Rose-bengal dichloran (King *et al*., 1979) and media for isolation of *Lipomyces* strains, which contained cyclohexamide and thymine as sole nitrogen source (Jansen van Rensburg, 1991). To prepare the latter the following were dissolved in 900 ml sterile distilled water: 5.0 g glucose, 1.0 g potassium phosphate (monobasic), 0.5 g magnesium sulphate, 0.1 g sodium chloride, 0.1 g calcium chloride, the volume was adjusted to 990 ml before adjusting the pH to 5.2. This was followed by the addition of 0.48 g thymine, 0.2 g chloramphenicol, 10.0 g

agar, 1.0 ml of stock solution of trace elements which had the following composition: (0.5 g boric acid, 0.04 g copper sulphate, 0.1 g potassium iodide, 0.2 g ferric chloride, 0.4 g zinc sulphate per litre). The medium was warmed in the microwave until all the agar had dissolved and cooled in a water bath to about 50^0C, followed by the addition of 10 ml vitamin solution (Van der Walt & Yarrow, 1984), 0.2 g cycloheximide which was firstly dissolved by gradual addition of approximately 1 ml acetone before adding to the medium which was mixed by gentle swirling before dispensing into petri-dishes and allowed to set. For the preparation of the liquid media, only the agar was omitted and all other components were prepared as explained above. The cultures were incubated at 25^0C for 14 days, in the case where no growth was observed after this incubation period, plates were incubated for a further seven days. Individual colonies were re-streaked on solid YM media for purity and pure cultures were maintained on YM agar slants.

2.2.3 Cryopreservation

Polypropylene straws (coloured drinking straws) were cut into lengths of 4 cm with one end sealed using a bunsen burner. The straws were placed in McCartney bottles with the open end facing up and autoclaved at 121^0C for 15 min. The culture was prepared by streaking the yeasts on (YM) agar, incubated at 25^0C for three to five days, depending on the isolate, to obtain single colonies and good growth. A single colony was used to inoculate 3 ml of LN broth medium (YNB-tryptone-glucose broth) and incubated on a rotary shaker for three days at 25^0C. A loop of culture was examined microscopically for morphology to ensure that there was no contamination. A

sterile 1 ml pipette was used to dispense 0.5 ml of 70% glycerol to each of the cultures and was thoroughly mixed before filling the autoclaved straws (E. Pretorius, personal communication).

The straws were three quarters filled with the glycerol culture mixture using a pasteur pipette and the open ends were sealed with the flame of a bunsen burner with the aid of a pair of unridged forceps. The sealed straws were carefully marked with a cryopen with the respective culture numbers. The cultures were placed in a -70^{0}C freezer for 2 h, and then placed on an aluminium ladder and immersed in liquid nitrogen (-196^{0}C).

2.2.4 Identification and characterization

The strains that were Diazonium Blue B (DBB) negative (Van der Walt & Hopsu-havu, 1976) and those with variable DBB reaction were included for identification. Identification of these isolates was carried out conventionally using morphological and physiological characterization as described by Van der Walt and Yarrow (1984). Assimilation and fermentation of carbon compounds were examined at 25^{0}C on a rollordrum rotating at 40 rph. The assimilation of carbon compounds were carried out by inoculating 3 ml sterile tap water in a test-tube with an actively growing yeast culture. The suspension was prepared such that black lines drawn on a white cardboard were visible as dark bands through the tube. A total of 40 tubes containing different carbon sources were inoculated with one drop of the suspension of the yeast culture to be identified. A control was included in each of the identification sets, made up of a basal medium without any carbon source and

inoculated with the same suspension of yeast culture to be identified. The tubes were incubated at 25^0C, and results were scored after three, seven, fourteen and twenty-one days of growth by recording the degree of turbidity using a white cardboard with black lines drawn and determining visibility of these lines through the tubes (Yarrow, 1998).

Fermentation of the carbon compounds was carried out using 2% w/v of the sugars to be tested with the exception of raffinose where 4% w/v was used (Yarrow, 1998; Barnett *et al*., 2000a). The sugars that were tested include glucose, galactose, inulin, lactose, maltose, melibiose, raffinose and trehalose. A durham tube was placed into each of the tubes containing the respective sugars. The same yeast suspension that had been prepared for the assimilation tests above was used to simultaneously inoculate the tubes for fermentation. The tubes were placed on a test-tube rack and incubated at 25^0C. The formation of gas was recorded over a period of seven days.

Nitrogen utilisation was determined by the auxanographic method (Lodder & Kreger-van Rij, 1952; Van der Walt & Yarrow, 1984). Yeast carbon base medium was used for the nitrogen assimilation tests. The media was prepared and dispensed into McCartney bottles. Prior to use the media were melted and cooled in a water bath to a temperature of between 40^0C and 45^0C. The actively growing yeast to be identified was used to prepare a suspension of cells in sterile tap water, and these were added to the melted medium and thoroughly mixed and poured into a petri dish. The agar was allowed to solidify and the agar surface to dry. The plate was divided into

three parts and at the periphery, sources of nitrogen were placed in the form of small crystals. Ammonium sulphate was used as a positive control and was placed on one of the three parts on the petri dish. Plates were incubated at 25^0C and observed for a week.

Ascospore morphology was investigated on 1/10 YM, Gorodkowa, 2% Malt extract and McClary acetate agar incubated at 25^0C for three weeks (Yarrow, 1998). The shape, ornamentation, colour, size of the ascospore and the number per ascus were investigated. Also, the formation of hyphae, chlamydospores and germ tubes were investigated.

2.2.5 Lipid analysis

2.2.5.1 Cultivation

A rapid extraction method was used where cultures were grown on YNB agar (YNB 6.7g, glucose 40g, agar 16g /litre) until stationary phase was reached (10 days). Cells were scraped off the agar, macerated with a pestle in a mortar whilst mixing with organic solvent mixture (chloroform: methanol, 2:1). They were left overnight, filtered and excess mixture was left to evaporate over a stream of nitrogen (Folch, 1957).

2.2.5.2 Fatty acid analysis

The fatty acid composition associated with total lipids was determined by addition of 200 µl of chloroform and trimethyl sulphonium hydroxide (TMSOH) to the lipid fraction according to the method of Butte (1983). The fatty acid methyl esters were analysed by gas chromatography (GC), with a flame

ionisation detector and a Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145^{o}C was increased by 3^{o}C/min to 225^{o}C and, following a 10 min isothermal period, increased to 240^{o}C at the same rate. The inlet and detector temperatures were 170^{o}C and 250^{o}C respectively. Nitrogen was used as a carrier gas at 5 ml/ min. Peaks were identified by reference to authentic standards.

2.2.6 Restriction Fragment Length Polymorphism

2.2.6.1 Strains analysed

Thirty strains representing 28 type species of the Lipomycetaceae and associated anamorphs were subjected to RFLP analyses (Table 4). These strains were obtained from the UFS yeast culture collection.

2.2.6.2 DNA extraction

YM broth (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1.0%) was inoculated with strains (Table 4) that were used for RFLP analysis. The flasks were incubated at 25^{0}C for 48 h. Genomic DNA was extracted by filling a 2 ml eppendorf tube with the culture. The tubes were centrifuged for a minute and the supernatant was removed by means of aspiration. The cells were resuspended in 500 µl DNA lysis buffer (100 mM Tris-HCl at pH 8.0; 50 mM EDTA; 1% SDS); 200 µl glass beads were added to the suspension and vortexed for 4 min with immediate cooling on ice. The liquid phase was removed including the foam part into a sterile 2 ml eppendorf tube. To this, 275 µl ammonium acetate (pH 7) was added, vortexed and incubated for 5 min at 65^{0}C, followed by another 5 min incubation on ice; 500 µl of chloroform

was added, vortexed and centrifuged for 2 min at 14, 000 x g at 4^0C. The supernatant was transferred to a new sterile eppendorf tube; DNA was precipitated with 750 µl isopropanol and incubated for 5 min at room temperature; followed by centrifuging for 2 min at 14,000 x g (4^0C). The pellet was washed with 70% ethanol, centrifuged again using the same conditions. The pellet was dried using a speed-vac and the pellet was resuspended in 40 µl of TE containing RNase and incubated in a 37^0C water-bath for 30 min that allowed the enzyme to digest the RNA.

2.2.6.3 PCR amplification of the ITS region

The primer pair used to amplify the ITS region (as described by White *et al.* 1990) and were as follows: ITS4 (5' –TCCTCCGCTTATTGATATGC-3') and ITS5 (5'- GGAAGTAAAAGTCGTAACAAGG –3'). The reaction mixture for PCR amplification for all the isolates was prepared using 50 µl volume under the following PCR conditions: a total of 25 cycles were performed and include initial denaturation at 94^0C for 2 min, denaturation at 94^0C, annealing at 58^0C for 30 s and extension at 72^0C for 30 s. A final extension was carried out at 72^0C for 7 min and a holding temperature of 4^0C for 5 min. PCR products were separated on a 0.8% agarose gel containing 15 µl of ethidium bromide (10 mg/ml) and visualized under UV light. The sizes of the PCR products were estimated by comparing them to a commercial molecular weight VIII marker (Roche).

2.2.6.4 DNA purification

The amplified DNA was purified using the GFX^{TM} – PCR DNA and gel band purification kit (Amersham, Biosciences) as follows: 500 µl of capture buffer was added to the GFX column, DNA solution was added and mixed with the capture buffer using a pipette, the column attached to the collection tube was centrifuged for 30 s, the flow-through was discarded, 500 µl of wash buffer was added to the column, centrifuged at full speed for 30 s, the collection tube was discarded and a sterile 1.5 ml microcentrifuge tube attached to the GFX column, finally 40 µl of elution buffer was applied directly to the glass fibre matrix in the GFX column, incubated at room temperature for 1 min and centrifuged at full speed for 1 min to recover the purified DNA.

2.2.6.5 Restriction digest

The purified DNA was restricted with each of the following enzymes, *Cfo*I, *Mbo*I and *Hae*III. The restriction digest was prepared in microcentrifuge tubes as follows: 5.0 µl of the PCR product was digested with the three restriction enzymes, according to the manufacturer's instructions. The reaction digests were incubated at 37^{0}C for 2 h, tubes were tapped every 30 min to ensure uniform digest of the PCR product. The fragments were separated by gel electrophoresis using a 1.5% gel of low melting point agarose containing Ethidium bromide. The run was carried out for 5 h, with pictures taken every hour to ensure that small sized bands/fragments were visualized, using UV transillumination and processed using the Gel Doc 1000 Video Gel Documentation System (Bio-Rad, Hercules, CA).

2.2.7 D1/D2 sequencing

DNA was extracted using the same method described for the RFLP analysis. The DNA was amplified using primers described by Kurtzman & Robnett, 1998). These were NL1 (5' –GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'- GGTCCGTGTTTCAAGACGG –3'). A total of 30 cycles were performed for each isolate, initial denaturation at 94^0C for 1 min, denaturation at 94^0C for 1 s, annealing at 52^0C for 55 s and extension at 72^0C for 1 min. A final extension was carried out at 72^0C for 10 min and a holding temperature of 4^0C for 5 min. PCR products were run on a 1.0% agarose gel with 15 µl of ethidium bromide (10 mg/ml) incorporated and visualized under UV light. The sizes of the PCR products were estimated by comparing them to a commercial 50 bp ladder (Promega).

The amplified DNA was purified with the GFX^{TM} – PCR DNA and gel band purification kit (Amersham, Biosciences) according to the manufacturer's instructions. The concentration of the DNA was estimated from the band intensity on the agarose gel after purification to be between 5 and 20ng. The concentration of the DNA estimated, enabled determination of the appropriate volume of DNA to be used for the sequencing reaction. The purified PCR product was prepared for ½ reactions by using 4 µl of premix, 2 µl of DNA and 4 µl of primer NL1 for the forward sequencing at a final concentration of 1.6 pmol/µl. The reverse sequencing reactions were prepared as for the forward sequencing reaction with the exception of the primer where NL4 was used instead of NL1. The cycling parameters for the sequencing reactions were performed with the following conditions: A total of 25 cycles were performed,

denaturation at 96^{0}C for 10 s, annealing at 50^{0}C for 1 s, extension at 60^{0}C for 4 min and the holding temperature at 4^{0}C for 5 min.

The post-reaction cleanup for the above reaction was carried out as follows: A sterile 1.5 ml eppendorf tube was used to which 2.0 µl of 3M sodium acetate was added and 50 µl of 100 % ethanol, the sequencing reaction obtained above was added to this tube; the mixture was vortexed briefly and incubated at room temperature for 15 min; centrifuged for 20 min at 4^{0}C with a speed of 14 000 x g; the supernatant was carefully removed by aspiration and 250 µl of 70% ethanol was added; vortexed briefly; centrifuged for 5 min at 4^{0}C with a speed of 14 000 x g; the supernatant was carefully removed by aspiration and the sample was dried in a speed-vac for 5 min. The sample was then submitted for sequencing.

2.2.7.1 Sequencing and alignment of sequence data

DNA sequencing was performed using the ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster city, CA), based on a modified Sanger's method using ABI V 3.0 cycle sequencing kit (Applied Biosystems). Sequences were analysed using sequence navigator V1.01 and assembled using Auto-assembler V1.4.0.

2.3 Results and discussion

2.3.1 Conventional identification

In total, 100 soil samples were collected from 10 regions comprising 10 sites each (Table 1). Identification of isolates was according to Kurtzman and Fell (1998) and Barnett *et al.* (2000a, b). A total of 165 strains with mostly ascomycetous affinity based on their DBB reaction were isolated from the 100 sites that were mostly humus rich with dense vegetation. The species *Lipomyces starkeyi* and *L. tetrasporus* were found distributed throughout the various habitats while *Debaryomyces hansenii*, *D. hansenii* var. *fabryi* and *D. occidentalis* were found in only 60% - 70% of the different regions. *Debaryomyces polymorphus*, *Dipodascus spicifer*, *Galactomyces geotrichum*, *G. reessii*, *Kluyveromyces lactis*, *L. kononenkoae*, *L. mesembrius*, *L. spencermartinsiae*, *Pichia anomala*, *P. fabianii*, *P. guilliermondii* and *Yarrowia lipolytica* were rather site specific (Table 1). No isolates representing the genus *Zygozyma* were found, which corroborates the assumption that these species are usually associated with plants and insects. Moreover, no isolates representing the *Lipomyces*-related anamorphic genus *Myxozyma* were recovered. Five isolates that were conventionally identified, as *Lipomyces* spp. could not be fully assigned due to atypical carbohydrate profiles obtained (Table 2). Consequently, their identification was confirmed through sequencing of their D1/D2 domain (Figures 1, 2) and comparing with sequences in Genbank at NCBI (http//www.ncbi.nlm.nih.gov).

2.3.2 Lipid analyses

The use of fatty acid composition as a rapid chemotaxonomic technique for identification of yeasts is well documented (Cottrell *et al.*, 1986; Kock *et al.*, 1986; Viljoen *et al.*, 1986a, b; Cottrell *et al.*, 1989; Miller *et al.*, 1989; Botha *et al.*, 1992; Kock & Ratledge, 1993; Van der Westhuizen *et al.*, 1994). The factors influencing the reproducibility of this technique were considered by Cottrell *et al.* (1985). These authors proposed that the cultivation conditions be standardized to include the type of medium, growth conditions, extraction and analyses of fatty acids. This method was then used as an important taxonomic tool in fungi (Botha & Kock, 1993). Consequently, the use of this phenotypic character enabled comparisons of the profiles obtained from yeasts isolated from Lesotho, a pristine environment to the authentic strains existing in the database.

All yeasts analysed were capable of producing 16:0, 16:1, 18:0, 18:1, 18:2 and most of the times 18:3 ω-3 fatty acids. No 18:3 ω-6 could be detected which is in accordance with literature (Kock & Botha, 1998). Here it was reported that only the Zygomycota and Protoctista are capable of producing 18:3 ω6 fatty acids and not the Dikaryomycota.

The lipid compositions of the isolated yeasts identified as *Cryptococcus albidus* showed the presence of 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 (Table 3). The most abundant fatty acids with similar distribution were found to be 18:1 and 18:2 and the lowest amounts were found in 16:1. The observed distribution of fatty acids in *C. albidus* is similar to that reported in literature

where some strains showed only trace amounts and others up to 7.5% of 18:3 ω3 (Smit *et al.*, 1988; Smit, 1991). *Cryptococcus laurentii* produced high amounts of 16:0 and 18:1 as were reported previously (Smit, 1991). One of the strains (52) produced significant amounts of 18:3 (Table 3) compared to the other strains of *C. laurentii* and was found to have the highest amount of 18:1. These profile variations for strains of *C. laurentii* are similar to those reported in literature (Smit, 1991).

Debaryomyces hansenii isolates produced about 60% of 18:1 as a predominant fatty acid, as well as relative high amounts of 16:0. Varying amounts of the other fatty acids, 16:1, 18:0, 18:2 and 18:3 were also present. This distribution is similar to that reported in literature (Merdinger & Devine, 1965; Saldanha-da-Gama *et al.*, 1997). *Debaryomyces hansenii* var. *fabryi* also showed a predominance of 18:1 followed by 16:0. The distribution of fatty acids observed for *D. hansenii* var. *fabryi* is similar to that reported in literature (Saldanha-da-Gama *et al.*, 1997). The predominant fatty acid obtained from most of the isolates of *D. occidentalis* was 18:1 reaching about 64% in isolate 63. Varying amounts of 18:3 were obtained in these isolates. The distribution of fatty acids in *D. polymorphus* also showed 16:0 and 18:1 as predominant fatty acids with the lowest amount obtained in 18:0. This distribution is also similar to that reported in literature (Saldanha-da-Gama *et al.*, 1997).

The fatty acids of *Dipodascus spicifer* were predominantly 16:0, 18:1 and 18:2 while smaller amounts of 16:1 and 18:0 were obtained. Isolate 67 produced

the highest amount of 18:3 (16.5%) compared to all the isolates in Table 3. The presence of 18:3 in *D. spicifer* has not been reported previously (Botha & Kock, 1993; Smith *et al*., 2003).

Galactomyces geotrichum and *G. reessii* both produced varying amounts of 16:0, 16:1, 18:0, 18:1 and 18:2 with 18:1 and 18:2 predominating. Trace amounts of 18:3 could be detected in both isolates, which is in accordance to that reported in literature (Kock, 1988; Botha & Kock, 1993; Pohl *et al.*, 1997).

The distribution of fatty acids in both *Issatchenkia orientalis* and *Kluyveromyces lactis* was similar, with the highest amount obtained being 18:1. Linolenic acid (18:3) was much higher in *I. orientalis* isolates i.e. 14.6% and 9.4% respectively than in *K. lactis* i.e. 3.4% (Table 3). The linolenic acid content in *K. lactis* is similar to that reported by Cottrell *et al.* (1985).

Isolates representing the genus *Lipomyces* are typically characterised (Table 3) by high percentages 16:0 and 18:1, which is in accordance with that reported by Jansen Van Rensburg (1994, 1995). All strains were also capable of producing trace amounts of 18:3 and low amounts of 16:1.

The fatty acids 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 were obtained in *P. anomala*, *P. fabianii* and *P. guilliermondii*. The amounts of 16:1 and 18:0 were the lowest in these species. This distribution of fatty acids in *P. anomala* is similar to that reported in literature (Noronha-da-Costa *et al.*, 1996; Malfeito-Ferreira *et al.*, 1997). The high 18:3 content of 7.4% and 5.5% found

in *P. fabianii* and *P. guilliermondii* respectively is similar to that reported in literature (Miller, 1989; Blignaut *et al.,* 1996).

The strains obtained from the traditional local brews were *Saccharomyces castellii* and *S. cerevisiae*. The most abundant fatty acids in *S. castellii* were 16:1 and 18:1. The observed distribution of fatty acids in *S. castellii* is similar to that reported by Augustyn *et al*., (1990). The predominant fatty acid in *S. cerevisiae* was 18:1, which is in accordance with literature. Strikingly, the presence of 18:3 obtained in this yeast has not been reported before in any of the many strains analysed in the past (Augustyn & Kock, 1989; Augustyn *et al*., 1990; 1991).

The fatty acids 16:0, 16:1, 18:0, 18:1 and 18:2 were obtained in similar amounts in *Y. lipolytica*. There was trace amount of 18:3 detected and this pattern is similar to that reported previously (Viljoen *et al.*, 1987).

2.3.3 PCR products of the ITS region

According to literature (Esteve-Zarzoso *et al.*, 1999; Egli & Henick-Kling, 2001; Petersen *et al.*, 2001; Van der Aa Kühle & Jespersen, 2003; Frutos *et al.*, 2004), phylogenetic analysis of closely related species is possible from the use of the region spanning the two intergenic transcribed spacers (ITS1 & ITS2) and the 5.8S ribosomal subunit. This is because the two non-coding ITS regions are very variable and the ribosomal subunit 5.8S displays a high interspecific divergence (Belloch *et al.*, 2002). This region was reported useful for differentiation of species within *Saccharomyces* (Fernandez-Espinar

et al., 2000), rapid identification of yeasts (Guillamón *et al.*, 1998), a rapid method for species identification and the differentiation of Flor yeasts (Fernandez-Espinar *et al.*, 2000). As a result of the successful use of this molecular technique to identify various yeasts and also to elucidate their taxonomic position, the method was used to study the type strains of the family, Lipomycetaceae including five atypical *Lipomyces* isolates from Lesotho (Table 4).

This region was found useful by enabling differentiation of species in the family Lipomycetaceae (Tables 4) while the sub-species and varieties could not be separated. Differentiation of genera was not possible since no one band size was specific to a particular genus (Table 4). In the genus *Dipodascopsis*, all species could be separated while the two varieties of *D. uninucleata* had a similar sized band. A similar pattern was observed for the genus *Lipomyces* i.e. *L. kononenkoae* subsp. *kononenkoae* and *L. kononenkoae* subsp. *spencermartinsiae* that produced similar band sizes. All the *Lipomyces* species could be separated with the exception of *L. lipofer* (*Waltomyces lipofer*) that had a similar band size to *L. tetrasporus*. In the genus *Myxozyma*, all species could be separated with the following exceptions *M. geophila*, *M. monticola* and *M. neotropica* showing a similar band size and *M. kluyveri* and *M. vanderwaltii* also with a similar band size. All the *Zygozyma* species could be separated with the exception of *Z. oligophaga* that had a similar band size to *Z. suomiensis*.

2.3.3.1 ITS region and D1/D2 domain

With some exceptions the findings of the PCR product of the ITS region are not consistent with the results of the D1/D2 sequence data of the family Lipomycetaceae.

2.3.3.1.1 *Dipodascopsis*

The two varieties of *D. uninucleata* are genetically close regarding both the D1/D2 domain data and the PCR product of the ITS region. *Dipodascopsis tóthii* had a PCR product of the ITS region of 680 bp while the two varieties of *D. uninucleata* both had 580 bp. This more distant relationship was also supported by the D1/D2 domain data. Both the D1/D2 sequence data and the PCR product of the ITS region for *B. anomala* and *D. tóthii* indicate a close relationship.

2.3.3.1.2 *Lipomyces*

In the genus *Lipomyces*, the D1/D2 sequence data indicate close relationships amongst species. The PCR product of the ITS region of these species are however different. The two varieties of *L. kononenkoae* with similar band size support the D1/D2 data. Both *L. lipofer* and *L. tetrasporus* had a similar size band for the ITS region, although the D1/D2 domain sequence data indicate a more distant relationship.

2.3.3.1.3 *Myxozyma*

Although the D1/D2 sequence data show some variation between several *Myxozyma* species, the PCR product of the ITS region indicate close phylogenetic relationships with *M. melibiosi* being an exception i.e. with 900 bp PCR product (Figures 3, 4, 5, 6).

2.3.3.1.4 *Zygozyma*

The D1/D2 sequence data indicate distant relationships among species in this genus while the PCR product of the ITS region suggest a closer relationship i.e. *Z. arxii* (600bp), *Z. oligophaga* (500bp), *Z. smithiae* (540bp) and *Z. suomiensis* (500bp).

2.3.3.2 Conventional approach and the ITS region

Taxonomic procedures used conventionally to analyse strain phenotypic characteristics include their ecological origin, morphology, physiology and sexual cycles (Yarrow, 1998; Barnett *et al.*, 2000a, b). However, it has been shown that phenotypic variation does not always correspond to genomic data (Vaughan-Martini, 2003).

2.3.3.2.1 *Babjevia* and *Dipodascopsis*

Babjevia anomala and *D. tóthii* differed by only 10 bp on the PCR product of the ITS region (Table 4, Figures 3, 7, 9). These two strains were previously found to exhibit a close relationship on the basis of chromosomal DNA bands that were larger than 1300 kilobases (Jansen van Rensburg *et al.*, 1995).

However, these species were different regarding morphological, sexual reproduction and physiological features (Kurtzman & Fell, 1998).

Dipodascopsis tóthii differ substantially from *D. uninucleata* var. *uninucleata* and *D. uninucleata* var. *wickerhamii* on the basis of morphological, sexual reproduction and physiological features while the two latter varieties are similar in this respect (Kurtzman & Fell, 1998). Similarly the PCR product of the ITS region for the two varieties for *D. uninucleata* (Table 4, Figures 3, 8, 9) was the same (580bp each) and differed from *D. tóthii* (680 bp).

2.3.3.2.2 *Lipomyces*

The size of the PCR product of the ITS region for the species (Table 4, Figures 3, 5, 6, 7, 8, 9) in this genus i.e. *L. doorenjongii*, *L. japonicus* (*Smithiozyma japonica*) *L. kockii*, *L. kononenkoae* subsp. *kononenkoae*, *L. kononenkoae* subsp. *spencermartinsiae*, *L. lipofer*, *L. mesembrius*, *L. starkeyi*, *L. tetrasporus* and *L. yamadae* show variation with the following exceptions that had same sized bands: the two subspecies of *L. kononenkoae* (580bp), *L. kockii* and *L. mesembrius* (910bp), *L. lipofer* and *L. tetrasporus* (1000bp). Variation in this genus is noted regarding ascospore morphology, whereby uneven surfaces are observed in the two subspecies of *L. kononenkoae*, irregular folds in *L. starkeyi* and longitudinal ridges in *L. tetrasporus* and also different physiological properties (Kurtzman & Fell, 1998).

2.3.3.2.3 *Myxozyma*

The PCR product of the ITS region (Table 4, Figures 3, 4, 5, 6, 10) in *M. geophila* (500bp), *M. kluyveri* (580bp), *M. lipomycoides* (550bp), *M. monticola* (500bp), *M. mucilagina* (510bp), *M. neglecta* (500bp) *M. neotropica* (500bp), *M. nipponensis* (600bp), *M. udenii* (600bp) and *M. vanderwaltii* (580bp) indicate a possible close relationship. This is confirmed by a few differences in the assimilation patterns for the key characters amongst the species that are morphologically similar with no sexual reproduction. The notable exceptions were *M. melibiosi* and *M. sirexii*, both with a band size of 900 bp each. For *M. melibiosi* this variation was in accordance with the finding by Cottrell and Kock (1990). They reported a unique electrophoretic karyotype with 11 chromosomal bands for *M. melibiosi*, which is different to all the *Myxozyma* species that were used in their study.

2.3.3.2.4 *Zygozyma*

In this genus, the species *Z. arxii*, *Z. oligophaga*, *Z. smithiae* and *Z. suomiensis* differ slightly on the basis of morphology, sexual reproduction and physiological characteristics (Kurtzman & Fell, 1998). This close relationship was also recognized from the similar sizes of the PCR product of the ITS region for these species (Figures 3, 4, 7, 8, 9) i.e. *Z. arxii* (600bp) *Z. oligophaga* (500bp) *Z. smithiae* (540bp) and *Z. suomiensis* (500bp). The same sized band for *Z. oligophaga* and *Z. suomiensis* correlates with a common CoQ8 system and the inability to assimilate sucrose and trehalose.

2.3.3.3 Lipid analysis and the ITS region

According to the model by Jansen Van Rensburg *et al.* (1995), a clear pattern was observed when comparing the oleaginicity and the percentage 16:1 (palmitoleic acid) in the neutral lipid fraction (NL) within the family Lipomycetaceae. In this model the different genera could be separated i.e. *Babjevia* (13.5% neutral lipids according to biomass (NLs) and 15.9% 16:1 in the NL fraction according to total FAs present in the NL), *Dipodascopsis* (small amounts of NLs and 1.5% to 3.9% 16:1), *Lipomyces* (7% to 39% NLs and less than 7% 16:1 in NL) while *Myxozyma* and the insect associated genus *Zygozyma* were in the same cluster and could not be separated (0.5% to 6% NLs and 7% to 22% 16:1).

2.3.3.3.1 *Dipodascopsis*

The model (Jansen Van Rensburg *et al.*, 1995) based on oleaginicity and the percentage 16:1 showed that *D. uninucleata* and *D. tóthii* are similar with the highest fatty acid being oleic acid (18:1). The percentage lipid fractions were about 55% (NL), 26% to 29% glycolipids (GL) and 15% to 18% phospholipids (PL) for these species. However, according to Kock *et al.* (1986), the percentage long-chain fatty acid composition of the two varieties of *D. uninucleata* and *D. tóthii* enabled differentiation on the basis of a higher percentage 18:1 for *D. tóthii* (52%) and the absence of 18:3 while *D. uninucleata* had 18:1 (39%) and 8% of 18:3. This finding was similar to that of the ITS region PCR product that also showed variation between the two closely related varieties of *D. uninucleata* (580bp each) and *D. tóthii* (680bp).

2.3.3.3.2 *Lipomyces*

According to the lipid model of Jansen van Rensburg *et al.* (1995), the *Lipomyces* species in general are characterized by the presence of a low percentage 16:1, (1.4% to 6.9%) according to total FAs present and high percentage NL (64% to 92%). However, *L. japonicus* was an exception and was found to be closely related to *Myxozyma* and *Zygozyma* in this respect. The PCR product of the ITS region (600bp) also showed variation to the other species of *Lipomyces* (580bp to 1200bp) and was closer in size to those of the species in both *Myxozyma* (500bp to 600bp) and *Zygozyma* (500bp to 600bp).

2.3.3.3.3 *Myxozyma*

Based on the model of Jansen Van Rensburg *et al.* (1995) the species in this genus are characterized by low percentages 0.5% to 6% NLs and between 7% to 22% 16:1 according to total FAs present in the NL fraction. The observed distribution of fatty acids in these species correlates with a close relationship inferred from the sizes of the PCR products of the ITS region.

2.3.3.3.4 *Zygozyma*

According to this model, the species in this genus could not be separated from those of *Myxozyma* and were characterized by similar profiles. The size of the ITS region PCR product also suggests close relationship among the species i.e. *Z. arxii* (600bp), *Z. oligophaga* (500bp), *Z. smithiae* (540bp) and *Z. suomiensis* (500bp).

2.3.3.3.5 Atypical *Lipomyces* species – PCR products of the ITS region

The five isolates obtained from Lesotho with atypical carbohydrate profiles show that isolates 52, 73 and 93 had a similar size (900bp) PCR product while isolate 58 is characterized by a 550bp size product and isolate 97 by a 1000bp product (Figure 11). Interestingly, the size of the ITS region for isolate 97 (Figure 11) was similar to that of *L. tetrasporus* (Table 4, Figure 3) which is in accordance with conventional methods.

2.3.4 RFLP analysis of the ITS region

Over the years, PCR-based techniques have emerged and gained popularity. This stems from the ease and speed with which they can be performed. According to Deák *et al.* (2000), restriction analysis of the internal transcribed spacer (ITS) that includes the more conserved 5.8S rRNA gene (ITS-PCR) has been found the most useful, since it allows species identification and typing of isolates (Oda *et al.*, 1997; Valente *et al.*, 1997; Guillamón *et al.*, 1998; Fernández *et al.*, 1999). This method has found amongst others, application in the rapid identification of yeasts involved in the spoilage of yoghurt (Caggia *et al*., 2001) and in rapid identification of medically important yeasts (Trost *et al.*, 2004).

2.3.4.1 RFLP analysis and the D1/D2 domain

RFLP analysis of the amplified products of the ITS region for the type species in the family Lipomycetaceae and the five atypical *Lipomyces* species from Lesotho have enabled differentiation of all species within genera by the three

restriction enzymes used in combination. This is in contrast to the D1/D2 sequence analysis, which was unable to distinctly differentiate some species.

2.3.4.1.1 *Dipodascopsis*

Both the D1/D2 and the restriction analysis of the ITS-PCR suggest a genetically close relationship for the two varieties of *D. uninucleata* although *Cfo*I was able to differentiate these while *Mbo*I and *Hae*III were unable to do so (Table 5). *Dipodascopsis tóthii* could be differentiated from the two varieties of *D. uninucleata* using *Cfo*I and *Mbo*I. *Hae*III did not digest the DNA in these species. However, two fragments i.e. 150 bp and 300 bp were common to the three species in this genus with the *Mbo*I digest.

In contrast to the D1/D2 sequence data that indicated a close relationship between *B. anomala* and *D. tóthii*, restriction analysis with *Cfo*I and *Mbo*I could clearly separate them. However, *Mbo*I digest showed two fragments 150 bp and 200 bp common to the two. *Hae*III digest had no recognition sites for any of the DNA of the three species in this genus including *B. anomala*.

2.3.4.1.2 *Lipomyces*

The strains of *L. doorenjongii* and the two subspecies of *L. kononenkoae* with similar sized ITS-PCR band (580bp) could not be separated using *Cfo*I and *Hae*III (Table 5). Using *Mbo*I it was possible to distinguish *L. doorenjongii* from *L. kononenkoae* subsp. *kononenkoae* while *L. kononenkoae* subsp. *spencermartinsiae* could not be differentiated from *L. doorenjongii* (Table 5).

The restriction analysis of the other species i.e. *L. japonicus*, *L. kockii*, *L. lipofer*, *L. mesembrius*, *L. starkeyi*, *L. tetrasporus* and *L. yamadae* yielded distinct restriction patterns that enabled separation when the three enzymes were used in combination (Table 5). The D1/D2 sequence data on the other hand showed only one or two bases difference among the species in the *Lipomyces* clade (Kurtzman & Robnett, 1998) suggesting a much closer relationship among these species i.e. *L. kononenkoae* subsp. *kononenkoae*, *L. kononenkoae* subsp. *spencermartinsiae*, *L. starkeyi* and *L. tetrasporus*.

2.3.4.1.3 *Myxozyma*

Sequence analysis of both the D1/D2 domain and the ITS-PCR indicate close genetic relationships among some species in this genus, although *M. kluyveri* and *M. udenii* were more distantly related to the other species on the basis of the D1/D2 sequence data whilst *M. geophila* and *M. kluyveri* shared common fragments with the three enzymes used (Table 6). The restriction patterns obtained enabled differentiation of all species (Table 6). *Myxozyma melibiosi* and *M. sirexii* with similar sized ITS-PCR products (900bp) could clearly be distinguished from the restriction patterns obtained (Table 6) with the exception of *Mbo*I that yielded similar sized fragments (Table 6).

2.3.4.1.4 *Zygozyma*

In this genus, both the D1/D2 sequence data and the distinct restriction patterns obtained with each of *Cfo*I, *Hae* III and *Mbo*I (Table 5) enzymes indicate only distant relationships between species.

2.3.4.2 Conventional approach and RFLP analysis of the ITS region

The most fundamental aspect of classification is the use of pure cultures. Procedures conventionally used for yeast identification include cellular morphology and distinctive reactions on standardized fermentation and assimilation tests (Barnett *et al.*, 1990, 1996, 2000a, b; Yarrow, 1998). These assays are laborious and sometimes lead to results that are not fully discriminative because of strain variability. Given these drawbacks, molecular approaches are explored, which include PCR-based techniques directed towards conserved regions in the ribosomal region, followed by restriction analysis (Belloch *et al.*, 2002).

2.3.4.2.1 *Babjevia* and *Dipodascopsis*

The ascospores in *B. anomala* are hyaline, spherical to ovoidal and smooth with a single layered wall while those in *D. tóthii* are also hyaline or subhyaline, broadly ellipsoidal and lack a slime sheath. They were also previously found to display a close relationship on the basis of chromosomal DNA bands that were larger than 1300 kilobases (Jansen Van Rensburg *et al.*, 1995). This closer relationship is also shown by the restriction patterns obtained with *Hae*III and *Mbo*I although *Cfo*I (Table 5) clearly enabled separation of the two. The latter corresponds with the observed differences regarding morphology and physiology (Kurtzman & Fell, 1998; Barnett *et al.*, 2000a).

Based on morphological and physiological features as well as mode of sexual reproduction, *D. tóthii* differs from the two varieties of *D. uninucleata*. In

Dipodascopsis uninucleata var. *uninucleata* and *D. uninucleata* var. *wickerhamii* asci are produced laterally on the hyphae and are acicular and ascospores are released by rupturing at the apex (Kurtzman & Fell, 1998) while in *D. tóthii*, asci arise after fusion of a terminal cell with its penultimate cell. The restriction patterns obtained are in line with this relationship whereby *D. tóthii* displayed distinct profiles different from the two varieties of *D. uninucleata*. However, digestion with *Mbo*I showed fragments sized 150bp and 300bp, which were common to the three species (Table 5). *Hae*III did not digest any of the ITS-PCR products for the species in this genus.

2.3.4.2.2 *Lipomyces*

The following species i.e. *L. doorenjongii*, *L. japonicus*, *L. kockii*, *L. kononenkoae* subsp. *kononenkoae*, *L. kononenkoae* subsp. *spencermartinsiae*, *L. lipofer*, *L. mesembrius*, *L. starkeyi*, *L. tetrasporus*, and *L. yamadae* displayed distinct restriction profiles with the three restriction endonucleases used in combination, which enabled differentiation among these species (Table 5). The well-defined differences noted among these species regarding morphological and physiological features as well as sexual reproduction such as variation in the ascospore wall, ridges in *L. tetrasporus*, smooth walled in the two varieties of *L. kononenkoae* and *L. lipofer* and warty walls in *L. japonicus* and *L. starkeyi* (Kurtzman & Fell, 1998; Barnett *et al.*, 2000a) agree with the distinct restriction profiles obtained.

2.3.4.2.3 *Myxozyma*

The anamorphic members of the family Lipomycetaceae (Table 6) could also be differentiated according to the distinct patterns obtained using *Cfo*I, *Hae*III or *Mbo*I. In general, there were more restriction fragments obtained with the use of *Mbo*I compared to those with *Cfo*I and *Hae*III indicating that there were more recognition sites for *Mbo*I (Table 6). The variation in restriction fragment sizes (Table 6) does not correlate with the few differences in the assimilation patterns and the inability to reproduce sexually (Kurtzman & Fell, 1998; Barnett *et al*., 2000a).

2.3.4.2.4 *Zygozyma*

Zygozyma arxii, *Z. oligophaga*, *Z. smithiae* and *Z. suomiensis* showed distinct restriction profiles with *Cfo*I, *Mbo*I or *Hae*III used in combination that enabled separation of the species (Table 5). This is in line with observed differences that are based on morphology, sexual reproduction and physiological characteristics (Kurtzman & Fell, 1998; Barnett *et al.*, 2000a). Although, *Z. oligophaga* and *Z. suomiensis* have a common CoQ8 system and are both unable to assimilate sucrose and trehalose, they could clearly be separated from the restriction profiles with *Cfo*I digest (Table 5). The restriction patterns for these two species with *Mbo*I yielded identical fragment sizes i.e. 50bp, 100bp and 350bp (Table 5) while *Z. oligophaga* could not be digested with *Hae*III.

2.3.4.3 Lipid and RFLP analysis

The distribution of fatty acids associated with the NL fraction was investigated by Van Rensburg *et al.* (1995). A useful pattern was drawn from their results when comparing oleaginicity and the % 16:1 (palmitoleic acid) in the NL fractions of the yeasts in the family Lipomycetaceae. In this model the different genera could be separated with *Babjevia* containing 13.5% NLs according to biomass and 15.9% 16:1 in the NL fraction, *Dipodascopsis* containing 4.6% NLs and 1.5% - 3.9% 16:1, *Lipomyces* with 7% - 39% NLs and less than 7% 16:1 in NL while *Myxozyma* and the insect associated genus *Zygozyma* were in the same cluster containing 0.5% to 6% NLs and 7 % to 22% 16:1.

2.3.4.3.1 *Dipodascopsis*

The Jansen Van Rensburg's Model (1995) based on oleaginicity and the % 16:1 shows that *D. uninucleata* and *D. tóthii* are similar. This is in contrast to results obtained where their restriction patterns were different (Table 5). Furthermore, restriction with *Cfo*I (Table 5) enabled clear separation of *D. uninucleata* var. *uninucleata*, *D. uninucleata* var. *wickerhamii* and *D. tóthii*.

2.3.4.3.2 *Lipomyces*

From the lipid model (Jansen Van Rensburg *et al.*, 1995), the *Lipomyces* species are generally characterised by the presence of a low percentage 16:1 and high percentage NL with the exception of *L. japonicus* that displays similar profiles to *Myxozyma* and *Zygozyma*. The isolated position of *L. japonicus* in *Lipomyces* is further highlighted by lower amounts of stearic acid

(18:0) than 16:1 (Van Rensburg *et al.*, 1995). The RFLPs of the species enabled differentiation when *Cfo*I, *Hae*III and *Mbo*I were used in combination (Table 5).

2.3.4.3.3 *Myxozyma*

Van Rensburg and co-workers (1995) showed that the species in this genus are recognised by the presence of a wide range of 16:1 (about 7% - 22% according to total FAs present in the NL fraction) and low percentages NLs (0.5% - 6%). The distinct RFLPs obtained with *Cfo*I, *Hae*III and *Mbo*I used in combination also shows similar variation among these species (Table 6).

2.3.4.3.4 *Zygozyma*

According to the lipid model of Jansen van Rensburg (1995), the species in this genus are characterized, as in *Myxozyma*, by a wide range of relative percentage 16:1 (6.5% - 22.0%). This is in accordance with the variation in restriction fragment patterns obtained in this genus when *Cfo*I, *Hae*III and *Mbo*I were used (Table 5).

2.3.4.3.5 RFLP analysis of atypical *Lipomyces* species

According to our results, *Cfo*I was unable to digest any of the five isolates i.e. 52(b), 58(b), 73, 93 and 97. *Hae* III displayed distinct patterns for isolate 97 and yielded identical patterns for isolate 52(b), 73 and 93 while isolate 58(b) could not be digested (Table 7). Using *Mbo*I, again isolates 52(b), 73 and 93 could not be separated (Table 7). Isolate 97 had a similar sized ITS-PCR to *L. lipofer* and *L. tetrasporus* (1000bp). Interestingly, these patterns were

different compared to any *Lipomyces* species tested (Table 5). The results are in line with previous work by (Williams *et al.*, 1995; Cirak *et al.*, 2003) and confirms that RFLP analysis of the ITS region is conserved at species level.

Consequently, we propose the use of restriction analysis of the ribosomal region spanning the ITS1, the 5.8S rRNA gene and the ITS2, for the identification of species within genera in the family Lipomycetaceae since the biologically distinct strains of *D. uninucleata* var. *uninucleata*, *D. uninucleata* var. *wickerhamii*, *L. kononenkoae* subsp. *kononenkoae*, *L. kononenkoae* subsp *spencermartinsiae*, *L. starkeyi* and *L. tetrasporus* which could not be separated with the D1/D2 analysis could be differentiated from the ITS analysis.

2.3.5 D1/D2 sequencing

The sequence of the D1/D2 domain of the 26S rRNA has recently been accepted as one of the main tools for yeast taxonomy and the available database includes many yeast species described to date (Kurtzman & Robnett, 1998; Fell *et al.*, 2000; Kurtzman, 2000; Scorzetti *et al*., 2002). The shift to molecular sequencing has been with the expectation that both close and distant relationships can be resolved by comparing sequences of the appropriate molecule (Kurtzman & Robnett, 1997). Previous work by Peterson and Kurtzman (1991) on the sequencing of the variable D2 region enabled resolution of various heterothallic species in the genera *Issatchenkia*, *Pichia* and *Saccharomyces*. They concluded that conspecific strains generally had less than 1% nucleotide substitutions in region D2 while

separate biological species had more than 1 % substitutions.

The regions that have been recently used for identification of species from different yeast genera include single copy nuclear genes (Kurtzman & Robnett, 2003), actin-1 (Daniel *et al.*, 2001; Kurtzman & Robnett, 2003); RNA polymerase II (Kurtzman & Robnett, 2003) and mitochondrially encoded genes (Kurtzman & Robnett, 2003). These sequence methods representing the multi-gene sequence approach are useful for phylogenetic studies because they use genes that are unlinked. The use of different genes would overcome the problem of many genera that are incorrectly placed as a result of individual datasets, which seem to resolve only the most closely related species (Kurtzman, 2001; 2003; Abliz *et al.*, 2004). This means that species that are less closely related are placed incorrectly or rather with uncertainty (Cadez *et al.*, 2002; Rokas *et al.*, 2003).

The size of the amplified D1/D2 region was found to be 600bp (Figure 12) for the five atypical isolates from Lesotho, which is the expected size of the D1/D2 variable domain of the large subunit. These isolates differ in phenotypic character from the available keys of Kurtzman & Fell (1998) and Barnett *et al.* (1990, 2000a, b).

Determination of the nucleotide sequence of the D1/D2 domain of the 26S rDNA of the isolates and comparison with the database of the sequences from all currently recognized yeasts confirmed that isolate (73) could be identified as *L. starkeyi* since the difference in nucleotide substitution did not exceed 1%

(Figure 1). There were six base substitutions obtained for this isolate and according to Kurtzman and Robnett (1998) strains with less than 1% are considered conspecific and therefore do not represent distinct biological species which are separated by more than 1% nucleotide substitutions.

The alignment of isolate (73) against *L. tetrasporus* showed nucleotide substitution exceeding 1% (Figure 2). There were eight base substitutions obtained for this isolate and according to Kurtzman and Robnett (1998) strains with more than 1% are considered separate biological species.

The alignment of the five isolates from Lesotho that had given atypical carbohydrate profiles when using conventional identification techniques were all confirmed as known *Lipomyces* species according to D1/D2 sequence data. Three of the isolates i.e. 52(b), 73 and 93 were confirmed as *L. starkeyi* whilst two i.e. 58(b) and 97 were confirmed as *L. tetrasporus*. Strikingly, this finding was in contrast to the RFLP profiles that were obtained for the five isolates (Table 7). *Cfo*I did not digest any of the five isolates. The use of *Hae*III yielded a unique profile for isolate 97 and another pattern identical among isolates 52(b), 73 and 93 while isolate 58(b) was not digested. *Mbo*I digest also displayed identical patterns for isolates 52(b), 73 and 93, and distinct patterns for isolates 97 and 58(b). Interestingly, the three isolates 52(b), 73 and 93 confirmed as *L. starkeyi* with the D1/D2 sequencing also displayed identical restriction patterns with the three enzymes used suggesting that they are probably the same species. However, the restriction patterns for the three are different to those obtained for the type strain of *L.*

starkeyi. It is now important to assess the variation in RFLP profiles between various strains from different habitats of a particular species in order to determine the conserved status of this genotypic character.

2.4 Conclusions

According to the results, the following conclusions can be drawn:

1) Soils from Lesotho are rich in the following yeasts: *L. starkeyi,* *L. tetrasporus*, *Debaryomyces hansenii*, *D. hansenii* var. *fabryi* and *D. occidentalis* .

2) *Debaryomyces polymorphus*, *Dipodascus spicifer*, *Galactomyces geotrichum*, *G. reessii*, *Kluyveromyces lactis*, *L. kononenkoae*, *L. mesembrius*, *L. spencermartinsiae*, *Pichia anomala*, *P. fabianii*, *P. guilliermondii* and *Yarrowia lipolytica* occupy restricted habitats.

3) The fatty acid profiles of the isolated lipomycetous yeasts are similar to those reported by Jansen Van Rensburg *et al.* (1995). This further corroborates the value of this phenotypic characteristic in the taxonomy of this group of yeasts.

4) *Dipodascus spicifer* isolated from the Lesotho highlands produced high amounts of 18:3 (16.5%) although it has been reported in literature that this organism does not produce any 18:3.

5) The brewing strains obtained from the local traditional brews also gave lipid profiles similar to other brewing strains with high amounts of 18:3 being observed in *Issatchenkia orientalis* (14.6% w/w).

6) The PCR products of the ITS region of some of the type strains of the family Lipomycetaceae showed high length variation that enabled rapid identification of those isolates.

7) The type strains, the sub-species and the varieties of the family Lipomycetaceae could be differentiated from each other using RFLP profiles obtained with the use of the three restriction enzymes in combination i.e. *Cfo*I, *Hae*III and *Mbo*I. It is now important to assess the variation in RFLP profiles between various strains from different habitats of a particular species in order to determine the conserved status of this genotypic character.

8) The RFLP profiles for the five isolates isolated from Lesotho that presented atypical carbohydrate patterns could be separated into three groups. The first group comprising of the three isolates i.e. 52(b), 73 and 93 gave similar restriction patterns with *Cfo*I, *Hae*III and *Mbo*I suggesting that they are the same species. Isolate 58(b) yielded a distinct profile while isolate 97 had similar sized ITS-PCR (1000bp) to that of *L. lipofer* and *L. tetrasporus*.

9) The D1/D2 sequence data enabled separating the five isolates into three groups. The first group comprising of three isolates showed about 1% nucleotide substitutions to *L. starkeyi* suggesting that these isolates in Group I

are known *Lipomyces* spp. The other two isolates yielded sequences that were 99% identical to *L. kononenkoae* subsp. *kononenkoae* (isolate 58(b)) and 100% identical to that of *L. tetrasporus* (isolate 97) further suggesting that these isolates are probably known *Lipomyces* species.

10) In conclusion, it is suggested that the D1/D2 sequence data is probably conserved at genus level and to a certain degree species level with the exception of closely related species that cannot be resolved with the latter method. However, more strains of a species representing the Lipomycetaceae should be subjected to similar RFLP analysis to further determine its conserved status.

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Table 1. Regions in Lesotho from which soil samples were obtained and the distribution of some of the yeasts therein.

	Regions									
Yeasts	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Debaryomyces hansenii	+	+	+	-	-	-	+	-	+	+
D. hansenii var, *fabryi*	+	+	+	+	+	+	+	-	-	-
D. occidentalis	+	+	+	+	+	+	+	-	-	-
D. polymorphus	-	-	+	-	-	-	-	-	-	-
Dipodascus spicifer	-	-	-	-	-	-	+	-	-	-
Galactomyces geotrichum	-	-	-	-	-	-	+	-	-	-
G. reessii	-	+	-	-	-	-	-	-	-	-
Kluyveromyces lactis	-	-	+	-	-	-	-	-	-	-
Lipomyces kononenkoae	-	-	-	-	-	-	-	+	-	-
L. mesembrius	-	-	-	-	+	-	-	-	-	-
L. spencermartinsiae	-	-	-	-	-	-	-	-	-	+
L. starkeyi	+	+	+	+	+	+	+	+	+	+
L. tetrasporus	+	+	+	+	+	+	+	+	+	+
Pichia anomala	-	-	-	-	+	-	-	-	-	-
P. fabianii	-	-	-	+	+	-	-	-	-	-
P. guilliermondii	-	-	-	-	+	-	-	-	-	-
Yarrowia lipolytica	-	+	-	-	-	-	-	-	-	-

1= Teya-teyaneng; 2 = Moletsane; 3 = Leribe; 4 = Joel's dam; 5 = Butha-buthe; 6 = Libono; 7 = Mothae; 8 = Tlokoeng; 9 = Mapholaneng; 10 = Mokhotlong.

Table 2. Carbon/Nitrogen assimilation profiles of the five atypical *Lipomyces* species compared to authentic *L. starkeyi* and *L. tetrasporus* (Barnett *et al.*, 2000).

C/N source	*L.starkeyi*	*L. tetrasporus*	52(b)	58(b)	73	93	97
2, keto D-gluc	+	+	-	-	-	-	-
Imidazole	+, -	+	+	-	+	+	-
Methyl-a-gluc	+	+	-	-	-	-	-
Ribitol	-	+	+	+	-	-	+
Starch	+, -	+	+	-	+	-	+
Succinate	+	+, -	-	-	-	-	-
Xylose	+	+	+	-	-	-	-

2, keto D- gluconate = 2, keto D-gluc

Methyl-a-glucoside = Methyl-a-gluc

Table 3. Yeast isolates and their corresponding fatty acid composition.

		% Fatty acids					
No	**Identified as**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**
1	*Cryptococcus albidus*	18.0	0.9	5.4	36.8	34.9	4.0
9	*C. albidus*	19.0	1.0	5.5	36.0	34.6	3.9
16	*C. albidus*	18.0	1.2	4.9	35.8	35.0	4.1
18	*C. albidus*	16.0	2.0	7.2	38.0	32.0	4.8
21	*C. albidus*	16.5	2.0	7.5	36.0	35.0	3.0
25	*C. albidus*	17.2	1.5	7.8	35.9	34.8	2.7
27	*C. albidus*	19.0	1.8	7.2	34.0	35.0	3.0
31	*C. albidus*	18.2	1.9	6.9	34.8	35.2	2.8
37	*C. albidus*	18.0	0.9	5.4	36.8	35.0	3.9
54	*C. albidus*	16.9	1.1	5.3	38.6	34.1	3.8
7	*Cryptococcus laurentii*	26.0	0.5	10.6	45.8	17.0	t
20	*C. laurentii*	25.5	1.1	2.9	40.3	30.2	t
52	*C. laurentii*	29.2	1.3	1.4	59.4	6.5	2.1
96	*C. laurentii*	35.5	6.4	5.6	47.5	5.1	t
5	*Debaryomyces hansenii*	20.2	12.3	1.2	57.2	7.0	2.3
17	*D. hansenii*	19.7	11.8	1.0	57.8	6.9	2.7
26	*D. hansenii*	18.5	8.8	t	66.3	4.2	2.2
44	*D. hansenii*	20.0	10.2	t	62.9	5.1	2.2
46	*D. hansenii*	21.4	9.2	0.9	60.9	4.8	2.8
56	*D. hansenii*	17.9	9.8	1.1	63.5	5.4	2.5

58	*D. hansenii*	18.0	10.8	0.8	62.5	4.9	2.4
98	*D. hansenii*	19.3	11.3	1.0	58.9	6.9	2.7
106	*D. hansenii*	19.0	10.3	1.0	59.4	7.3	2.0
109	*D. hansenii*	19.6	11.0	1.2	58.6	6.7	2.8
118	*D. hansenii*	19.5	11.5	1.3	58.5	6.6	2.5
126	*D. hansenii*	19.2	9.9	1.5	59.5	7.0	2.9
129	*D. hansenii*	18.8	12.5	1.1	58.0	7.6	2.0
153	*D. hansenii*	18.5	12.0	1.5	58.5	6.8	2.8
157	*D. hansenii*	18.4	11.8	1.4	59.0	6.4	2.7
127	*D. hansenii* var. *fabryi*	31.8	5.6	5.1	51.0	5.9	0.5
3	*Debaryomyces occidentalis*	22.6	8.6	0.1	60.0	5.7	3.1
4	*D. occidentalis*	22.0	9.0	1.0	62.0	4.0	2.0
10	*D. occidentalis*	20.6	2.7	3.5	42.9	30.3	t
13	*D. occidentalis*	23.5	9.2	3.4	58.5	3.2	2.2
19	*D. occidentalis*	19.9	9.1	1.2	56.7	8.6	4.4
23	*D. occidentalis*	19.5	9.0	1.8	56.2	8.8	4.5
29	*D. occidentalis*	22.2	8.4	0.1	59.8	5.5	3.0
30	*D. occidentalis*	19.9	8.8	1.2	57.8	7.6	4.2
34	*D. occidentalis*	20.5	8.5	1.8	56.4	8.2	4.4
38	*D. occidentalis*	22.2	8.0	0.2	60.2	5.5	3.1
40	*D. occidentalis*	22.8	7.6	0.1	59.6	5.8	3.2
49	*D. occidentalis*	21.0	8.6	1.0	62.0	6.2	1.2
57	*D. occidentalis*	20.8	8.2	1.0	60.5	7.2	1.8
63	*D. occidentalis*	19.5	8.3	5.7	64.1	2.4	t
66	*D. occidentalis*	22.0	7.5	1.0	60.2	6.5	2.0
91	*D. occidentalis*	22.0	9.0	1.0	62.0	4.0	2.0
93	*D. occidentalis*	21.5	8.0	2.0	61.5	4.9	2.1
95	*D. occidentalis*	21.8	8.5	1.0	63.0	5.1	0.6
97	*D. occidentalis*	23.8	7.5	1.0	62.0	5.0	0.7
117	*D. occidentalis*	23.0	8.5	0.8	61.0	6.2	0.5
121	*D. occidentalis*	22.2	8.2	2.0	59.2	7.4	0.8
136	*D. occidentalis*	20.0	8.5	1.0	62.0	6.5	2.0
51	*D. polymorphus*	21.8	9.2	0.2	61.9	5.2	1.8
60	*D. polymorphus*	20.9	9.5	0.1	63.1	4.6	1.6
67	*Dipodascus spicifer*	22.1	2.0	3.7	35.5	20.2	16.5
115	*D. spicifer*	14.3	2.3	2.6	50.5	30.3	t
114	*Galactomyces geotrichum*	15.0	1.6	2.0	42.7	38.7	t
35	*G. reessii*	20.1	2.6	3.0	43.5	30.8	t
88	*Issatchenkia orientalis*	21.9	17.4	3.4	31.7	10.9	14.6
89	*I. orientalis*	15.2	14.3	3.6	44.0	13.3	9.4
42	*Kluyveromyces lactis*	17.0	7.4	0.6	62.9	8.7	3.4
100	*Lipomyces kononenkoae*	48.9	0.3	0.9	49.6	0.3	t
132	*L. kononenkoae*	46.5	0.8	1.7	49.5	1.5	t
135	*L. kononenkoae*	45.5	0.5	0.8	51.5	1.7	t
137	*L. kononenkoae*	48.9	0.8	1.9	45.6	2.3	t
138	*L. kononenkoae*	45.8	0.5	1.0	50.8	1.9	t
158	*L. kononenkoae*	49.8	0.1	0.9	48.2	0.2	t
160	*L. kononenkoae*	45.0	2.0	2.8	48.0	2.2	t
163	*L. kononenkoae*	44.9	0.3	3.8	49.6	1.4	t
101	*L. mesembrius*	43.3	2.4	6.2	44.3	3.8	t

150	*L. mesembrius*	45.4	2.6	5.8	43.2	3.0	t
151	*L. mesembrius*	41.0	2.5	7.8	45.8	2.9	t
164	*L. spencermartinsiae*	45.8	1.2	1.0	50.0	2.0	t
6	*L. starkeyi*	34.1	9.8	4.9	40.1	11.1	t
12	*L. starkeyi*	32.5	9.5	5.2	42.0	10.8	t
61	*L. starkeyi*	25.8	5.3	10.0	50.9	7.9	t
99	*L. starkeyi*	26.0	5.0	9.9	52.2	6.8	t
103	*L. starkeyi*	43.1	5.6	13.4	28.1	9.8	t
108	*L. starkeyi*	30.7	5.5	9.8	48.7	5.3	t
111	*L. starkeyi*	41.7	6.9	7.3	31.4	12.7	t
124	*L. starkeyi*	30.5	5.3	8.9	49.5	5.8	t
130	*L. starkeyi*	30.8	5.0	9.0	49.7	5.1	t
140	*L. starkeyi*	33.2	9.9	3.9	41.5	11.5	t
141	*L. starkeyi*	33.0	9.5	5.2	45.0	7.3	t
146	*L. starkeyi*	32.0	10.8	3.5	42.0	11.7	t
148	*L. starkeyi*	32.5	5.8	14.0	35.9	11.8	t
149	*L. starkeyi*	33.8	5.4	15.2	35.5	10.0	t
152	*L. starkeyi*	33.0	5.2	14.9	36.0	10.8	t
154	*L. starkeyi*	32.0	4.5	15.0	35.8	12.6	t
155	*L. starkeyi*	30.5	6.2	14.5	36.5	12.3	t
2	*L. tetrasporus*	27.0	1.5	2.9	38.1	30.4	t
8	*L. tetrasporus*	38.7	7.5	6.9	41.3	5.6	t
11	*L. tetrasporus*	39.8	7.2	7.9	34.8	10.2	t
14	*L. tetrasporus*	40.2	6.7	7.1	34.5	11.4	t
15	*L. tetrasporus*	32.0	4.9	6.2	51.8	5.1	t
22	*L. tetrasporus*	39.1	5.9	7.2	42.0	5.8	t
24	*L. tetrasporus*	37.9	6.2	7.5	42.5	5.9	t
28	*L. tetrasporus*	38.5	6.0	6.9	43.2	5.4	t
32	*L. tetrasporus*	39.0	6.4	7.0	42.8	4.8	t
33	*L. tetrasporus*	32.8	8.5	4.8	45.8	7.8	0.2
36	*L. tetrasporus*	32.0	7.1	4.5	48.9	7.5	t
39	*L. tetrasporus*	38.2	6.0	7.8	42.1	5.8	t
41	*L. tetrasporus*	36.2	7.2	8.0	43.5	5.1	t
43	*L. tetrasporus*	32.4	8.8	4.8	45.6	7.8	t
45	*L. tetrasporus*	35.5	7.4	4.4	44.8	5.8	2.0
47	*L. tetrasporus*	36.8	8.6	4.6	42.8	4.6	2.5
48	*L. tetrasporus*	22.1	5.0	5.0	63.9	6.6	2.1
50	*L. tetrasporus*	23.0	7.0	4.3	58.4	7.3	t
53	*L. tetrasporus*	36.4	7.5	5.6	44.5	6.0	t
55	*L. tetrasporus*	28.5	5.4	6.6	54.1	4.6	0.8
59	*L. tetrasporus*	36.6	6.0	6.3	44.8	6.3	t
62	*L. tetrasporus*	30.2	5.4	2.0	59.5	2.0	0.9
64	*L. tetrasporus*	35.4	8.5	4.8	45.2	6.1	t
68	*L. tetrasporus*	36.0	7.2	6.4	44.5	5.5	0.4
69	*L. tetrasporus*	33.0	5.0	2.5	56.9	2.5	0.1
71	*L. tetrasporus*	30.2	5.4	2.0	59.8	2.0	0.6
72	*L. tetrasporus*	33.5	5.2	2.8	56.0	2.2	0.2
73	*L. tetrasporus*	30.5	4.8	2.9	58.5	1.5	1.8
74	*L. tetrasporus*	35.8	7.5	5.8	45.8	5.0	0.1
75	*L. te trasporus*	32.5	6.9	2.8	55.8	2.0	t

77	*L. tetrasporus*	33.6	4.2	2.2	56.4	2.8	0.8
80	*L. tetrasporus*	35.2	7.2	4.8	46.0	6.8	t
82	*L. tetrasporus*	30.8	7.5	4.4	49.0	7.3	1.0
90	*L. tetrasporus*	28.0	5.2	6.2	54.8	4.4	0.9
92	*L. tetrasporus*	29.5	5.8	5.2	53.9	4.2	1.3
94	*L. tetrasporus*	25.8	6.4	6.1	55.4	4.8	1.5
102	*L. tetrasporus*	35.6	6.5	4.9	47.9	5.1	t
104	*L. tetrasporus*	32.2	6.8	4.4	48.2	7.2	1.2
105	*L. tetrasporus*	30.5	7.0	4.0	49.8	7.8	0.9
107	*L. tetrasporus*	37.2	5.6	6.2	45.2	5.8	t
110	*L. tetrasporus*	36.7	6.0	6.3	44.8	6.2	t
112	*L. tetrasporus*	39.4	2.5	8.8	43.0	4.8	1.5
116	*L. tetrasporus*	41.8	3.8	9.6	41.2	3.3	0.2
119	*L. tetrasporus*	30.8	7.1	4.4	49.0	7.3	1.4
120	*L. tetrasporus*	42.2	3.4	9.2	42.0	3.2	t
122	*L. tetrasporus*	36.9	5.9	6.2	44.2	6.8	t
123	*L. tetrasporus*	42.0	3.8	9.4	41.4	3.2	0.2
125	*L. tetrasporus*	38.0	3.9	9.8	43.0	3.8	1.5
128	*L. tetrasporus*	26.5	4.8	6.3	58.2	3.6	0.6
131	*L. tetrasporus*	38.4	3.4	8.8	43.8	3.8	1.8
133	*L. tetrasporus*	35.2	4.0	11.0	41.8	4.2	3.8
134	*L. tetrasporus*	41.8	3.5	9.2	41.5	3.4	0.5
139	*L. tetrasporus*	38.8	3.2	9.8	42.9	3.5	1.6
142	*L. tetrasporus*	39.5	3.4	9.4	43.0	3.2	1.2
143	*L. tetrasporus*	27.5	5.3	5.8	56.0	5.4	t
144	*L. tetrasporus*	29.0	7.2	7.0	51.5	5.3	t
145	*L. tetrasporus*	38.5	6.9	4.9	36.9	12.8	t
147	*L. tetrasporus*	29.0	7.2	7.0	51.5	5.3	t
156	*L. tetrasporus*	35.0	8.0	4.4	43.9	7.4	1.3
159	*L. tetrasporus*	28.2	4.5	7.5	53.5	6.3	t
161	*L. tetrasporus*	38.5	6.9	6.4	43.5	3.8	0.5
162	*L. tetrasporus*	26.8	3.5	8.1	56.8	4.8	t
165	*L. tetrasporus*	28.0	4.2	7.2	54.6	6.0	t
78	*Pichia anomala*	25.0	6.4	3.4	38.8	21.2	5.2
83	*P. anomala*	30.3	6.5	3.8	33.7	20.5	5.2
86	*P. anomala*	29.8	6.1	5.2	31.6	21.4	5.9
76	*P. fabianii*	30.8	6.5	4.6	34.0	18.5	5.6
81	*P. fabianii*	28.0	5.1	6.5	30.8	22.2	7.4
113	*P. guilliermondii*	18.5	16.5	1.2	32.8	25.5	5.5
85	*Saccharomyces castellii*	17.9	49.2	4.1	27.9	0.9	t
87	*S. castellii*	12.6	30.6	4.6	47.8	3.2	1.2
84	*S. cerevisiae*	23.7	12.9	3.6	36.4	10.8	12.6
65	*Yarrowia lipolytica*	16.2	15.8	2.2	45.8	20.0	t
70	*Y. lipolytica*	15.8	15.6	2.6	45.1	20.8	t
79	*Y. lipolytica*	15.5	15.4	2.5	45.6	21.0	t

16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = a-linolenic acid; t = trace.

Table 4. Strains used for RFLP analyses and corresponding PCR product sizes of the ITS region.

Yeast	UOFS No:	Size of ITS region (bp)
Babjevia anomala	Y-7931	690
Dipodascopsis tothii	Y-12690T	680
D. uninucleata var. *uninucleata*	Y-17583T	580
D. uninucleata var. *wickerhamii*	Y-2181T	580
Lipomyces doorenjongii	Y-27504T	580
L. kockii	Y-27505T	910
L. kononenkoae subsp. *kononenkoae*	Y-11553T	580
L. kononenkoae subsp. *spencermartinsiae*	Y-7042T	580
L. lipofer	Y-11555T	1000
L. mesembrius	Y-27506T	910
L. starkeyi	Y-11557T	1200
L. tetrasporus	Y-11562T	1000
L. yamadae	Y-27507T	590
Myxozyma geophila	Y-17252T	500
M. kluyveri	Y-17277T	580
M. lipomycoides	Y-17253T	550
M. melibiosi	Y-11781T	900
M. monticola	Y-17726T	500
M. mucilagina	Y-11823T	510
M. neglecta	Y-27508T	500
M. neotropica	Y-17859T	500
M. nipponensis	Y-2051T	600
M. sirexii	Y-2054T	900
M. udenii	Y-17387T	600
M. vanderwaltii	Y-17727	580
Smithiozyma japonica	Y-17848T	600
Zygozyma arxii	Y-17921T	600
Z. oligophaga	Y-17247T	500
Z. smithiae	Y-17922T	540
Z. suomiensis	Y-17356T	500
Lipomyces sp. (52b)–atypical isolates from Lesotho)	Y- 2183	900
L. sp. (58b)	Y- 2187	550
L. sp. (73)	Y- 2198	900
L. sp. (93)	Y- 2210	900
L. sp. (97)	Y- 2214	1000

Table 5. Size in base pairs (bp) of PCR products of the ITS region and restriction fragments of teleomorphic members of the Lipomycetaceae.

	Size of the restriction fragment (bp)		
Yeast	***Cfo* I**	***Mbo* I**	***Hae* III**
B. anomala	690	150, 200, 350	690
D. tothii	50, 100, 150, 350	150, 200, 300	680
D. uninucleata var. *uninucleata*	580	100, 150, 300	580
D. uninucleata var. *wickerhamii*	200, 350	100, 150, 300	580
L. doorenjongii	580	150, 400	580
L. kockii	125, 175, 250, 350	900	100, 200, 600
L. kononenkoae subsp. *konone*	580	50, 200, 300	580
L. kononenkoae subsp. *spencer*	580	150, 400	580
L. lipofer	1000	100, 150, 250, 450	150, 250, 600
L. mesembrius	900	175, 250, 450	200, 700
L. starkeyi	100, 300, 800	1200	150, 250, 350, 400
L. tetrasporus	400, 550	100, 150, 250, 450	100, 250, 600
L. yamadae	150, 400	50, 125, 175, 250	550
L. japonicus	200, 400	75, 525	150, 450
Z. arxii	600	50, 150, 400	50, 100, 450
Z. oligophaga	50, 450	50, 100, 350	500
Z. smithiae	150, 400	125, 400	550
Z. suomiensis	50, 200, 250	50, 100, 350	50, 450

L. kononenkoae subsp. *konone* = *L. kononenkoae* subsp. *kononenkoae*

L. kononenkoae subsp. *spencer* = *L. kononenkoae* subsp. *spencermartinsiae*

Table 6. Size in base pairs (bp) of PCR products of the ITS region and restriction fragments of anamorphic members of the Lipomycetaceae.

	Size of the restriction fragment (bp)		
Yeast	***Cfo* I**	***Mbo* I**	***Hae* III**
M. geophila	50, 200, 250	50, 100, 350	50, 450
M. kluyveri	100, 200, 225	50, 100, 150, 250	100, 450
M. lipomycoides	550	50, 125, 375	550
M. melibiosi	200, 700	100, 350, 450	250, 600
M. monticola	75, 175, 250	50, 100, 350	500
M. mucilagina	200, 300	100, 400	50, 450
M. neglecta	125, 375	100, 150, 250	500
M. neotropica	200, 300	100, 400	500
M. nipponensis	600	50, 150, 350	50, 550
M. sirexii	900	100, 350, 450	100, 250, 550
M. udenii	50, 125, 425	50, 150, 400	600
M. vanderwaltii	550	100, 450	550

Table 7. Size in base pairs (bp) of PCR products of the ITS region and restriction fragments for the five isolates from Lesotho with atypical carbohydrate profiles.

	Size of the restriction fragment (bp)		
Yeast	***Cfo* I**	***Mbo* I**	***Hae* III**
L. sp. (52b)	900	50, 125, 325, 400	100, 200, 600
L. sp. (58b)	550	100, 125, 325	550
L. sp. (73)	900	50, 125, 325, 400	100, 200, 600
L. sp. (93)	900	50, 125, 325, 400	100, 200, 600
L. sp. (97)	1000	100, 200, 300, 400	100, 150, 200, 550

```
S73                     1       GAACACGTCT-GCCTTAGTAACTGCGAGTGAAGCGGCAAGAGCTC 44
Lipomyces starkeyi D1D2 1   AAACCAACAGGGATTGCCTTAGTAACTGCGAGTGAAGCGGCAAGAGCTC 49
S73                     45  AAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGTAGAAGC 93
Lipomyces starkeyi D1D2 50  AAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGTAGAAGC 98

S73                     94  ATCTTTTGGATTGGTCCTTGCATATGTTCCTTGGAACAGGACATCATAG 142
Lipomyces starkeyi D1D2 99  ATCTTTTGGATTGGTCCTTGCATATGTTCCTTGGAACAGGACATCATAG 147

S73                     143 AGGGTGAGAATCCCGTTCATGGTGGGGAATCCAATTCTTTGTAAAGTGC 191
Lipomyces starkeyi D1D2 148 AGGGTGAGAATCCCGTTCATGGTGGGGAATCCAATTCTTTGTAAAGTGC 196

S73                     192 TTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAA 240
Lipomyces starkeyi D1D2 197 TTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAA 245

S73                     241 TTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACA 289
Lipomyces starkeyi D1D2 246 TTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACA 294

S73                     290 GTGATGGAAAGATGAAAAGAACTCCGAAAGGAGAGTGAAAAAGGACGTG 338
Lipomyces starkeyi D1D2 295 GTGATGGAAAGATGAAAAGAACTCCGAAAGGAGAGTGAAAAAGGACGTG 343

S73                     339 AAATTGTTGAAAGGGAAGTGCTTGAGATCAGTCTTAAGGTTTAGTGATC 387
Lipomyces starkeyi D1D2 344 AAATTGTTGAAAGGGAAGTGCTTGAGATCAGTCTTAAGGTTTAGTGATC 392

S73                     388 AGTCTTCCTTTTGGTTGGTGCACTCACTATTTCTTAGGCCAACATCAGT 436
Lipomyces starkeyi D1D2 393 AGTCTTCCTTTTGGTTGGTGCACTCACTATTTCTTAGGCCAACATCAGT 441

S73                     437 TTTGGCGGCAGGATAATGACATTGGAATGTGGCTCTTTCGGGAGTGTTA 485
Lipomyces starkeyi D1D2 442 TTTGGCGGCAGGATAATGACATTGGAATGTGGCTCTTTCGGGAGTGTTA 490

S73                     486 TAGCCTTTGTTGATACTGCTTGCTTAGACTGAGGACTGCGCTTTGGCTA 534
Lipomyces starkeyi D1D2 491 TAGCCTTTGTTGATACTGCCTGCTTAGACTGAGGACTGCGCTTTTGCTA 539

S73                     535 G                               535
Lipomyces starkeyi D1D2 540 GGATGTTGGCATAATGATCTTAAGCAGC 567
```

Figure 1. Sequence alignment of the isolate *Lipomyces* sp. (73) against D1/D2 sequence data of *L. starkeyi* showing six base substitutions.

```
S73                          1       GAACACGTCT-GCCTTAGTAACTGCGAGTGAAGCGGCAAGAG 41
Lipomyces tetrasporus D1D2   1   AAACCAACAGGGATTGCCTTAGTAACTGCGAGTGAAGCGGCAAGAG 46

S73                         42  CTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGT 87
Lipomyces tetrasporus D1D2  47  CTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGT 92

S73                         88  AGAAGCATCTTTTGGATTGGTCCTTGCATATGTTCCTTGGAACAGG 133
Lipomyces tetrasporus D1D2  93  AGAAGCATCTTTTGGATTGGTCCTTGCATATGTTCCTTGGAACAGG 138

S73                        134 ACATCATAGAGGGTGAGAATCCCGTTCATGGTGGGGAATCCAATTC 179
Lipomyces tetrasporus D1D2 139 ACATCATAGAGGGTGAGAATCCCGTTCATGGTGGGGAATCCAATTC 184

S73                        180 TTTGTAAAGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTC 225
Lipomyces tetrasporus D1D2 185 TTTGTAAAGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTC 230
S73                        226 TAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGAC 271
Lipomyces tetrasporus D1D2 231 TAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGAC 276

S73                        272 CGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTCCGAA 317
Lipomyces tetrasporus D1D2 277 CGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTCCGAA 322

S73                        318 AGGAGAGTGAAAAAGGACGTGAAATTGTTGAAAGGGAAGTGCTTGA 363
Lipomyces tetrasporus D1D2 323 AGGAGAGTGAAAAAGGACGTGAAATTGTTGAAAGGGAAGTGCTTGA 368

S73                        364 GATCAGTCTTAAGGTTTAGTGATCAGTCTTCCTTTGGTTGGTGCA 409
Lipomyces tetrasporus D1D2 369 GATCAGTCTTAAGGTTTAGTGATCAGTCTTCCTTTTGGTTGGTGCA 414

S73                        410 CTCACTATTTCTTAGGCCAACATCAGTTTTGGCGGCAGGATAATGA 455
Lipomyces tetrasporus D1D2 415 CTCACTATTTCTTAGGCCAACATCAGTTTTCGCGGCAGGATAATGA 460

S73                        456 CATTGGAATGTGGCTCTTTCGGGAGTGTTATAGCCTTTGTTGATAC 501
Lipomyces tetrasporus D1D2 461 CATTGGAATGTGGCTCTTTCGGGAGTGTTATAGCCTTTGTTGATAC 506

S73                        502 TGCTTGCTTAGACTGAGGACTGCGCTTTGGCTAG            535
Lipomyces tetrasporus D1D2 507 TGCCTGCTTAGACTGAGGACCGCGCTTTTGCTAGGATGTTGGCATA 552

S73                        536                 535
Lipomyces tetrasporus D1D2 553 ATGATCTTAAGCAGC 567
```

Figure 2. Sequence alignment of the isolate *Lipomyces* sp. (73) against D1/D2 sequence data of *L. tetrasporus* showing eight base substitutions.

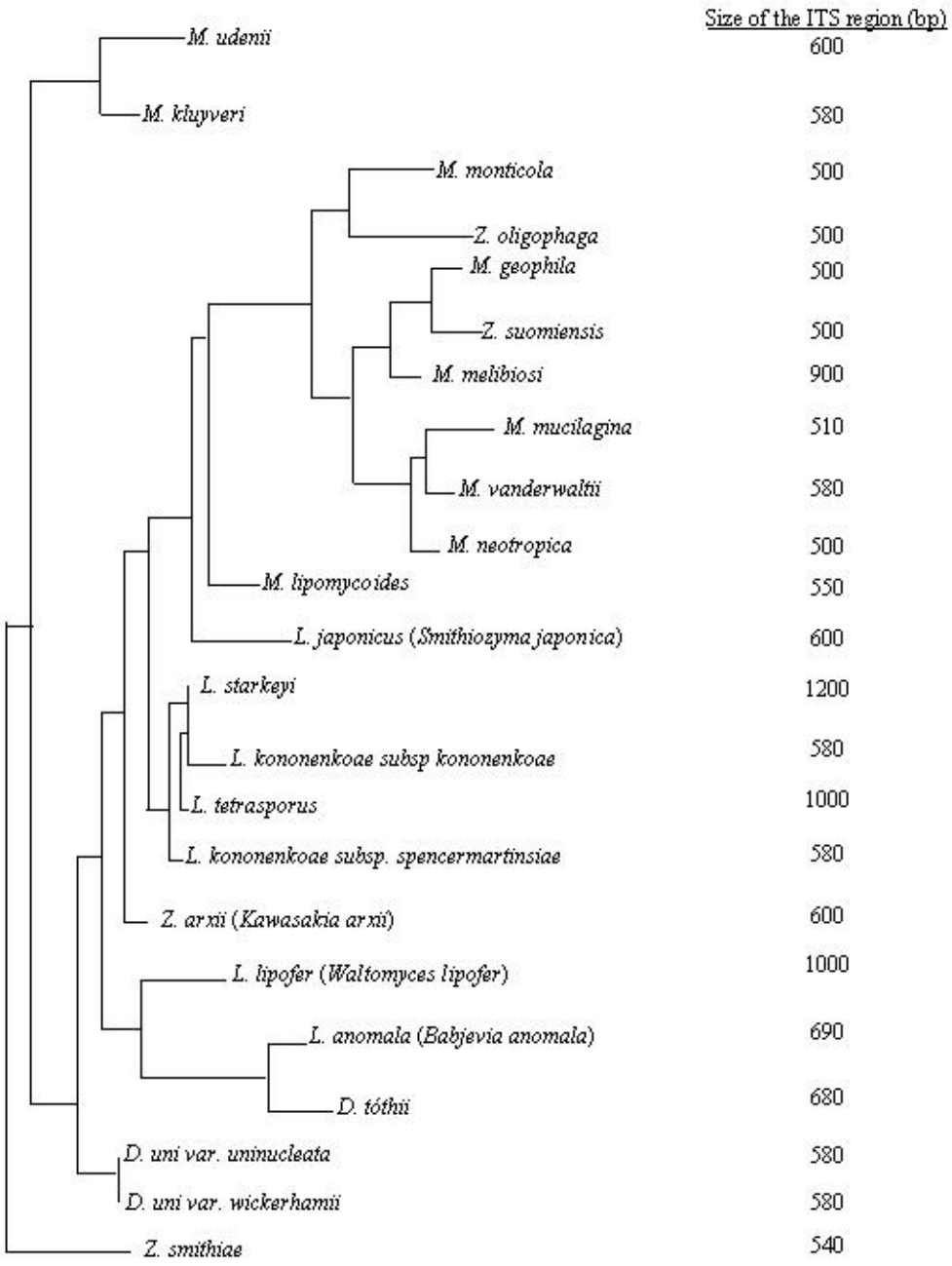


Figure 3. Phylogenetic tree of the *Lipomyces* clade based on analysis of LSU 26S domain D1/D2 reproduced from Kurtzman & Robnett (1998). The sizes of the ITS region for the species illustrated are represented by Figures 4 to 10.

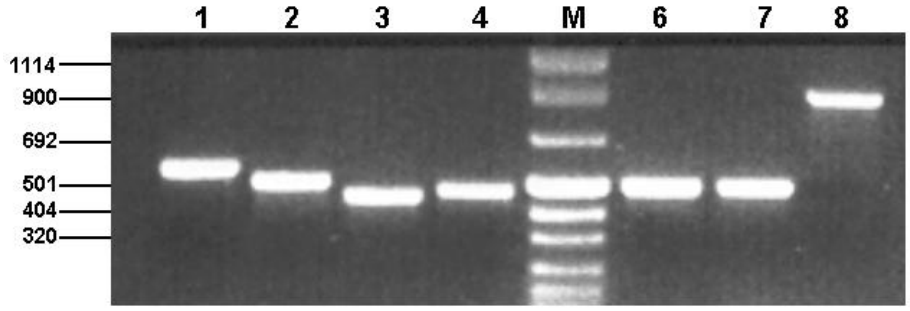


Figure 4. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 *M. udenii*; Lane 2 *M. kluyveri*; Lane 3 *M. monticola*; Lane 4 *Z. oligophaga*; Lane 5 Mol wt VIII (Roche); Lane 6 *M. geophila*; Lane 7 *Z. suomiensis* and Lane 8 *M. melibiosi*.

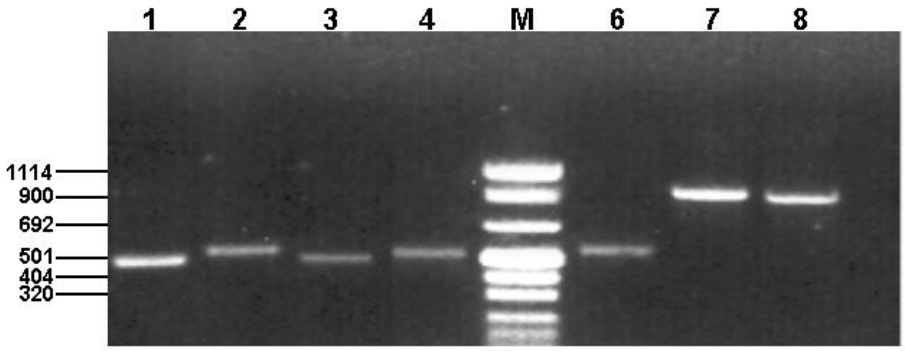


Figure 5. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 *M. mucilagina*; Lane 2 *M. vanderwaltii*; Lane 3 *M. neotropica*; Lane 4 *M. lipomycoides*; Lane 5 Mol wt VIII (Roche); Lane 6 *L. yamadae*; Lane 7 *M. sirexii* and Lane 8 *L.* spp. (73).

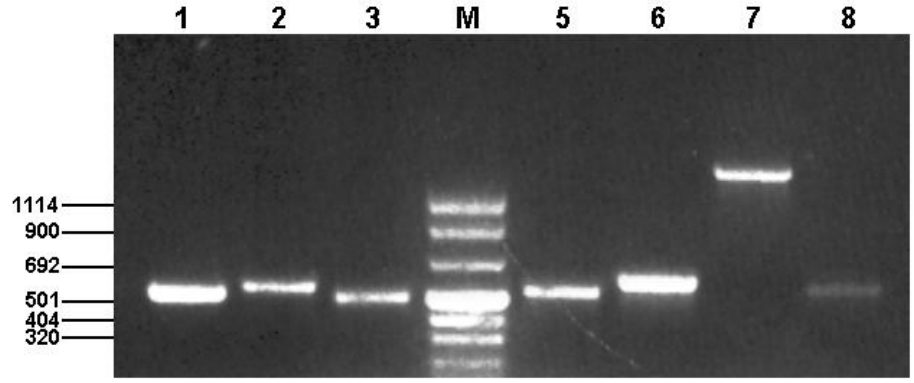


Figure 6. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 *M. mucilagina*; Lane 2 *M. vanderwaltii*; Lane 3 *M. neotropica*; Lane 4 Mol wt VIII (Roche); Lane 5 *M. lipomycoides* ; Lane 6 *S. japonica*; Lane 7 *L. starkeyi* and Lane 8 *L. kononenkoae* subsp. *kononenkoae*.

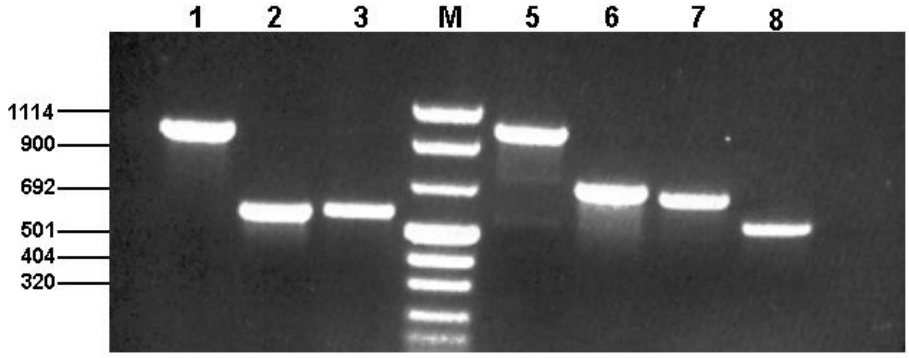


Figure 7. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 *L. tetrasporus* ; Lane 2 *L. kononenkoae subsp. spencermartinsiae* ; Lane 3 *Z. arxii*; Lane 4 Mol wt VIII (Roche); Lane 5 *L. lipofer*; Lane 6 *B. anomala*; Lane 7 *D. tothii* and Lane 8 *D. uninucleata* var. *uninucleata*.

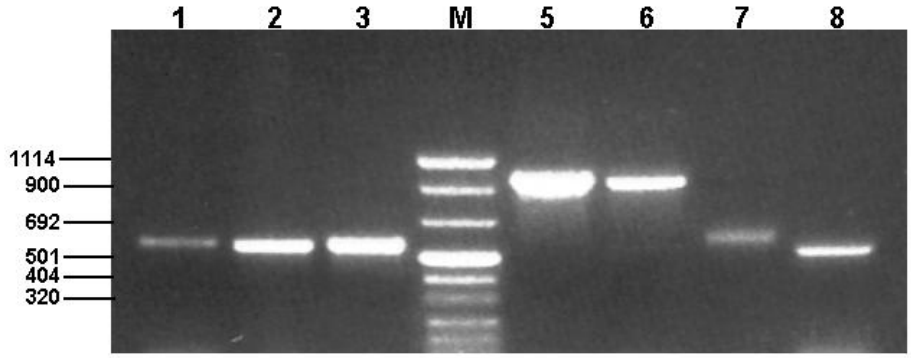


Figure 8. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 *D. uninucleata* var. *wickerhamii*; Lane 2 *Z. smithiae*; Lane 3 *L. doorenjongii*; Lane 4 Mol wt VIII (Roche); Lane 5 *L. kockii*; Lane 6 *L. mesembrius*; Lane 7 *L. yamadae* and Lane 8 *M. neglecta*.

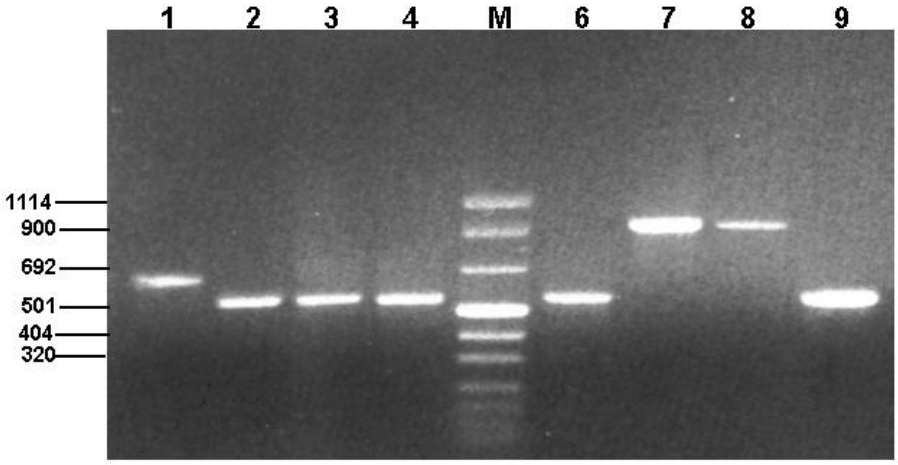


Figure 9. Amplified DNA of the ITS region with primer pair ITS4 and ITS5 purified using Amersham DNA purification kit, Lane 1 *D. tothii*; Lane 2 *D. uninucleata* var. *uninucleata*; Lane 3 *D. uninucleata* var. *wickerhamii*; Lane 4 *Z. smithiae*; Lane 5 Mol wt VIII (Roche); Lane 6 *L. doorenjongii*; Lane 7 *L. kockii*; Lane 8 *L. mesembrius* and Lane 9 *L. yamadae*.

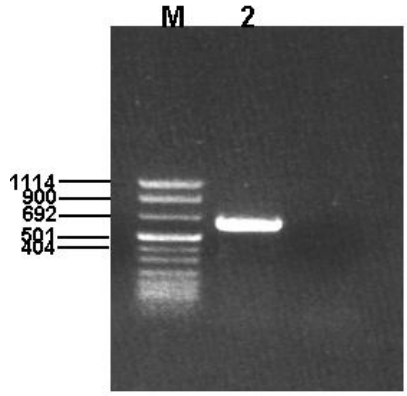


Figure 10. Amplified DNA of the ITS region with primer pair ITS4 and ITS5, Lane 1 Mol wt VIII (Roche), Lane 2 *M. nipponensis*.

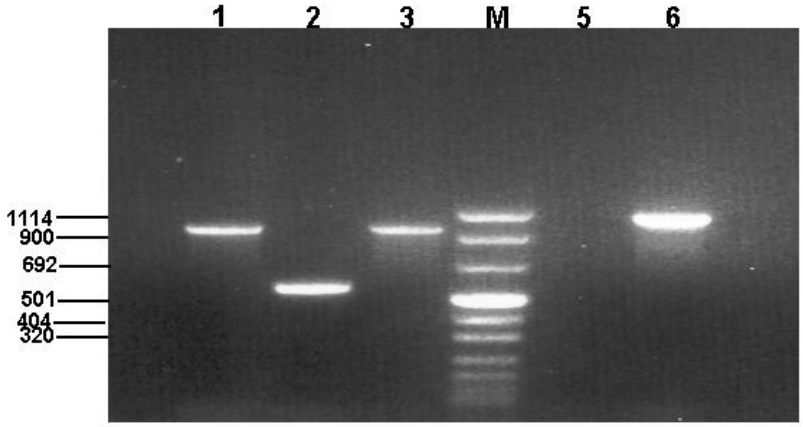


Figure 11. Amplified DNA of the ITS region with primer pair ITS4 and ITS5 for the *Lipomyces* isolates isolated from Lesotho with atypical carbohydrate profiles; Lane 1 *Lipomyces* sp. 52(b); Lane 2 *Lipomyces* sp. 58(b); Lane 3 *Lipomyces* sp. 73; Lane 4 Mol wt VIII (Roche); Lane 5 *Lipomyces* sp. {93 - No product} and Lane 6 *Lipomyces* sp. 97. In a subsequent amplification the PCR product of isolate 93 was found to be the same size as that for isolate 52(b) and 73 (Lanes 1, 3); result not shown.

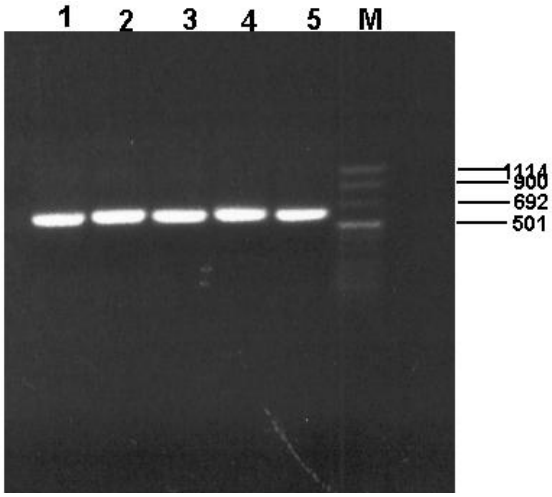


Figure 12. Amplified DNA of the D1/D2 domain with primer pair NL1 and NL4 Lane 1 *Lipomyces* sp. 52(b); Lane 2 *Lipomyces* sp. 58(b); Lane 3 *Lipomyces* sp. 73; Lane 4 *Lipomyces* sp. 93; Lane 5 *Lipomyces* sp. 97 and Lane 6 Mol wt VIII (Roche).

Chapter 3

The isolation of ascomycetous yeasts with the ability to utilize derivatives of cyclohexane or chlorobutane

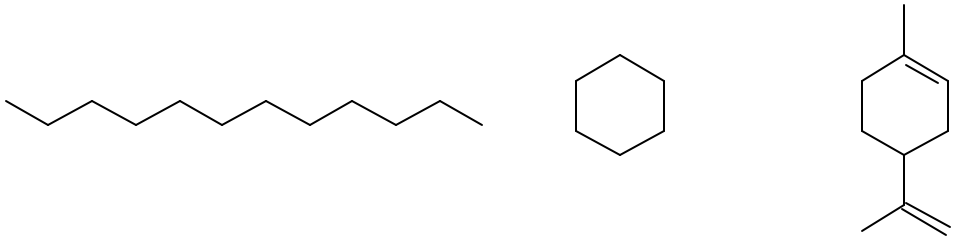
3.1 Introduction

In the laboratory, growth of microorganisms is usually studied on media that contain glucose or other carbohydrates as carbon and energy sources. The ability of microorganisms to utilize non-carbohydrate carbon sources such as alkanes and low molecular weight aromatic compounds is also well established (Van Beilen *et al.*, 1998; 2003). Microbes with the ability to degrade alkanes include bacteria, yeasts, fungi and algae. Studies of growth on cyclic compounds have largely been restricted to aromatic substrates, although growth of bacteria on alicyclic compounds such as cyclohexane and cyclododecane has been investigated (Schumacher & Fakoussa, 1999).

The growth of basidiomycetous yeasts on a range of low molecular weight aromatic compounds, which are products of lignin degradation, have also been reported (Sampaio, 1999; Romero *et al.*, 2002; Rapp & Gabriel-Jurgens, 2003). However, reports on growth of ascomycetous yeasts on aromatic compounds are limited. Interestingly, a number of alkane utilizing ascomycetous yeasts can also utilize phenol (Scheller *et al.*, 1998). We found no records of ascomycetous yeasts that have the ability to degrade saturated cyclic compounds such as cyclohexanes.

In order to investigate the isolation of ascomycetous yeasts grown on cyclic alkanes it was important to consider firstly the utilization of simple straight chain alkanes and understand their mechanisms. This is because there is very little information available concerning the utilization of cyclic alkanes by ascomycetous yeasts and because we expect similar mechanisms to be

involved.



Dodecane Cyclohexane Limonene

Figure 1. Chemical structures of examples of hydrocarbons (i.e. straight chain alkane (dodecane), a cyclic alkane (cyclohexane) and a cyclic monoterpene (limonene).

3.1.1 Alkanes

Alkanes (Figure 1) or saturated hydrocarbons also known as paraffins are chemically inert (Labinger & Bercaw, 2002). Microbial degradation of n-alkanes in most cases proceeds via a rate-limiting cytochrome P450 monooxygenase mediated terminal hydroxylation forming the corresponding primary alcohol. This is followed by further oxidation by long-chain alcohol oxidases, alcohol and aldehyde dehydrogenases. The resulting fatty acids enter the ß-oxidation cycle (Van Beilen *et al*., 2003) to form acetyl-CoA, which is eventually degraded to CO_2 and water with the concomitant production of energy.

3.1.1.1 Utilization of n-alkanes

The utilization of n-alkanes and 1-alkenes as carbon sources by yeasts such as *Candida tropicalis*, *Debaryomyces hansenii* and *Yarrowia lipolytica* has long been established (Middelhoven, 1993; Iida *et al.*, 1998; Scheller *et al.*,

1998; Yadav & Loper, 1999; Schmitz *et al.*, 2000; Sumita *et al.*, 2002a; Middelhoven *et al.*, 2003; Smit *et al.*, 2004). A number of cytochrome P450 isozymes that are implicated in alkane assimilation have been cloned from yeasts such as *C. maltosa* (Zimmer *et al.*, 1996; Ohkuma *et al.*, 1998), *D. hansenii* (Yadav & Loper, 1999) and *Y. lipolytica* (Iida *et al.*, 2000; Sumita *et al.*, 2002b). These enzymes belong to the CYP52 gene family of the microsomal class II P450s (Van Beilen *et al.*, 2003) and have different substrate specificities. These allow them to either act on alkanes (C12 or C16) or to oxidize fatty acids at the ?-position resulting in the degradation of alkanes that may be present in environments contaminated with oil.

3.1.2 Monoterpenes

We found no reference to ascomycetous yeasts that have the ability to degrade cyclic compounds such as cyclohexanes (Figure 1). However, growth of basidiomycetous yeasts on limonene, (Figure 1) a cyclic monoterpene, has recently been described (Thanh *et al*, 2004). Monoterpenes fall under a class of compounds known as isoprenoids. They are C10 hydrocarbons built from two isoprene units joined in a head to tail manner, and can be acyclic, monocyclic or bicyclic (Croteau, 1987; Sikkema *et al.*, 1995; Van der Werf *et al.*, 1999). These compounds are constituents of the essential oils of plants (Colocousi *et al.*, 1996; Van der Werf & de Bont, 1998; King & Dickinson, 2000; 2003). They are the components responsible for flavour or aroma of many plants and their function in nature is not completely understood. The biological functions suggested include

competitive phytotoxicity, defence against herbivores and microbial infections, to name but a few.

3.1.2.1 Degradation of monoterpenes

The microbial degradation of monoterpenes is hampered by their toxicity. A few yeast strains, all belonging to the alkane-utilizing yeasts, can hydroxylate monoterpenes (Van Rensburg *et al.*, 1997), but we found no reference to ascomycetous yeast strains with the ability to utilize monoterpenes as the only carbon source. Attempts to isolate yeasts in selective media, with monoterpenes as carbon source always failed (M.S. Smit personal communication, 2004). This is probably due to the toxicity of the monoterpenes. In a previous study, limonene (Figure 1) utilizing basidiomycetous yeasts were isolated from soil and plant residues from monoterpene-rich environments using an enrichment technique with less toxic monoterpene analogs (cyclohexanedioic acid or cyclohexanedimethanol) as sole carbon source (Thanh *et al.*, 2004).

3.1.3 Halogenated hydrocarbons

Chlorophenols belong to another class of hydrophobic substrates that can be degraded by alkane utilizing ascomycetous yeasts (Hofmann & Schauer, 1988; Middelhoven *et al.*, 1992; Middelhoven, 1993). Environmental problems arising from the production and use of compounds such as halogenated hydrocarbons has prompted investigation into their fate, which resulted in various searches for microorganisms that are able to degrade these toxic compounds (Sikkema *et al.*, 1994; Copley, 1998; Fetzner, 1998;

Sun *et al.*, 2000). These pollutants are recognised as being potentially mutagenic, carcinogenic and toxic to vital organs such as liver, kidney or neural systems (Brown-Woodman *et al.*, 1998; Akers *et al.*, 1999).

Halogenated organic compounds are environmental pollutants resulting from widespread environmental contamination due to the use and disposal of halogenated aromatic compounds (Mohn & Tiedje, 1992). The halogenated compounds are used for industrial and agricultural applications and they are often present in industrial waste. They are resistant to both abiotic and biotic degradation resulting in a need for studies of the microbial degradation of these recalcitrant compounds. Previous studies have focused on the physiological processes responsible for their mineralisation, as well as on the enzymes involved in the cleavage of the carbon-halogen bond (Copley, 1998; Fetzner, 1998).

3.1.3.1 Biodegradation of halogenated hydrocarbons

The microbial biodegradation of these compounds is facilitated by the presence of dehalogenase enzymes that are able to remove for instance chlorine atoms from the aromatic rings of chlorinated compounds and hence detoxify them (Chang & Alvarez-Cohen, 1995; Mehmood *et al.*, 1997; Johan *et al.*, 2001).

This dehalogenation is the key reaction in microbial degradation of halogenated compounds. It results in the removal of the halogen that is responsible for the toxic and xenobiotic character of the compound, by

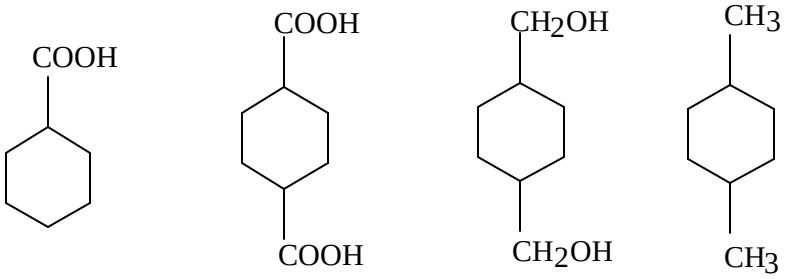
replacing it with a hydrogen or a hydroxyl group (Janssen *et al*, 2001). These enzymes have been identified in organisms that have the ability to grow on compounds such as 1-chloroalkanes (Jesenska *et al*, 2000). A few microorganisms that can degrade such compounds have been isolated from heavily contaminated sites (Olaniran *et al.*, 2004).

3.1.4 Aim of this study

Given the above information pertaining to mostly basidiomycetous yeasts and bacteria, we thought it worthwhile to pursue the isolation of ascomycetous yeasts that can utilize unusual substrates such as cyclohexanes, monoterpenes as well as low molecular weight halogenated compounds, which might be similar to intermediates in the degradation of chlorophenols. In this case, we aim to establish whether it would be possible to isolate yeasts with such unusual metabolic activities from soil obtained from pristine environments in Lesotho. This study was different from most published studies in that organisms with the ability to utilize non-carbohydrate substrates are usually isolated from polluted environments.

3.2 Materials and Methods

3.2.1 Chemicals and media used



1 2 3 4

The following chemicals were used: 1 = cyclohexanecarboxylic acid (CHC), 2 = 1,4-cyclohexanedicarboxylic acid (1,4-CHDA), 3 = 1,4-cyclohexanedimethanol (1,4-CHDM), 4 = 1,4-dimethylcyclohexane (1,4-DMCH), dodecane, pristane, (+) limonene (Fluka), (-) α-pinene (Fluka), (-) β-pinene (Fluka), 2-chlorobutyric acid (2-CBA) and 1,2-dichlorobutane (1,2 DCB). Yeast extract/malt extract agar (YM) contained per litre: 16.0 g agar, 10.0 g glucose, 3.0 g malt extract, 5.0 g peptone, 3.0 g yeast extract. Yeast nitrogen base glucose medium (YNB) contained per litre: 5.0 g glucose, 6.7 g YNB (Difco). Yeast peptone medium (YP) contained per litre: 10.0 g glucose, 5.0 g peptone and 5.0 g yeast extract.

3.2.2 Growth conditions

Isolation experiments were performed in 500 ml shake flasks containing 50 ml broth while growth experiments were conducted in 500 ml flasks containing 25 ml broth. The flasks were incubated at 25^0C on a horizontal shaker for five days.

3.2.3 Yeasts capable of utilizing substituted cyclohexanes

The substituted cyclohexanes i.e. CHC, 1,4-CHDA or 1,4-CHDM were used in enrichment cultures to enable isolation of ascomycetous yeasts capable of utilizing them.

3.2.3.1 Isolation

Soil samples were collected from 10 regions on the northeast side of Lesotho representing different habitats. YNB broth without glucose was supplemented

with initially 0.1 % (w/v) of CHC, 1,4-CHDA or 1,4-CHDM as sole carbon source. Flasks were inoculated with soil samples (ca. 1 g) from each of the ten regions. Substrate concentrations were gradually increased to a final concentration of 1 % (w/v). At each successive increase in concentration, 1 ml of the culture from the previous concentration was used as inoculum for the next concentration. Following incubation, at the highest concentration the suspension was plated on YM agar and incubated at 25^0C. Individual colonies were plated on YM agar in order to obtain pure cultures.

3.2.3.2 Identification

Microscopy of the isolates was carried out to determine the morphology of the yeast isolates as well as ensuring purity of the cultures. All isolates were subjected to a Diazonium Blue B test (Yarrow, 1998). Seven isolates from enrichment cultures containing 1% (w/v) of the various substrates were classified as ascomycetous yeasts based on the DBB test and selected for further identification. The selected isolates (Table 1) could, based on morphology, already be distinguished as different species. Conventional methods as described by Yarrow, (1998) were used for further identification.

3.2.3.3 Growth on 1,4 disubstituted cyclohexanes and other hydrocarbons

Cultures of the isolates that had been grown for three days on YM plates were inoculated into YNB without glucose broth that was supplemented with either 1,4-CHDA, 1,4-CHDM, 1,4-DMCH, dodecane or pristane as indicated in the legends. Total cell counts were determined for each of the isolates at 24 hr intervals over a five-day period. Experiments were repeated at least three

times and similar results were obtained. Growth on some of the carbon sources was also confirmed by transferring inocula (1 ml) to the same media containing the corresponding carbon sources. Cell counts were performed using an improved Neubauer haemacytometer.

3.2.4 Yeasts capable of utilizing monoterpenes

The yeast isolates were tested for growth on the monoterpenes (limonene, α-pinene and β-pinene) because these yeasts were isolated using substrates that previously yielded limonene utilizing *Rhodotorula* species (Thanh *et al.*, 2004).

3.2.4.1 Growth of isolates on monoterpenes

Each yeast isolate was streaked on four YNB agar plates and the plates were placed in four dessicators. A vial containing either (+) limonene (Fluka), (-) α-pinene (Fluka) or (-) β-pinene (Fluka) was placed in three of the dessicators together with another vial containing water to maintain a moist environment thus avoiding drying of the cultures. A negative control was included with only culture plates and a vial of water placed in the dessicator without any of the monoterpenes. The plates in the dessicators were incubated for one week at 25^0C.

3.2.5 Yeasts capable of utilizing chlorocompounds

The chlorocompounds 1,2 DCB and 2-CBA was used as carbon sources to enable isolation of yeasts with the ability to utilize them.

3.2.5.1 Isolation

Yeasts were isolated from soil samples as previously described for the 1,4-di-substituted cyclohexanes (3.2.3.1). The carbon source was either 1,2 DCB or 2-CBA. Concentrations were gradually increased as explained for the cyclohexanes (3.2.3.1) starting with 0.1% (w/v) and increasing to a final concentration of 1% (w/v). The yeast isolates obtained with the highest concentration of 1% (w/v) were purified and identified using conventional techniques as previously described.

3.2.5.2 Growth on chlorocompounds

YNB media was inoculated with the yeasts obtained above. 1,2 DCB or 2-CBA were added to the media at a concentration of 1% (w/v). Cell counts were determined for the yeasts growing in the presence of 1,2 DCB or 2-CBA.

In order to establish the mechanism of detoxification, (i.e. to monitor the disappearance of either 1,2 DCB or 2-CBA over time and the simultaneous formation of a product), samples were drawn as follows: 500 µl of the sample was drawn and filtered through a Millex syringe driven filter unit (Millipore, USA). Samples were analysed using the HPLC with a Phenomenex synergi 4 micron Hydro-PP 80A (250 X 4.60 mm) column and 0.05 M KH_2PO_4 in 1% acetonitrile at pH 2.5 as mobile phase. The flow rate was 0.8 ml/min and detection was at a wavelength of 220 nm.

3.3 Results and discussion

3.3.1 Isolation of the yeast isolates

Conventional morphological identification techniques (Yarrow, 1998; Barnett *et al.*, 2000) were used to preliminarily classify 135 isolates obtained from the different enrichments i.e. cyclohexanecarboxylic acid (CHC), 1,4-cyclohexanedicarboxylic acid (1,4-CHDA), 1,4-cyclohexanedimethanol, (1,4-CHDM), 1,2-dichlorobutane (1,2 DCB) or 2-chlorobutyric acid (2-CBA). The yeast isolates were confirmed to be both ascomycetes (30) (Table 1) and basidiomycetes (105) by their DBB reactions.

3.3.1.1 Identification of yeast isolates

The basidiomycetous yeast isolates outnumbered the ascomycetous yeasts. Based on cell and colony morphology the ascomycetous isolates obtained from enrichments with the cyclohexane derivatives (CHC, CHDA, CHDM) could be divided into five groups (possibly five different species), while the isolates from enrichments with chloro derivatives (2-CBA, 1,2 DCB) could be divided into two groups (possibly two different species) (Table 1). One representative from each of these seven groups were subjected to conventional identification methods (Yarrow, 1998; Barnett *et al*., 2000). The five isolates from the cyclohexane derivatives were identified as *D. hansenii*, *P. anomala*, *P. fabianii*, *P. guilliermondii* and *Y. lipolytica*. The two isolates obtained from the isolations with the 2-CBA were identified as *P. anomala* and *Y. lipolytica*. The *Pichia anomala* isolates obtained from both CHC and 2-CBA utilized lactose while authentic strains do not (Table 2). *Yarrowia lipolytica* from 1,4 CHDA was able to utilize arabinitol while the strain from 2-

CBA could not. According to Barnett *et al.* (2000), *Y. lipolytica* does not utilize arabinitol. However, the physiological tests of the two strains from 2-CBA were complemented by sequencing of the D1/D2 domain of the 26S rDNA, which confirmed their identification as 100% accurate. *Debaryomyces hansenii* was able to utilize lactose and butane 2,3 diol, *P. anomala* strains and *P. fabianii* could utilize lactose, *P. guilliermondii* was able to utilize butane 2,3 diol while the *Y. lipolytica* strains were able to assimilate lactose and arabinitol was utilized by the *Y. lipolytica* from the CHDA. According to Barnett *et al.* (2000), *D. hansenii*, *P. anomala*, *P. fabianii* and *Y. lipolytica* do not normally utilize lactose.

There are no reports in the literature of ascomycetous yeasts isolated from media supplemented with these substrates as carbon sources. Thanh *et al.* (2004) recently reported the isolation of novel *Rhodotorula* spp. from enrichments done using the 1,4-disubstituted cyclohexanes as carbon sources. *Debaryomyces hansenii*, *P. guilliermondii* and *Y. lipolytica* have been reported to utilize a wide range of non-carbohydrate carbon sources that include n-alkanes as well as phenol (Hofmann & Schauer, 1988) and in the case of *D. hansenii* also other hydroxybenzenes (Middelhoven, 1993).

3.3.2 Growth on 1,4-disubstituted cyclohexanes

Total cell counts were determined for each yeast strain i.e. *D. hansenii*, *P. anomala*, *P. fabianii*, *P. guilliermondii* and *Y. lipolytica* growing in YNB media supplemented with the following substrates as only carbon source: 1,4-CHDA (Table 3), 1,4-CHDM (Table 4) or 1,4-DMCH (Table 5). Tween 20 was added

as emulsifier. Flasks containing only Tween 20 without the cyclohexane derivatives served as negative controls. These flasks showed significantly less growth when compared with the flasks supplemented with the cyclohexane derivatives. However, when Tween 20 was omitted from the media, no growth was observed on the cyclohexane derivatives (results not shown) indicating that utilization of these substrates depended on the presence of an emulsifier. Previous studies (O'Sullivan *et al.*, 2004) have shown that Tween 40 and Tween 80 improved the emulsification of lipophilic compounds, thus increasing the availability of the substrate to the cells in culture.

Except in the case of *D. hansenii*, growth on 1,4-DMCH (Table 5) was better than growth on the other two substrates (Tables 3, 4). *Debaryomyces hansenii* only showed significant growth on 1,4-CHDA (Table 3), the substrate from which it had been isolated. It might lack the ability to oxidise the diol to the dicarboxylic acid and or the ability to hydroxylate dimethylcyclohexane. The other four yeasts are probably able to hydroxylate dimethylcyclohexane to the diol, even though they had been isolated from 1,4-CHDM. This suggests that these yeasts have monooxygenases that can hydroxylate the methyl groups and / or the ring to yield the diol and / or the corresponding cyclohexanol derivatives. It has been shown that degradation of cyclohexane proceeds via a ring hydroxylation (Schumacher & Fakoussa, 1999).

The ability of microbes to adapt to toxic compounds such as alcohols is ascribed to an ability to alter the lipid composition of the membrane to prevent

disruption of membrane organisation, which would subsequently lead to increased permeability resulting in cell death. The adaptation to the presence of toxic compounds is explained by a mechanism called homeoviscous adaptation whereby microbes maintain their membranes at a constant fluidity by changing the degree of saturation and the average chain length of the fatty acids in their membranes or by isomerising their cis-unsaturated fatty acids to trans-unsaturated fatty acids. Both these mechanisms have been encountered in the organisms that exhibit the greatest resistance to alcohols (Rubin & Rottenberg, 1982; Veld *et al.*, 1993; Heipieper *et al.*, 1994; Erdal *et al.*, 2003).

3.3.3 Growth on other hydrocarbons as C-source

Pichia anomala and *Y. lipolytica* were also tested for growth on other hydrocarbons. The C-source mostly favoured by these yeasts (Figures 2, 3) was dodecane, followed in the decreasing order by pristane and 1,4-CHDM. Growth in cultures supplemented with these three substrates was significantly better than in control flasks without C-source.

The ability of the *P. anomala* strain isolated from 1,4-CHDM to grow on dodecane and pristane is very unusual. This is because ascomycetous yeasts able to grow on n-alkanes and phenol have a coenzyme Q9, while those unable to grow have CoQ7. According to literature, (Kurtzman & Fell, 1998; Barnett *et al.*, 2000) *P. anomala* has a coenzyme Q7 and would therefore, not be expected to grow on either of these compounds. Previous work by Hofmann & Schauer (1988) showed that *P. anomala* (*Hansenula*

anomala) could not utilize either hexadecane or phenol. In addition, a well studied alkane-utilizer, *C. tropicalis*, showed in other studies minimal growth on branched hydrocarbons such as pristane (Gilewicz *et al.*, 1979).

The growth of both the *Y. lipolytica* and the *P. anomala* strains on pristane is thus very interesting, since growth on branched chain hydrocarbons is not very common and pristane is often used as an inert co-solvent when growth on solid or toxic hydrocarbons is investigated (Mauersberger *et al.*, 1996). Growth of *Y. lipolytica* strains on pristane has however previously been reported (Mauersberger *et al.*, 1996).

The finding of the different genera of yeasts able to grow on these unusual substrates agrees with the study by Middelhoven and Kurtzman (2003) where they reported that ascomycetous yeasts able to utilize unusual carbon sources such as uric acid or amines and hexadecane are widely distributed across the ascomycete phylogenetic tree. In addition, ascomycetous yeasts able to utilize the above mentioned compounds occur in clusters closely related phylogenetically. It is not surprising therefore, that the species isolated from the unusual substrates belonged to the genera *Debaryomyces* and *Pichia* both of which are in the family Saccharomycetaceae. It has, also been shown that yeasts able to utilize unusual carbon sources have in common physiological characteristics that require complicated enzymatic pathways that depend on enzymes, which occur as multigene families that are apparently shared by species involved (Middelhoven & Kurtzman, 2003).

3.3.4 Growth of yeasts on monoterpenes

Thanh *et al.* (2004) reported that *Rhodotorula* spp. isolated on the 1,4-disubstituted cyclohexanes were also able to utilize limonene supplied in the vapour phase as only carbon source. However, the isolates of *Y. lipolytica*, *P. anomala*, *P. fabianii*, *P. guilliermondii* and *D. hansenii* showed no growth after seven days on either limonene, α-pinene or β-pinene supplied in the vapour form as only carbon and energy source. The possible explanation for this observed inability to grow in the presence of monoterpenes might be due to toxicity of these compounds to yeasts especially in an oxygen limited environment like the dessicator, since slight growth was observed on the control plates placed in the dessicator without monoterpenes. Previous work by Uribe and co-workers (1985) showed that ß-pinene inhibits yeast cell respiration and that this was further influenced by the number of yeast cells present in the culture. This suggests that the size of the inoculum that was used could also have influenced the ability of the yeasts to grow in the presence of these compounds. It has also been shown (Uribe *et al.*, 1990; Busmann & Berger, 1994) that monoterpenes inhibit oxygen consumption and thus uncouple oxidative phosphorylation in the yeast mitochondria.

3.3.5 Growth of yeasts on chlorocompounds

The cell counts obtained suggest that *Y. lipolytica* was perhaps able to grow in the presence of 2-CBA (Figure 4) with the growth in the control being significantly lower. However, the utilization of 2-CBA could not be confirmed using HPLC, because the peak corresponding to this compound increased over time (results not shown), suggesting that some product was formed that

eluted at the same retention time as the substrate. This finding may be confirmed by using GC-MS analysis.

The growth of *P. anomala*, was rather poor, with cell counts being higher in the control media where *P. anomala* was inoculated in YNB without addition of 2-CBA (results not shown). Attempts to determine whether *P. anomala* would be able to degrade the 2-CBA were also not successful. The substrate concentration remained constant. These results for *P. anomala* suggest that the organism does not grow well in the presence of 2-CBA despite the fact that it had been isolated from the media containing 2-CBA as a carbon source. The inability of both *P. anomala* and *Y. lipolytica* to grow in the presence of 2-CBA could be due to toxicity of the acid to the yeasts at acid pH (Middelhoven, 1997). It has been reported that organic acids are more toxic to microorganisms at pH values that are less than that of soil (pH of ~ 6.5) (Garcia *et al*., 2000, 2002; Cabral *et al.*, 2003).

3.4 Conclusions

1) There were more basidiomycetes than ascomycetes isolated when derivatives of cyclohexane or chlorobutane were used in enrichments. This observation is supported by literature, which shows a number of reports that have been on isolation of basidiomycetous yeasts from unusual carbon sources whereas this is the first report on isolation of ascomycetous yeasts able to grow on these unusual substrates. The substrate that yielded the highest number of isolates was 1,4-CHDM.

2) Molecular identification by sequencing of the D1/D2 domain of the 26S rDNA of the *P. anomala* and *Y. lipolytica* strains isolated from 2-CBA confirmed that the conventional identification was accurate. The ability of the *P. anomala* strain isolated from 1,4-CHDM to grow on dodecane and pristane is very unusual, and this strain should be subjected to sequencing to confirm identification.

3) The yeast strains were able to grow on the cyclohexane derivatives although they seemed to prefer a shorter straight chain hydrocarbon as confirmed by higher growth rates in the presence of dodecane.

4) The isolates obtained could not grow in the presence of monoterpenes, although they had been isolated from substrates that were supposed to mimic the structures of monoterpenes and which previously had yielded limonene utilizing *Rhodotorula* species. The inability of these strains to grow on monoterpenes might be due to toxicity of the monoterpenes, because of their hydrophobicity and lipophilicity resulting in them partitioning into the lipid bilayer of cell membranes.

5) The inability of *P. anomala* and *Y. lipolytica* to degrade the 2-CBA might be due to toxicity of low molecular weight organic acids at low pH (Middelhoven, 1997; Cabral *et al.*, 2003).

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Table 1. The frequency with which different yeast isolates was obtained from enrichment cultures with various carbon sources. Cyclohexanecarboxylic acid = CHC, 1,4-cyclohexanedicarboxylic acid = 1,4 CHDA, 1,4-cyclohexanedimethanol = 1,4 CHDM, 2-chlorobutyric acid = 2-CBA and 1,2 dichlorobutane = 1,2 DCB.

Isolate	CHC	CHDA	CHDM	2-CBA	1,2 DCB
Debaryomyces hansenii	-	+(2)	+(6)	-	-
Pichia anomala	+(3)	+(3)	+(5)	+(1)	+(1)
Pichia fabianii	-	-	+(2)	-	-
Pichia guilliermondii	-	-	+(1)	-	-
Yarrowia lipolytica	-	+(2)	+(2)	+(1)	+(1)

The number in () refers to the number of isolates that were obtained using the various carbon sources, No isolates obtained = -, Isolates obtained = +.

Table 2. Carbon assimilation profiles of yeast isolates compared to that of the authentic strains (Barnett *et al.*, 2000) (+ = reactions for isolates different to authentic strains).

Isolate	UOFS strain No	Isolated from	Biochemical tests showing differences			
			Lac	Arabin	Prop-1,2	But-2,3
Debaryomyces hansenii	Y-1977	CHDA	**+**	v	v	**+**
Pichia anomala	Y-2220	CHC	**+**	v	v	v
Pichia fabianii	Y-1990	CHDM	**+**	-	+	+
Pichia guilliermondii	Y-2186	CHDM	-	v	v	**+**
Yarrowia lipolytica	Y-2221	CHDA	**+**	**+**	v	-
Pichia anomala	Y-2188	2-CBA	**+**	v	v	v
Yarrowia lipolytica	Y-2355	2-CBA	**+**	-	v	-

Lactose = Lac, Arabinitol = Arabin, Propane 1,2 diol = Prop-1,2; Butane 2,3 diol = But-2,3.

Table 3. Growth of new isolates in YNB broth supplemented with 0.1% (v/v) Tween 20 and 1% (v/v) 1,4-cyclohexanedicarboxylic acid (CHDA). Controls were only supplemented with 0.1% (v/v) Tween 20. The cells were grown over a 120 hour period, with counts recorded daily.

Strain	Initial count ($\times 10^4$ cells)		Count after 120 hrs ($\times 10^4$ cells)	
	Without CHDA	With CHDA	Without CHDA	With CHDA
Debaryomyces hansenii	150	175	775	1050
Pichia anomala	125	125	650	1250
Pichia fabianii	135	175	675	1100
Pichia guilliermondii	100	150	685	1125
Yarrowia lipolytica	100	120	640	1000

Table 4. Growth of new isolates in YNB broth supplemented with 0.1 % (v/v) Tween 20 and 1 % (v/v) 1,4-cyclohexanedimethanol (CHDM). Controls were supplemented with 0.1 % (v/v) Tween 20. The cells were grown over a 120 hour period, with counts recorded daily.

Strain	Initial count (x 10^4 cells)		Count after 120 hrs (x 10^4 cells)	
	Without CHDM	With CHDM	Without CHDM	With CHDM
Debaryomyces hansenii	130	125	750	990
Pichia anomala	135	125	850	1275
Pichia fabianii	145	150	775	1200
Pichia guilliermondii	140	150	875	1250
Yarrowia lipolytica	135	130	990	1100

Table 5. Growth of new isolates in YNB broth supplemented with 0.1 % (v/v) Tween 20 and 1 % (v/v) 1,4-dimethylcyclohexane (1,4-DMCH). Controls were supplemented with 0.1 % (v/v) Tween 20. The cells were grown over a 120 hour period, with counts recorded daily.

Strain	Initial count (x 10^4 cells)		Count after 120 h (x 10^4 cells)	
	Without DMCH	With DMCH	Without DMCH	With DMCH
Debaryomyces hansenii	125	120	725	980
Pichia anomala	130	125	890	1350
Pichia fabianii	120	125	750	1290
Pichia guilliermondii	125	120	910	1325
Yarrowia lipolytica	135	130	920	1410

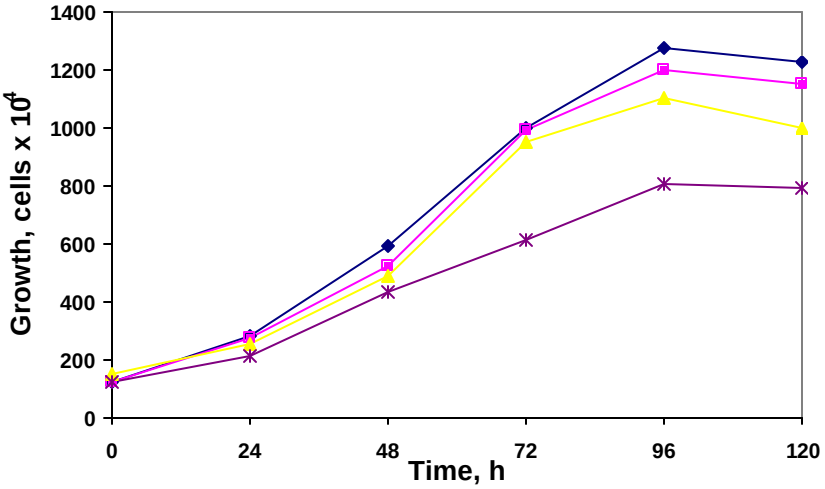

Figure 2. Total cell counts obtained for *P. anomala* when grown in YNB medium supplemented with different carbon sources: v/v 1% dodecane (?), pristane (?), 1,4-cyclohexanedimethanol (?) and no carbon source (×) in YNB respectively.

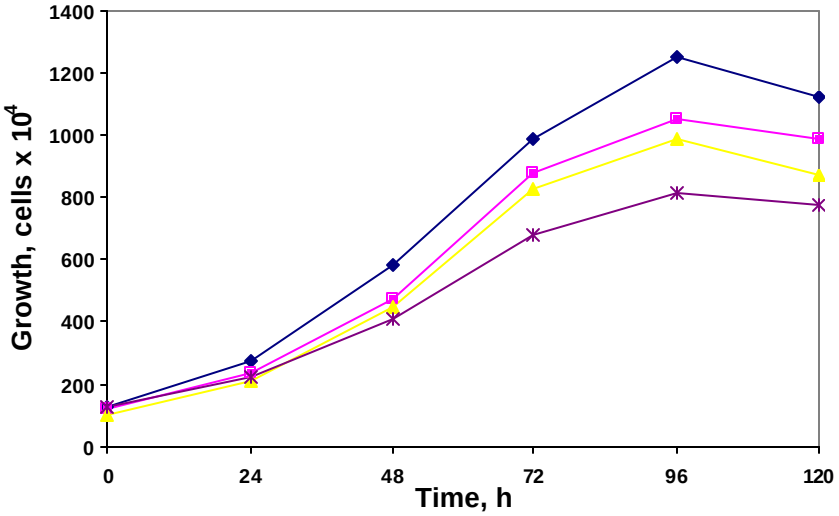

Figure 3. Total cell counts obtained for *Y. lipolytica* when grown in YNB medium supplemented with different carbon sources: 1% v/v dodecane (?), pristane (?), 1,4-cyclohexanedimethanol (?) and no carbon source (×) in YNB respectively.

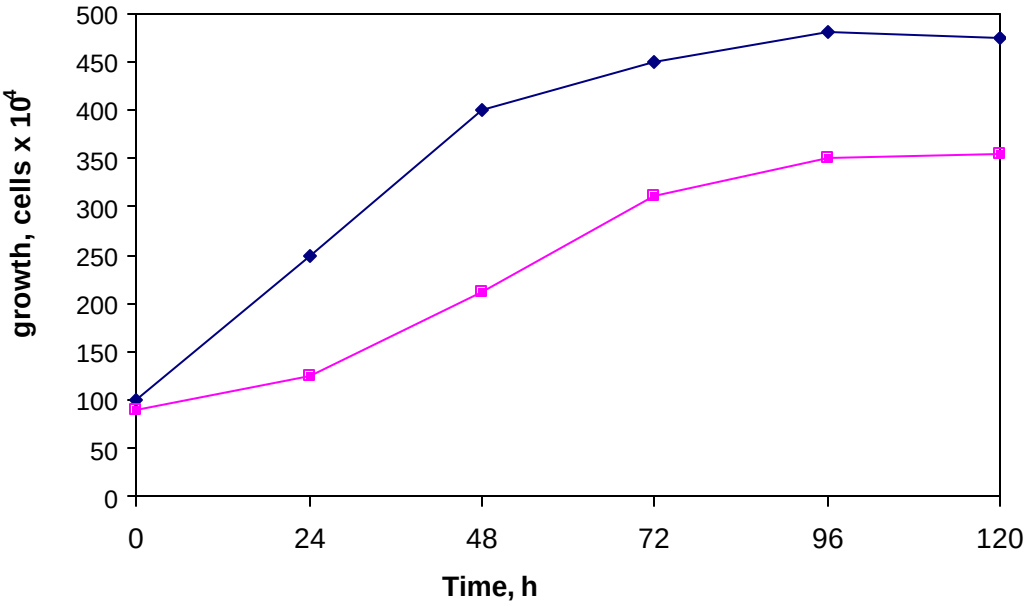

Figure 4. Total cell counts of *Y. lipolytica* in YNB medium supplemented with 1% v/v 2-chlorobutyr ic acid (?) and in its absence (?) monitored over a five-day period.

SUMMARY

In view of the decline of natural habitats due to urban and industrial development, the need to search for new yeasts is pressing since few natural habitats have been thoroughly investigated for yeast species. Yeasts have not been isolated from Lesotho before despite being an ideal environment for yeasts. In addition, isolation of yeasts able to utilize complex substrates, similar to intermediates in the degradation of chlorophenols would be important in their detoxification. Yeasts with these abilities are usually isolated from polluted environments and their presence from the pristine environment in Lesotho would be unexpected.

The species *Lipomyces starkeyi* and *L. tetrasporus* were found distributed throughout the various habitats while *Debaryomyces hansenii*, *D. hansenii* var. *fabryi* and *D. occidentalis* were found in 60% - 70% of the different regions. *Debaryomyces polymorphus*, *Dipodascus spicifer*, *Galactomyces geotrichum*, *G. reessii*, *Kluyveromyces lactis*, *L. kononenkoae*, *L. mesembrius*, *L. spencermartinsiae*, *Pichia anomala*, *P. fabianii*, *P. guilliermondii* and *Yarrowia lipolytica* were site specific. The fatty acid profiles of the isolated lipomycetous yeasts are similar to those reported in literature. This corroborates the value of this phenotypic characteristic in the taxonomy of these yeasts.

The PCR products of the ITS region of some of the type strains of the family Lipomycetaceae showed high length variation enabling rapid identification. The type strains, the sub-species and the varieties of the family Lipomycetaceae could be differentiated from each other using RFLP profiles

obtained with the restriction enzymes used in combination i.e. *Cfo* I, *Hae*III and *Mbo*I. The RFLP profiles for the five Lesotho isolates with atypical carbohydrate patterns could be separated into three groups. The first group, comprising of three isolates (52b, 73, 93), gave similar restriction patterns suggesting that they are the same species. Isolate 58b yielded a distinct profile while isolate 97 had similar sized ITS-PCR (1000bp) to that of *L. lipofer* and *L. tetrasporus*. It is important to assess the variation in RFLP profiles between various strains from different habitats of a particular species in order to determine the conserved status of this genotypic character. More strains of a species representing the Lipomycetaceae should be subjected to similar RFLP analysis to further determine its conserved status.

The D1/D2 sequence data enabled separation of the five isolates into three groups. The first group, comprising of three isolates, showed 1% nucleotide substitutions to *L. starkeyi* suggesting that these isolates are probably known *Lipomyces* spp. The other two isolates yielded sequences that were 99% identical to *L. kononenkoae* subsp. *kononenkoae* (isolate 58b) and 100% identical to that of *L. tetrasporus* (isolate 97) further suggesting that these isolates are probably known *Lipomyces* species.

Isolation of basidiomycetous yeasts from unusual carbon sources has been reported, however, this is the first report of isolation of ascomycetous yeasts able to grow on unusual substrates. The substrate that yielded the highest number of isolates was 1,4 cyclohexanedimethanol.

Pichia anomala and *Y. lipolytica* strains (identity confirmed with D1/D2 sequencing) isolated from 2-chlorobutyric acid could not degrade the 2-chlorobutyric acid probably due to toxicity of low molecular weight organic acids at low pH. The ability of *P. anomala* isolated from 1,4 cyclohexanedimethanol to grow on dodecane and pristane is unusual and this strain should also be subjected to sequencing to confirm identification. *Debaryomyces hansenii*, *P. anomala*, *P. fabianii*, *P. guilliermondii* and *Y. lipolytica* could grow on the cyclohexane derivatives although they preferred a shorter straight chain hydrocarbon as confirmed by higher growth rates in the presence of dodecane.

The isolates could not grow in the presence of monoterpenes, although they had been isolated from substrates that were supposed to mimic the structures of monoterpenes and which previously had yielded limonene utilizing *Rhodotorula* species. The inability of these strains to grow on monoterpenes might be due to toxicity of the monoterpenes, because of their hydrophobicity and lipophilicity resulting in partitioning into the lipid bilayer of cell membranes.

OPSOMMING

Gesien in die lig van die verdwyning van natuurlike habitatte a.g.v. stedelik en industriële ontwikkeling, is dit noodsaaklik om nuwe giste te soek aangesien min natuurlike habitatte reeds deeglik ondersoek is vir gisspesies. Giste is nog nie voorheen uit Lesotho geïsoleer nie ten spyte van die feit dat dit ‘n ideale omgewing vir giste bied. Verder kan die isolasie van giste wat komplekse substrate, soos die afbraakprodukte van chlorofenole, kan benut, belangrik wees in hul detoksifikasie. Giste met hierdie vermoëns word gewoonlik uit besoedelde omgewings geïsoleer en hul teenwoordigheid in onbesoedelde omgewings in Lesotho sou onverwags wees.

Die spesies *Lipomyces starkeyi* en *L. tetrasporus* kom verspreid voor in verskeie habitatte terwyl *Debaryomyces hansenii*, *D. hansenii* var. *fabryi* en *D. occidentalis* in 60% - 70% van die verskillende streke voorkom. *Debaryomyces polymorphus*, *Dipodascus spicifer*, *Galactomyces geotrichum*, *G. reessii*, *Kluyveromyces lactis*, *L. kononenkoae*, *L. mesembrius*, *L. spencermartinsiae*, *Pichia anomala*, *P. fabianii*, *P. guilliermondii* en *Yarrowia lipolytica* was meer spesifiek. Die vetsuurprofiele van die geïsoleerde lipomisete giste is soortgelyk aan die in die literatuur. Dit ondersteun die waarde van hierdie fenotipiese eienskap in die taksonomie van hierdie giste.

Die PKR-produkte van die “ITS” gebied van sommige van die tipestamme van die familie Lipomycetaceae het ‘n hoë mate van variasie in lengte getoon, wat vinnige identifikasie moontlik gemaak het. Die tipestamme, sub-spesies en variëteite van die familie Lipomycetaceae kon van mekaar onderskei word d.m.v. “RFLP” profiele verkry met die kombinasie van beperkingsensieme: d.i.

*Cfo*I, *Hae* III en *Mbo* I. Die “RFLP” profiele vir die vyf Lesotho isolate met ongewone koolhidraatpatrone, kon in drie groepe verdeel word. Die eerste groep, bestaande uit drie isolate (52b, 73, 93), het soortgelyke beperkingspatrone gelewer. Dit het daarop gedui dat hulle tot dieselfde spesie behoort. Isolaat 58b het ‘n unieke profiel gelewer, terwyl isolaat 97 ‘n PKR produk gelewer het met ‘n grootte (1000 bp) soortgelyk aan *L. lipofer* en *L. tetrasporus*. Dit is belangrik om die variasie in “RFLP” profiele tussen verskillende stamme van ‘n spesifieke spesie vanaf verskillende habitatte te ondersoek om sodoende die gekonserveerde status van hierdie genotipiese eienskap te bepaal. Meer stamme van spesies wat die Lipomycetaceae verteenwoordig behoort aan dieselfde “RFLP” analise onderwerp te word om verder die gekonserveerde status te bepaal.

Die D1/D2 basis-opeenvolgings-data het dit moontlik gemaak om die vyf isolate in drie groepe te verdeel. Die eerste groep, bestaande uit drie isolate, het ‘n 1% nukleotiedsubstitusie met *L. starkeyi* getoon, wat dui daarop dat hierdie isolate moontlik behoort tot bekende *Lipomyces* spp. Die ander twee isolate het basis-opeenvolgings gelewer wat 99% identies was aan *L. kononenkoae* subsp. *kononenkoae* (isolaat 58b) en 100% identies was aan *L. tetrasporus* (isolaat 97) wat verder dui daarop dat die isolate waarskynlik behoort tot die bekende *Lipomyces* spesies.

Isolasie van basidiomisete giste vanuit ongewone koolstofbronne is voorheen gerapporteer. Dit is egter die eerste rapportering van die isolasie van askomisete giste wat op ongewone koolstofbronne kan groei. Die substraat wat die hoogste getal isolate gelewer het, was 1,4 sikloheksaandimetanol.

Pichia anomala en *Y. lipolytica* stamme (identiteit bevestig d.m.v. D1/D2 basis-opeenvolging-bepaling) geïsoleer vanaf 2-chlorobottersuur kon nie 2-chlorobottersuur afbreek nie, moontlik a.g.v die toksisiteit van organiese sure met ‘n lae molekulêre massa by ‘n lae pH. Die vermoë van *P. anomala,* geïsoleer vanaf 1,4 sikloheksaandimetanol, om op dodekaan en pristaan te groei is ongewoon en hierdie stam behoort aan basis-paar-opeenvolging-bepaling onderwerp te word om sy identiteit te bevestig. *D. hansenii*, *P. anomala*, *P. fabianii*, *P. guilliermondii* en *Y. lipolytica* kon op die sikloheksaanafgelydes groei alhoewel hulle ‘n korter reguitketting koolhidraat verkies het, soos bevestig deur hul hoër groeisnelhede in die teenwoordigheid van dodekaan.

Die isolate kon nie in die teenwoordigheid van monoterpene groei nie, alhoewel hulle geïsoleer is uit substrate wat veronderstel was om soortgelyk te wees aan die strukture van monoterpene en waaruit die limoneenbenuttende *Rhodotorula* spesie voorheen geïsoleer is. Die onvermoë van hierdie stamme om op die monoterpene te groei kan toegeskryf word aan die toksisiteit van monoterpene a.g.v. hul hidrofobiese en lipofiliese aard wat lei tot die vernietiging van die lipied-dubbellaag van selmembrane.

KEYWORDS:

Yeasts; Lesotho; Identification; Fatty acids; ITS region; RFLP analysis, D1/D2 domain, Cyclohexane; Monoterpenes; Chlorobutane.

SLEUTELWOORDE:

Giste, Lesotho, identifikasie, Vetsure, ITS gebied, RFLP analise, D1D2 gebied, Sikloheksaan, Monoterpene, Chlorobutaan.