PSYCHROTOLERANT MUCORALEAN FUNGI PRESENT IN PRISTINE MOUNTAIN FYNBOS SOIL AND VINEYARD SOIL FROM THE STELLENBOSCH REGION

By

Heidi E. Samson

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at the University of Stellenbosch

Supervisor: Dr. A. Botha

February 2001

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Mucoralean fungi are mostly saprotrophs that are frequently encountered in soil habitats. Using an isolation temperature of *circa* 25°C, other workers obtained these fungi from a wide diversity of geographical areas in southern Africa. However, it is known that psychrotolerant mucoralean fungi, able to grow at 25°C as well as at 5°C, occur in pristine Alti Mountain Grassland. Nothing is known about the diversity of these psychrotolerant soil fungi in other vegetation types of South Africa.

Consequently, in this study, the psychrotolerant fungal taxa and numbers in soil from a vineyard and from pristine Mountain Fynbos were determined using an incubation temperature of 4°C and a complex isolation medium. The latter contained agar, malt extract, peptone, yeast extract, penicillin and streptomycin sulphate. Soil samples were analysed in late summer, autumn and mid-winter. It was found that, for the samples taken in late summer and autumn, the diversity of mucoralean species in the soil differed between fynbos and vineyard. In winter however, no significant difference was detected between the Shannon's diversity indices of mucoralean species in the soil samples taken from the two habitats. It was found that in both soil types, the percentage mucoralean fungi on the plates increased from summer to winter. In addition, the numbers of detectable *Mortierella* subgenus *Mortierella* on the plates were higher in winter than in late summer. The diversity of mucoralean species obtained during winter in fynbos and vineyard soil was significantly less than the diversity of these species in Alti Mountain Grassland soil.

To determine if the *Mortierella* subgenus *Mortierella* isolates from the fynbos and vineyard soil, and those obtained from Alti Mountain Grassland, differ in the ability to grow at low temperatures, the radial growth rate on malt extract agar at 4°C and 8°C was determined for each isolate. The results indicate that not only did seasonal changes occur in the taxa within *Mortierella* subgenus *Mortierella,* but that the isolates dominating the soil in different seasons also differed in the ability to grow at low temperatures. The percentage of isolates that had reached greater colony diameters after 8 days of incubation at 4°C, was higher

for the isolates obtained in the cold wet month of July than for those obtained in the warmer dryer month of February. Similar results were obtained with the radial growth experiments conducted at 8°C. The *Mortierella* subgenus *Mortierella* isolates obtained in winter from fynbos and vineyard soil showed less variation in low temperature growth rate than the isolates of this taxon obtained in winter from Alti Mountain Grassland soil during a previous study. This variation corresponds to the greater number (20) of *Mortierella* subgenus *Mortierella* species found in the grassland soil. Altogether only seven species of this subgenus was detected during the present study in the fynbos and vineyard soil samples. It was speculated that this difference in diversity between the fynbos and vineyard isolates, and the grassland isolates obtained in a previous study, might have been as a result of differences in the habitat or the enumeration methods used.

The phylogenetic relationship between different psychrotolerant isolates of *Mortierella* subgenus *Mortierella* originating from the soil of the fynbos, vineyard and Alti Mountain Grassland, was subsequently determine through comparison of ITS regions, within ribosomal RNA repeats. Consequently, 45 psychrotolerant *Mortierella* subgenus *Mortierella* isolates originating from the three soil habitats was compared on the basis ITS 1 nucleotide sequence composition and radial growth rate at 4°C. Phylogenetic analyses showed that the isolates could be grouped into two clusters correlating with the ability to grow at low temperatures. Each cluster was further subdivided into two subgroups. It was found that except for one subgroup and the reference strain occurring in another subgroup, all the subgroups contain isolates originating from a single soil habitat. Therefore, the ITS 1 sequence of these fungi seems to indicate the original habitat and ability to grow at low temperatures. This correlation of the ITS sequence with the ecological habitat of a fungus has also been observed by other workers for other fungal groups.

OPSOMMING

Mucoraliese fungi is meestal saprotrofe wat dikwels in grondhabitatte aangetref word. Deur gebruik te maak van 'n isolasietemperatuur van *circa* 25°C, het ander werkers dié fungi van 'n wye verskeidenheid geografiese gebiede in suidelike Afrika verkry. Dit is egter bekend dat die psigrotolerante mucoraliese fungi, wat in staat is om by 25°C en ook by 5°C te groei, in ongeskonde Alti Berg-Grasland voorkom. Niks is egter bekend oor die diversiteit van dié psigrotolerante grondfungi in ander veldtipes van suidelike Afrika nie.

Die psigrotolerante fungustaksa en -getalle in grond van 'n wingerd en van ongeskonde Berg Fynbos is gevolglik in dié studie bepaal deur gebruik te maak van 'n inkubasietemperatuur van 4°C en 'n komplekse isolasiemedium. Laasgenoemde het agar, moutekstrak, peptoon, gisekstrak, penisillien en streptomisiensulfaat bevat. Grondmonsters is in die laatsomer, herfs en midwinter geanaliseer. Daar is 'n verskil gevind tussen die diversiteit van die mucoraliese spesies in die grond van fynbos en dié van wingerd in die monsters wat in die laatsomer en midwinter geneem is. In die winter is daar egter geen beduidende verskil gevind tussen die Shannon diversiteitsindekse van mucoraliese spesies in die grondmonsters wat uit die twee habitatte getrek is nie. In albei grondtipes is daar gevind dat die persentasie mucoraliese fungi op die plate toegeneem het van somer tot winter. Daarby was die aantal waarneembare *Mortierella* subgenus *Mortierella* op die plate meer in die winter as in die laatsomer. Die diversiteit van mucoraliese spesies wat in die winter uit fynbos- en wingerdgrond verkry is, was beduidend minder as die diversiteit van dié spesies in Alti Berg-Grasland grond.

Om te bepaal of die *Mortierella* subgenus *Mortierella* isolate van die fynbos- en wingerdgrond en dié van Alti Berg-Grasland van mekaar verskil ten opsigte van hul vermoë om by lae temperature te groei, is die radiale groeitempo op moutekstrak by 4°C en 8°C vir elke isolaat bepaal. Die resultate dui aan dat daar nie alleen seisoenale veranderinge in die taksa binne *Mortierella* subgenus *Mortierella* voorkom nie, maar dat die isolate wat tydens verskillende seisoene uit die grond verkry is, ook ten opsigte van hul groeivermoë by lae temperature

van mekaar verskil. Die persentasie isolate wat groter kolonie diameters bereik het ná 8 dae inkubasie by 4°C, was hoër vir die isolate van die koue, nat Juliemaand as vir dié wat in die warmer en droër Februariemaand verkry is. Soortgelyke resultate is verkry met radiale groei-eksperimente wat by 8°C gedoen is. Die *Mortierella* subgenus *Mortierella* isolate wat in die winter uit fynbos- en wingerdgrond verkry is, het 'n kleiner variasie in hul groeitempo by lae temperature getoon as die isolate in dié takson wat tydens 'n vorige studie in die winter uit Alti Berg-Grasland grond verkry is. Dié variasie stem ooreen met die groter aantal (20) *Mortierella* subgenus *Mortierella* spesies wat in die graslandgrond gevind is. Slegs sewe spesies van dié subgenus is gedurende die huidige studie in die fynbos- en wingerdgrondmonsters waargeneem. Daar is gespekuleer dat dié verskil in diversiteit tussen die fynbos- en wingerdisolate en die graslandisolate van die vorige studie die gevolg mag wees van verskille tussen die habitat of die enumerasiemetodes wat gebruik is.

Die filogenetiese verwantskap tussen verskillende psigrotolerante isolate van *Mortierella* subgenus *Mortierella* uit die grond van die fynbos, wingerd en Alti Berg-Grasland, is vervolgens bepaal deur 'n vergelyking van interne getranskribeerde spasieerder (ITS) areas, binne ribosomale RNS herhalings. Daar is gevolglik 45 psigrotolerante *Mortierella* subgenus *Mortierella* isolate uit die drie grondhabitatte met mekaar vergelyk op grond van die basis ITS 1 nukleotied opeenvolgingsamestelling en radiale groeitempo by 4°C. Filogenetiese analises het die isolate in twee groepe verdeel op grond van hul vermoë om by lae temperature te groei. Elke groep is verder in twee subgroepe verdeel. Daar is gevind dat behalwe vir een subgroep en die verwysingstam wat in 'n ander subgroep voorgekom het, elkeen van die subgroepe bestaan het uit isolate wat van 'n enkele grond habitat verkry is. Dit wil dus voorkom of die ITS 1 opeenvolging van dié fungi 'n aanduiding gee van die oorspronklike habitat en die vermoë om by lae temperature te groei. Dié korrelasie tussen die ITS opeenvolging en die ekologiese habitat van 'n fungus is ook deur ander werkers vir ander fungusgroepe waargeneem.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following:

Dr. A. Botha for his guidance, advice and the valuable contributions he made towards this study.

The National Research Foundation (NRF) for financial support.

Nicolene Botha for technical assistance.

My wonderful family and friends for their love, support and encouragement throughout this study.

The staff and students of the Department of Microbiology at the University of Stellenbosch for their support and advice.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. Motivation

A number of industrially important bio-products such as alcohol, high value fatty acids, chitin, enzymes and intermediates in steroid synthesis, are produced by mucoralean fungi (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Hansson & Dostalek, 1988; Tsuchiura & Sakura, 1988; Hering *et al.*, 1991; Botha *et al.*, 1995; Eroshin *et al.*, 1996, Streekstra, 1997). In addition, these fungi have been used for millennia in the preparation of fermented Asian foods (Hesseltine, 1965). Consequently, the morphology of the mucoralean fungi as well as the physiological processes involved in the production of the above-mentioned products were thoroughly researched in the past decades (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Ratledge, 1989). Also, much is known about the occurrence of the Mucorales in different habitats from various geographical regions the world over (Domsch *et al.*, 1980).

A few mucoralean species were found to be parasitic on mammals (Hesseltine & Ellis, 1973; Alexopoulos & Mims, 1979; Domsch *et al.*, 1980). However, generally mucoralean fungi are saprotrophs, which live in association with decaying plant material, dung or other organic debris in soil (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Seabi *et al.*, 1999). These fungi are able to rapidly utilise the limited number of simple carbohydrate molecules present in dead plant material, before other fungi take over the mineralisation of carbon (Hesseltine & Ellis, 1973; Botha *et al.*, 1997; Seabi *et al.*, 1999).

Knowledge on the distribution of these fungi in the indigenous vegetation types of southern Africa however, is limited to the coastal regions of Kwazulu-Natal, the Karoo, Savannah plains and grasslands (Eicker, 1974; Roux & Van Warmelo, 1997; Botha *et al.*, 1999). Relatively few studies were conducted in fynbos (Allsopp *et al.*, 1987). Most of these studies were performed using an incubation temperature of *circa* 25°C. However, it is known that certain mucoralean species are psychrotolerant and can also be isolated at temperatures as low as 0°C (Carreiro & Koske, 1992). Using the soil plate technique of Warcup (1950), it was found that 92.4% of the isolates obtained at 5°C in mid-winter from Alti Mountain Grassland (Low & Rebelo, 1996) soil, were

representatives of *Mortierella* subgenus *Mortierella* (Botha *et al.*, 1999). However, nothing is known about the occurrence of psychrotolerant mucoralean fungi in soil from the fynbos of the Western Cape.

With the above as background the aim of this study was: 1) To determine the diversity of psychrotolerant mucoralean fungi present in vineyard soil and soil from pristine Mountain Fynbos (Low & Rebelo, 1996) in the Western Cape, South Africa. 2) To determine the phylogenetic relationship between different psychrotolerant isolates of *Mortierella* subgenus *Mortierella* through comparison of internal transcribed spacer regions (ITS), within ribosomal RNA repeats (Hseu *et al.*, 1996; de Koker, 2000).

1.2. The Mucorales

1.2.1. General characteristics

Mucoralean fungi are typical chemoorganotrophic heterotrophs, which utilise reduced organic molecules as carbon, energy and hydrogen sources. These fungi are able to utilise a variety of carbon sources such as hexoses, pentoses, di- and trisaccharides, polysaccharides, glucosides, alcohols, as well as organic acids (Botha *et al.*, 1997a; Botha *et al.*, 1997b; Botha & Du Preez, 1999; Pohl, 1999). Mucoralean fungi are able to grow on simple media containing an inorganic nitrogen source, minerals and one of the above carbon sources, but it is known that certain members, such as *Pilobolus* require additional growth factors (i.e. siderophores) to survive (Hesseltine & Ellis, 1973). Generally mucoralean fungi can be cultivated at *circa* 25°C, but certain species, such as *Mortierella alpina* is also able to grow at temperatures as low as 0°C, while others such as *Rhizomucor tauricus* can grow up to 55°C (Schipper, 1978; *Domsch et al.*, 1980).

Mucoralean fungi are characterised by eucarpic, mostly coenocytic thalli with an extensive mycelium which contain haploid nuclei (Benjamin, 1979). Asexual

reproduction occurs by means of nonmotile sporangiospores borne on few or many spored sporangia, which may contain columellae (Hesseltine & Ellis, 1973). During sexual reproduction two similar gametangia conjugate to form a thick walled zygospore (Benjamin, 1979). The Mucorales consist of thirteen families (Table1.1), which differ mainly from one another with regard to the nature of the asexual reproduction structures, specifically the characteristic features of the sporangiospores, sporangia, columellae and sporangiophores (Hawksworth *et al.*, 1995; Botha & Du Preez, 1999). However, some differences also exist in the morphology of the sexual reproductive structures such as the zygospores and suspensor cells. To illustrate the morphological diversity within this fungal order, some characteristic features of the mucoralean families are depicted in figure 1.1.

The Chaetocladiaceae, of which the genus *Dichotomocladium* is a member, is characterised by sporangiophores that terminate in sterile spines (Fig. 1.1a). Pedicillate, unispored sporangiola are borne on the sporangiophores (Benny & Benjamin, 1993; Strauss, 1997). Members of this family frequently produce rough walled zygospores, which are suspended between opposite aligned suspensor cells (Benny & Benjamin, 1993). Members of the Choanephoraceae (Fig. 1.1b), such as the genus *Choanephora* are characterised by sporangia and sporangiola borne on separate sporangiophores. When zygospores are formed they are striate and are borne between tong-like suspensor cells (Hawksworth *et al.*, 1995). The Cunninghamellaceae is known for the absence of sporangia, but has unispored sporangiola (Fig. 1.1c), which are borne on a swollen vesicle at the tip of the sporangiophore (Hawksworth *et al.*, 1995). Zygospores are generally warty and borne between opposite aligned suspensors cells (Benjamin, 1979). Members of the Gilbertellaceae (Fig. 1.1d) are characterised by columellate sporangia containing sporangiospores with appendages. When zygospores are formed they are generally rough walled and are situated between opposite aligned suspensor cells (Hawksworth *et al.*, 1995). Representatives of the Mortierellaceae typically have sporangia and sporangiola without columellae, or with rudimentary columellae (Fig. 1.1e). Zygospores are smooth or angular, and tend to get inwebbed in sterile hyphae

Table 1.1. The families of the Mucorales (Hawksworth *et al.*, 1995)

Chaetocladiaceae:	A. Fischer (1892), 2 genera, 7 species
Choanephoraceae:	J. Schröt. (1894), 3 gen. (+ syn.), 5 spp.
Cunninghamellaceae:	Naumov ex R.K. Benjamin (1959), 1 gen. (+3 syn.), 7 spp.
Gilbertellaceae:	Benny (1991), 1 gen., 2 sp.
Mortierellaceae:	A. Fischer (1892), 7 gen. (+7 syn), 106 spp.
Mucoraceae:	Dumort. (1822), 20 gen. (+27 syn), 102 spp.
Mycotyphaceae:	Benny & R.K. Benjamin, (1985). 2 gen. (+1 syn.), 6 spp.
Phycomycetaceae:	Arx (1982), 1 gen., 3 spp.
Pilobolaceae:	Corda (1842), 3 gen. (+3 syn), 13 spp.
Radiomycetaceae:	Hesseltine & J.J. Ellis (1974), 2 gen. (+1 syn.), 4 spp.
Saksenaeaceae:	Hesseltine & J.J. Ellis (1974), 1 gen., 1 sp.
Syncephalastraceae:	Naumov ex R.K. Benjamin (1959), 1 gen., 2 spp.
Thamnidiaceae:	Fitzpatrick (1930), 12 gen. (+9 syn.), 22 spp.

Abbreviations: gen., genus/era; syn., synonym; spp./sp., species

and are borne between tong-like or opposite aligned suspensor cells (Hesseltine & Ellis, 1973).

Members of the Mucoraceae (Fig. 1.1f) are characterised by columellate multi-spored sporangia, while specialised sporangiola are absent (Hesseltine & Ellis, 1973). Rhizoids and stolons may be produced. When zygospores are formed they are generally smooth to warty, borne between opposite aligned or tong-like suspensor cells, which may be naked or may have appendages (Hawksworth *et al.*, 1995). The Mycotyphaceae (Fig. 1.1g) is characterised by sporangiola, which are borne on dehiscent pedicles (Alexopoulos & Mims, 1979). Members of Phycomycetaceae are characterised by large, unbranched sporangiophores (Fig. 1.1h) and zygospores with coiled tong-like suspensor cells bearing branched appendages (Alexopoulus & Mims, 1979). Representatives of the Pilobolaceae (Fig. 1.1i) are known to have columellate sporangia containing specialised liberation mechanisms. Zygospores are smooth and characteristically borne between tong-like or opposite aligned suspensor cells (Hawksworth *et al.*, 1995).

Members of the Radiomycetaceae are characterised by sporangiola, which are borne on secondary ampullae (Fig. 1.1j). Sporangiophores are often stoloniferous, while many spored sporangia are absent. Zygospores are characteristically smooth and borne on opposite aligned, appendaged suspensor cells. In the Saksenaeaceae (Fig. 1.1k) sporangia are lageniform and columellate (Hesseltine & Ellis, 1973).

Members of the Syncephalastraceae produce merosporangia (Fig. 1.1l) and warty zygospores, which are borne between opposite aligned suspensor cells (Hesseltine & Ellis, 1973; Benjamin, 1979). Representatives of the Thamnidiaceae produce columellate sporangia of which the walls are diffluent (Fig. 1.1m). These fungi are also characterised by columellate sporangiola with persistent walls. Zygospores are warty and are borne between opposite aligned suspensor cells (Hesseltine & Ellis, 1973; Bejamin, 1979; Hawksworth *et al.*, 1995).

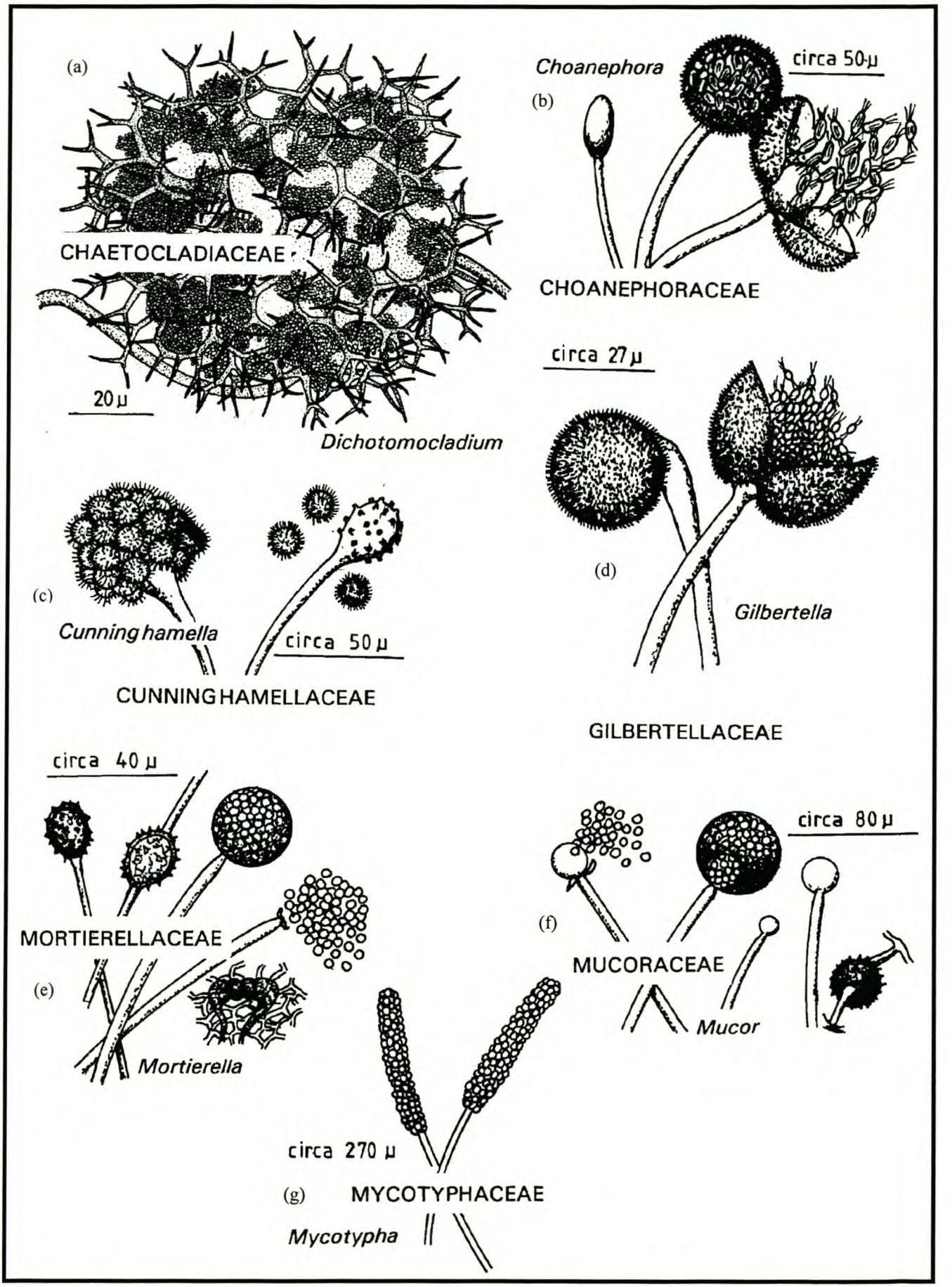

Figure 1.1. Morphological diversity among the families of the Mucorales. To illustrate differences, sketches of the reproductive structures of a representative genus in each family are given (Adapted from Strauss, 1997).

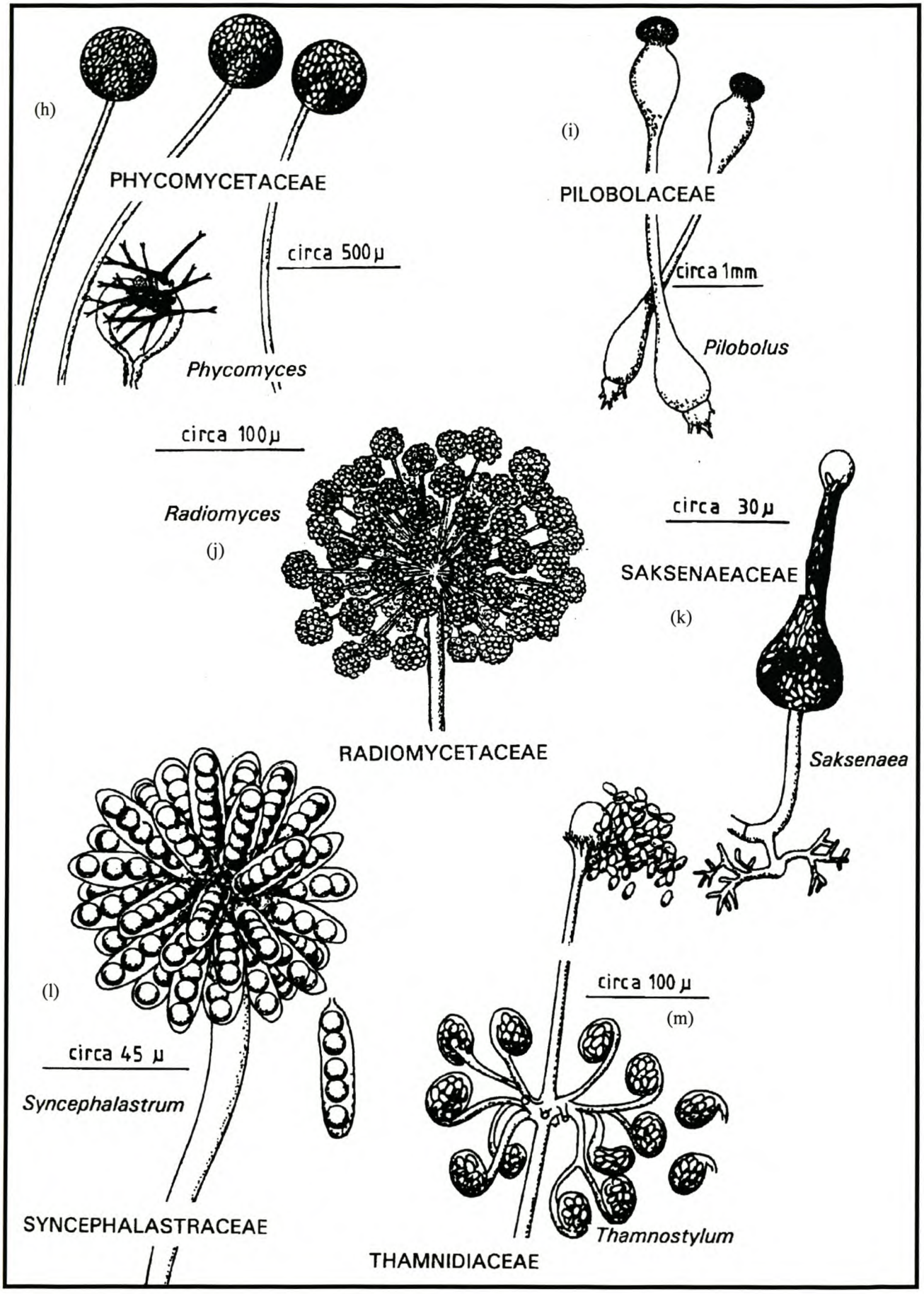

Figure 1.1. Continues.

1.2.2. Habitats of the Mucorales

Mucoralean fungi are the first saprotrophic colonisers responsible for the decay of organic material in soil (Alexander, 1961). Although most of these fungi are saprotrophs, a few species have been identified as being parasites of animals, fungi and plants (Hesseltine & Ellis, 1973; Alexopoulos & Mims, 1979; Ross, 1979; Domsch *et al.*, 1980; Hawksworth *et al.*, 1995). *Parasitella parasitica* is a known parasite of fungi, while *Absidia corymbifera* and *Absidia ramosa* are fungal pathogens of domestic animals, and *Sporodinella umbellata* is an insect parasite (Hesseltine & Ellis, 1973; Evans & Samson, 1977).

Mucoralean fungi may also act as opportunistic pathogens of humans causing mucormycosis in people with diabetes, leukaemia or an immune deficiency (Alexopoulos & Mims, 1979; Zabel, 1997). This is mainly caused by species of the genera *Rhizopus*, *Rhizomucor*, *Absidia* (Marshall *et al.*, 1997), *Cunninghamella* and *Mucor* (Voigt *et al.*, 1999). *Mortierella wolfii* is the only *Mortierella* species, which show restricted growth at 20°C, but good growth at temperatures near the body temperature of mammals, rendering them, among the *Mortierella* species, the most pathogenic to mammals (Domsch *et al.*, 1980). This species is known to cause mycotic abortion, pneumonia and systemic mycosis in cattle.

Mucoralean fungi such as *Rhizopus* and *Mucor* are almost always present in unspoiled foods such as nuts, spices, flour, protein-rich foods such as milk products, dog food, meats, fresh vegetables and fresh and dried fruit (Hesseltine & Ellis, 1973; Botha & Du Preez, 1999). These fungi however are also known to be agents of food spoilage (Hesseltine & Ellis, 1973; Schipper, 1978; Bartschi *et al.*, 1991), because of their ability to secrete saccharolytic and proteolytic enzymes (Michailides *et al.*, 1992; Díaz-Minguez & López-Matas, 1999). *Rhizopus stolonifer*, for example, causes a serious transit disease of strawberries, known as leak and a soft rot of sweet potatoes in storage. However, the Mucorales does not only cause spoilage in food, but certain species are also used in the preparation of Oriental foods such as sufu, tempeh, ragi and meju (Alexopoulos & Mims, 1979; Botha & Du Preez, 1999). In these

cases, strains of *Actinomucor, Mucor* or *Rhizopus* are used in combination with each other, or in combination with other microbes, to ferment substrates such as soybeans, rice or other cereals to produce more palatable products (Hesseltine, 1965).

Mucoralean fungi may cause severe diseases of certain economically important plants (Ross, 1979). *Choanephora cucurbitarum* colonise squash blossoms and fruits, which sometimes leads to considerable damage (Alexopoulos & Mims, 1979). *Rhizopus* species, such as *Rhizopus arrhizus*, *Rhizopus stolonifer* and *Rhizopus oryzae*, which are known for the production of high quantities of lipases are pathogenic to sunflowers causing the disease head rot (Hayes & Gulari, 1992; Cruz *et al.*, 1993; Talaro & Talaro, 1993). This disease is of economical importance since it reduces seed oil and yield (Kolte, 1985). Oil from infected seeds also has an increased amount of undesirable free fatty acids, from 0.8% (w/w) in oil from healthy seeds to 19.4% (w/w) in oil from infected seeds (Weiss, 1983; Kolte, 1985).

Not only do mucoralean fungi occur in food habitats, or certain habitats typical of pathogens, but these fungi can almost always be isolated from soil, air, dung or decaying plant material (Ross, 1979). Although mucoralean fungi occur mostly in moist environments, studies have indicated that these fungi may also be found in soil and on plant material from arid or semi-arid regions (Bokhary & Parvez, 1991; Steinman *et al.*, 1995; Guiraud *et al.*, 1995; Roux & Van Warmelo, 1997).

In most of the above-mentioned studies, isolation temperatures of *circa* 25°C were used, however it is known that certain mucoralean species can be isolated from soil at temperatures as low as 0°C (Carreiro & Koske, 1992). These fungi are therefore able to continue to utilise organic matter as the temperature decreases during winter. Similarly, it was found that certain mucoralean fungi are able to rapidly colonise fruit in cold storage (Alexopoulos & Mims, 1979; Spotts & Cervantes, 1986). It is especially pears, tomatoes and strawberries that are often spoiled in such a manner. The spoilage agents usually are *Mucor mucedo, Mucor piriformis* or *Mucor racemosus* (Botha & du Preez, 1999). All

these fungi, including *Mucor flavus* and *Mucor plasmaticus* are capable of growth at temperatures ranging from *circa* 25°C to as low as 0°C (Domsch *et al.*, 1980; Botha & du Preez, 1999).

As in the case of *Thamnidium*, a spoilage agent of meat in cold storage, the above-mentioned *Mucor* species are psychrotolerant, and hence able to grow at 5°C as well as at 20°C and above (Benny & Benjamin, 1975; Jannasch, 1998; Botha *et al.*, 1999). This characteristic distinguishes these fungi from true psychrophylic mucoralean fungi, which includes members of *Chaetocladium* and *Helicostylum*, that grow poorly at temperatures of 20°C and above (Brooks & Hansford, 1923; Hesseltine & Anderson, 1957; Benny, 1995). The ability of mucoralean fungi to grow in these cold temperature habitats seems to be as a result of their ability to produce significant quantities of polyunsaturated fatty acids, which increase membrane fluidity at low temperatures (Manocha & Campbell, 1978; Botha *et al.*, 1999). Recently it was found that *Mortierella,* a genus that occurs in soil habitats and that may produce up to 20 % (w/w) polyunsaturated fatty acids in its phospholipids (Kendrick & Ratledge, 1992), can selectively be isolated from soil, when an isolation temperature of 5°C is used (Botha *et al.*, 1999).

1.2.3. Geographical distribution of *Mortierella* in southern Africa

Although it is known that *Mortierella* commonly occurs in soil the world over (Domsch *et al.*, 1980), records of representatives of this genus in southern African soils are relatively scarce. This, despite a number of surveys that were conducted on the soil fungi of indigenous vegetation types.

During a survey of soil fungi in a coastal forest situated in Kwazulu-Natal, it was found that dikaryomycotan genera such as *Penicillium*, *Trichoderma* and *Humicola*, as well as the mucoralean genus *Cunninghamella,* were the most common fungi present (Eicker, 1970). Other mucoralean genera that were found were *Absidia*, *Gongronella*, *Mortierella* and *Mucor.* Although it is generally known that species of *Mortierella* are commonly found in forest soils

(Warcup, 1951), *Mortierella isabellina* was the only species of this genus encountered in the survey by Eicker (1970). In addition, it was infrequently obtained from the soil.

In a study of fungi present in alkaline soil from open-savannah in the Pretoria district of Transvaal (Eicker, 1974), it was found that *Mucor spinosus* was the most abundant mucoralean species followed by *Cunninghamella echinulata*. Other mucoralean fungi that were found were representatives of *Absidia*, *Actinomucor*, *Gongronella*, *Mortierella* and *Zygorhynchus*. In a study of the microorganisms present in soil of the Giribes plains in northern Namibia it was found that *Aspergillus* and *Penicillium* were the most prevalent fungi, while 10% of the total number of fungal isolates were mucoralean fungi (Eicker *et al.*, 1982). The mucoralean genera were *Absidia*, *Cunninghamella* and *Rhizopus*. No representatives of *Mortierella* were encountered during this survey.

Opperman & Wehner (1994) conducted a survey of fungi associated with grass roots on the Springbok Flats. They found that the dikaryomycotan species *Periconia mamocrospinosa* and *Fusarium nygamai* were the most prevalent colonisers of the grass roots. *Gongronella* was the only mucoralean genus that was encountered during this study and it comprised only 1% of the total number of fungal isolates. As with the previous study, no representatives of *Mortierella* were found during the study.

Roux & Van Warmelo (1997) did a survey on microorganisms associated with plants and their roots, as well as leaf litter in a natural Karoo pasture near Middelburg in the Eastern Cape. During this survey 135 genera were isolated of which 46% represented hyphomycetes and 35% coelomycetes. The mucoralean isolates only comprised 4% of the total number of fungi isolated and belonged to the genera *Actinomucor*, *Cunninghamella*, *Mortierella*, *Mucor*, *Rhizopus* and *Rhizomucor*.

A study conducted on soil from Dry Sandy Highveld Grassland in an arid region in southern Africa revealed that 12% of the total number of fungal isolates were mucoralean fungi comprising of the genera *Mortierella*, *Mucor* and *Rhizopus*

(Strauss, 1997). These results, as well as the results of the previous surveys, were obtained on relatively non-selective media containing malt extract and/or yeast extract. When Strauss (1997) added benomyl to a series of isolation media with different carbon sources, *Absidia*, *Cunninghamella* and *Gongronella* were also found to be present in the soil sample from Dry Sandy Highveld Grassland.

Seabi *et al.* (1999) used non-selective as well as benomyl containing media to determine the fungi in soil from Upper Nama Karoo near De Aar in the Northern Cape. Only four mucoralean species were detected. *Actinomucor elegans* and *Rhizopus oryzae* were the most abundant followed by *Mortierella isabellina* and *Mucor circinelloides*.

It must be noted that all of the above surveys were conducted at temperatures ranging from 20°C to 30°C. Psychrotolerant fungi, able to grow at 5°C as well as at 25°C were not tested for. In a survey conducted by Paul (1999) on the psychrotolerant fungi present during mid-winter in soil from the Alti Mountain Grassland in Kwazulu-Natal, it was found that 92.4% of the total number of fungi obtained was *Mortierella* isolates while 6% were *Mucor* isolates. Altogether 20 *Mortierella* species were detected in a single soil sample. The most abundant identifiable *Mortierella* species in the soil was *Mortierella alpina* followed by *Mortierella antarctica* and *Mortierella basiparvispora*.

It therefore seems that the isolation temperatures used during most of the above mentioned surveys were bias against selection of psychrotolerant fungi such as members of *Mortierella*. In addition, it seems that the dominant fungal genus in soil at low temperatures, is *Mortierella.* This finding however must be confirmed by more studies on the psychrotolerant fungi in different soil types.

1.2.4. Characteristics of the genus *Mortierella*

The genus *Mortierella*, represents a group of ubiquitous saprotrophic soil fungi, that has been classified in the family Mortierellaceae, because of several

unusual features (De Ruiter *et al.*, 1993). The reproductive structures of this genus are generally more delicate than the reproductive structures of other members of the Mucorales. Some of the species also produce sporangia with indistinct or no columellae. These sporangia can be grouped into those that contain numerous, few or single spores. In the latter case these structures are known as sporangioles, especially if they contain only one or two spores (Domsch *et al.*, 1980; Fa-jun, 1992).

Another distinctive feature of some *Mortierella* species among the Mucorales, is the garlic-like odour of their branched mycelium (Domsch *et al.*, 1980; De Ruiter *et al.*, 1993). A further characteristic that is used to differentiate some *Mortierella* species from the rest of the Mucorales, are the cellular fatty acid and sterol compositions. Most Mucorales produce ergosterol, while desmosterol and the polyunsaturated fatty acid arachidonic acid are not produced (Weete & Gandhi, 1999). In contrast, it was found that some *Mortierella* species form a well-defined taxon within the Mortierellaceae, in that they not only share common morphological features, but also produce arachidonic acid and desmosterol. However, traditionally the taxonomy of *Mortierella* is not based on the cellular chemical composition, but on morphological features.

In 1863 Coemans established the genus *Mortierella* based on the morphology of the type species *Mortierella polycephala* Coem. (Fig. 1.6) and it was placed in the family Mucoraceae (Fa-jun, 1992; De Ruiter *et al.*, 1993). Later in 1875, Tieghem proposed the tribe Mortierellees of the family Mucoraceae to accommodate the genus Mortierella Coem., while in 1888 Berlese and Toni proposed the subfamily Mortierellae to include *Mortierella* Coem. and *Herpocladium* J. Schröt (Fa-jun, 1992). In 1892 Fisher raised this subfamily to the family Mortierellaceae (Fa-jun, 1992; De Ruiter *et al.*, 1993). Subsequently, the genus *Mortierella* was monographed by Linneman in 1941 and later again by Zycha *et al.* in 1970 (Domsch *et al.*, 1980). Zycha *et al.* considered this genus to compromise of 83 species, which are grouped into 11 sections. Currently the Mortierellaceae consists of over a hundred species and 7 genera, of which *Mortierella* Coem. is the major genus and contains species that are sometimes difficult to distinguish from each other (Hawksworth *et al.*, 1995).

Subsequently, for practical reasons, a taxonomic key was constructed for the genus *Mortierella* in which the species were grouped in two subgenera (Gams, 1977). *Mortierella* subgenus *Micromucor* W. Gams and *Mortierella* subgenus *Mortierella*, (Benjamin, 1979; Domsch *et al.*, 1980) are mainly distinguished from one another on the basis of morphological and cultural characteristics (Gams, 1977; Amano *et al.*, 1992). Members of the subgenus *Micromucor* are characterised by slow-growing, velvety colonies and pigmented sporangia with small columellae (Fig. 1.2). This subgenus contains several species with distinct *Mucor*-like characteristics. The true taxonomic position of the subgenus *Micromucor* however is uncertain because no sexual stage has been reported for this taxon (Gams, 1977; Benjamin, 1979; Domsch *et al.*, 1980; Amano *et al.*, 1992).

The subgenus *Mortierella* is characterised by the production of white cottony aerial hyphae and a thin spreading mycelium containing hyaline sporangia (Gams, 1977). These sporangia may also have no columellae. The mycelium of this subgenus has a distinctive garlic-like odour. Also, unlike *Mortierella* subgenus *Micromucor*, members of *Mortierella* subgenus *Mortierella* are characterised by the formation of arachidonic acid and desmosterol in the cellular lipids (Weete & Gandhi, 1999).

Gams (1977) recognised nine sections within *Mortierella* subgenus *Mortierella,* which can be distinguished from each other mainly on the basis of the morphology of the sporangiophores and sporangiospores. The first section, that he recognised, Section *Simplex* W. Gams, is characterised by unbranched sporangiophores exceeding 200 μm in length (Fig. 1.3), while Section *Alpina* Linnem. is characterised by shorter (less than 150 μm) sporangiophores with distinctive widening bases and sometimes many-spored sporangia (Fig. 1.4). The other section containing shorter unbranched sporangiophores, Section *Schmuckeri* W. Gams, is characterised by the formation of very slender sporangiophores arising in dense rows from aerial hyphae, each ending in a single-spored sporangium (Fig. 1.5). Members of the Section *Mortierella* Linnem. *emend.* W. Gams produce racemosely branched sporangiophores with

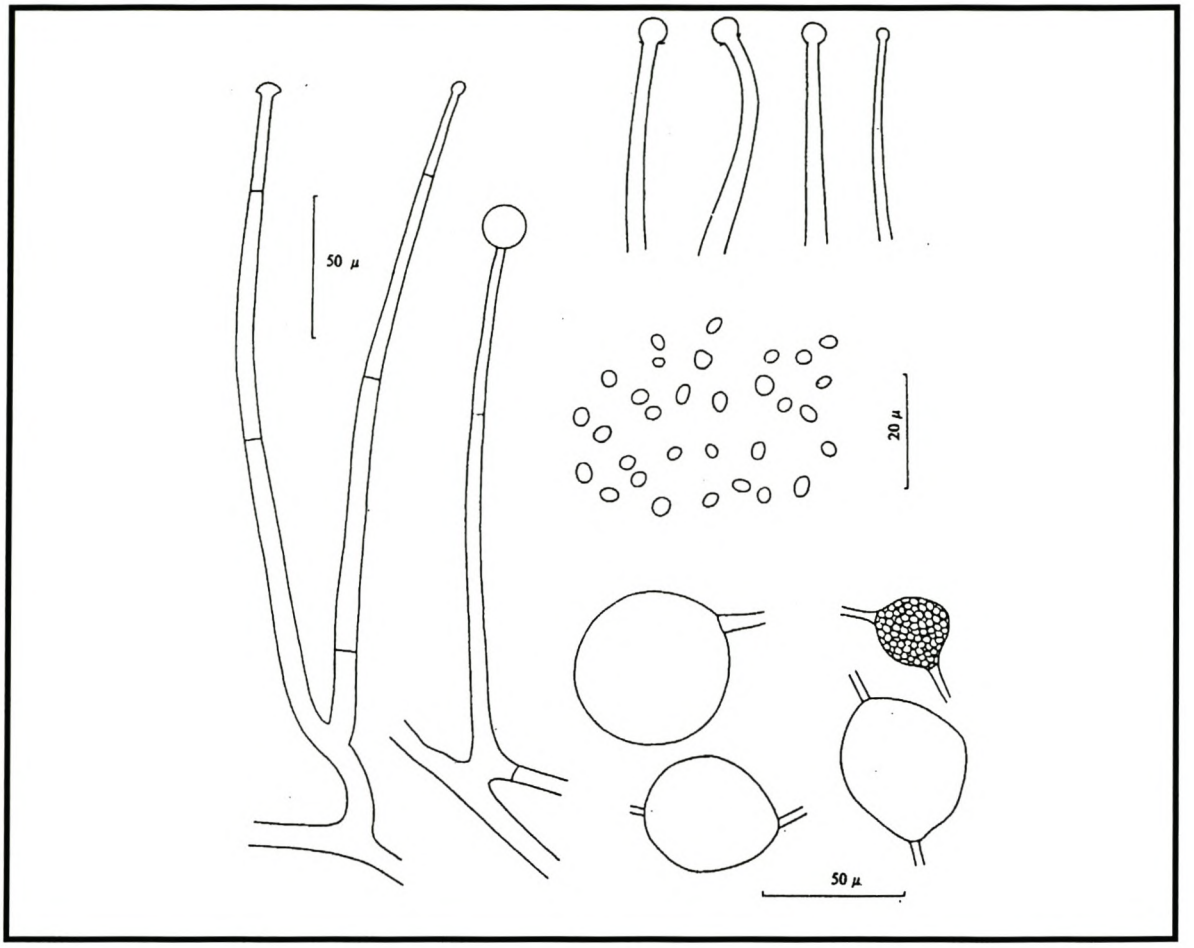

Figure 1.2. Morphological features of *Mortierella ramanniana* var. *ramanniana* (Möller) Linnem., a member of *Mortierella* subgenus *Micromucor*. The dimensions and morphology of sporangiophores, columellae, sporangiospores and chlamydospores are given (Fa-jun, 1992).

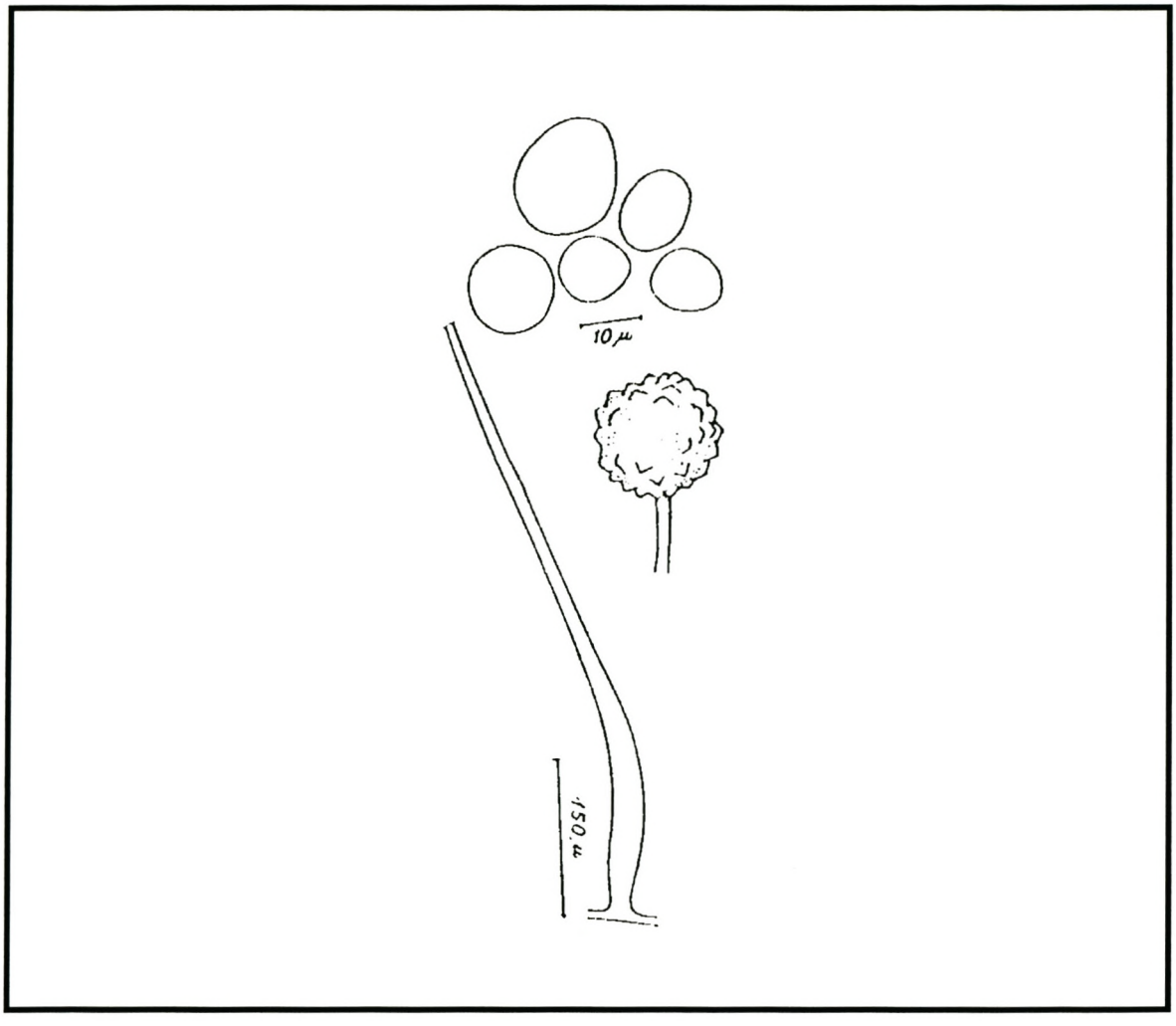

Figure 1.3. Morphological features of *Mortierella angusta* (Linnem.) Linnem., a member of *Mortierella* subgenus *Mortierella* Section *Simplex*. The dimensions of the sporangiospores, a chlamydospore and sporangiophore are illustrated (Zycha *et al.*, 1969).

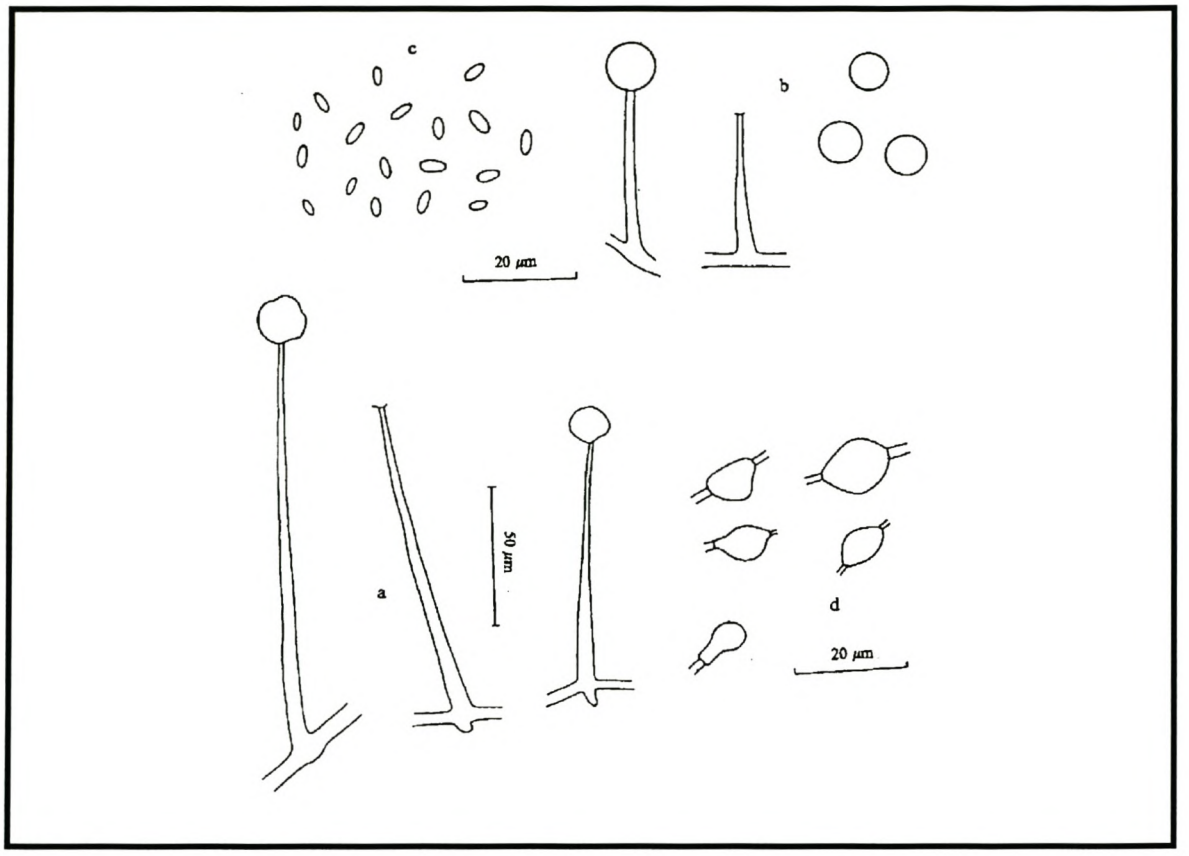

Figure 1.4. Morphological characteristics of *Mortierella alpina* Peyronel, a member of *Mortierella* subgenus *Mortierella* Section *Alpina*. The dimensions and morphology of the following structures are illustrated: (a) sporangiophores with sporangia, (b) deciduous single spored sporangiophores, (c) sporangiospores and (d) chlamydospores (Fa-jun, 1992).

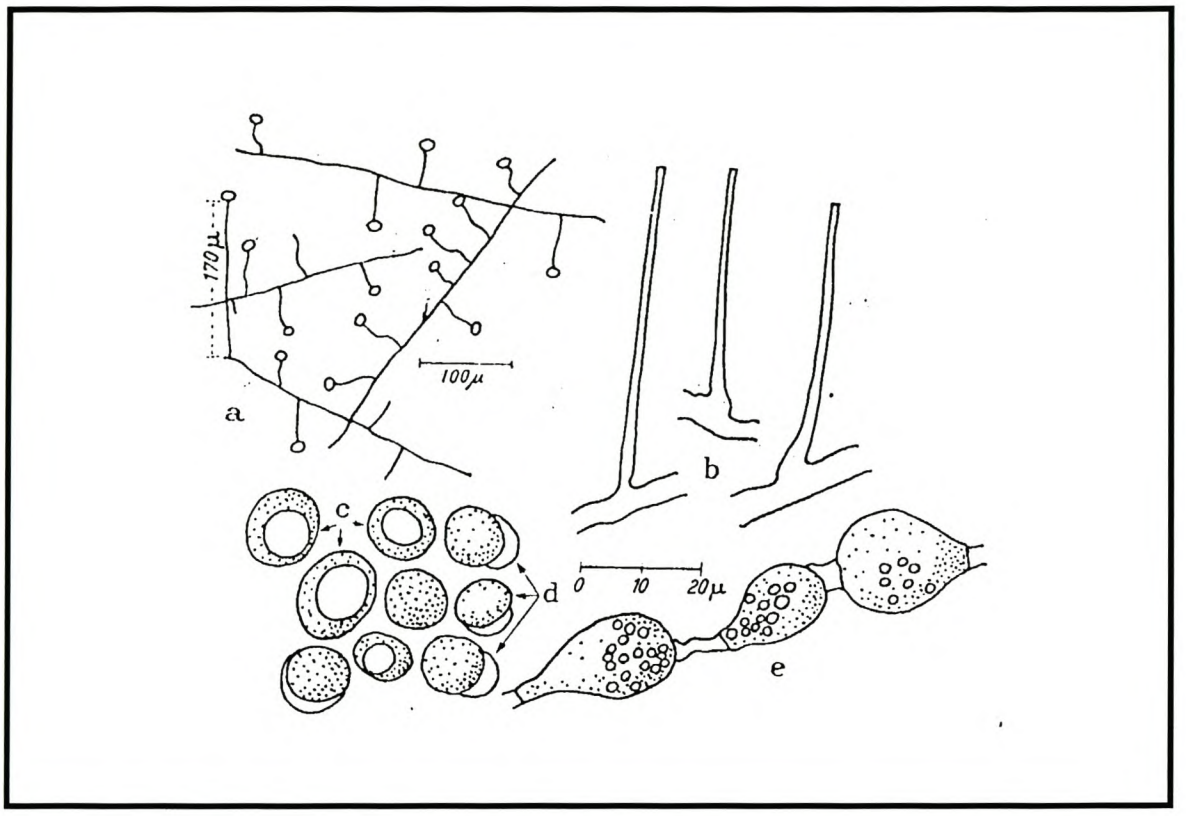

Figure 1.5. Morphological features of *Mortierella schmuckeri* Linnem., a member of *Mortierella* subgenus *Mortierella* Section *Schmuckeri*. The morphology and dimensions of the following structures are illustrated: (a) growth habit of sporangiophores, (b) sporangiophores, (c) young single spored sporangia with oil droplet in centre, (d) mature single spored sporangia with oil droplet on the exterior and (e) chlamydospores (Zycha *et al.*, 1969).

a thick main stem and thin, short branches arising from above the middle of the main stem (Fig. 1.6). In the Section *Actinomortierella* (Chalabuda) W. Gams, these short branches arise from an inflated region at the uppermost part of the sporangiophore (Fig. 1.7). The Section *Hygrophila* Linnem. is characterised by branches arising from the lower part of the sporangiophore, while each sporangiophore or branch terminates in a sporangium containing several sporangiospores (Fig. 1.8). Similarly, members of the Section *Stylospora* Linnem. produce sporangiophores with branches at the lower end, however, these sporangiophores end in single-spored sporangia (Fig. 1.9).

The last two sections recognised by Gams (1977) are both characterised by sporangiophores with branches arising from the middle or upper part of the sporangiophore. The sporangiophores in the Section *Spinosa* Linnem. are often bent upwards above an ascendant basal part and the many-spored sporangia may contain minute columellae (Fig. 1.10). While the sporangiophores of the Section *Haplosporangium* (Thax.) W. Gams are short with a broad base, strongly tapered in the middle part and arising in dense rows from aerial hyphae (Fig. 1.11). The sporangia of this last section are one- or two spored.

A problem in the classification of species within *Mortierella* subgenus *Mortierella* however, is the inability of many strains to produce sporangiophores, sporangia or even characteristic ornamented chlamydospores in culture (Gams, 1977). In these cases, only smooth-walled chlamydospores, or no chlamydospores at all are produced, making species identification impossible (Gams, 1977; Botha *et al.*, 1999). It is therefore not surprising that alternative taxonomic criteria are investigated to elucidate taxa within the Mucorales (Zhou *et al.*, 1991).

1.3. Molecular sequence analyses in the taxonomy of the Mucorales

As indicated above, difficulties may be encountered in the identification of some mucoralean fungi (Zhou *et al.*, 1991). In addition, classical criteria that are used

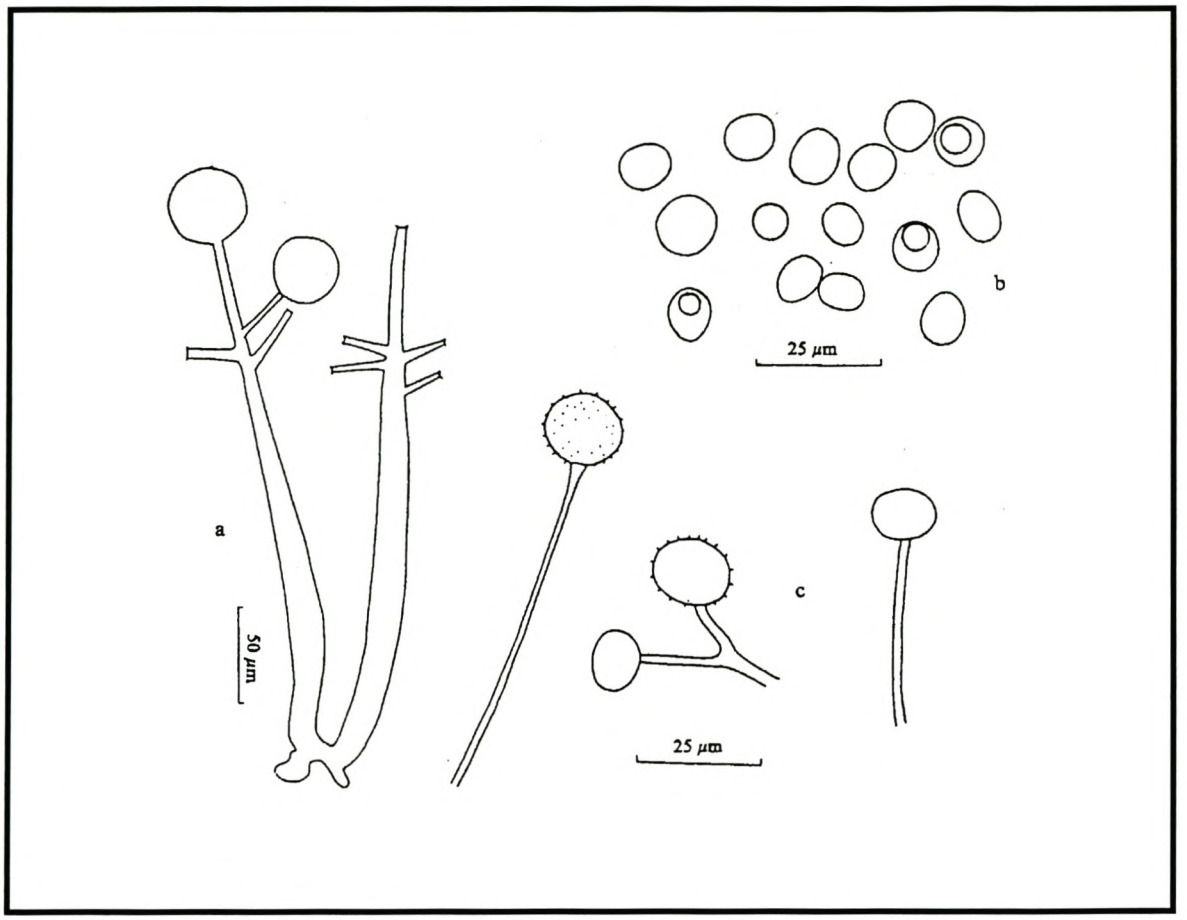

Figure 1.6. Morphological characteristics of *Mortierella polycephala* Coem., a member of *Mortierella* subgenus *Mortierella*, Section *Mortierella*. The morphology and dimensions of the following structures are illustrated: (a) sporangiophores, (b) sporangiospores and c) stylospores (Fa-jun, 1992).

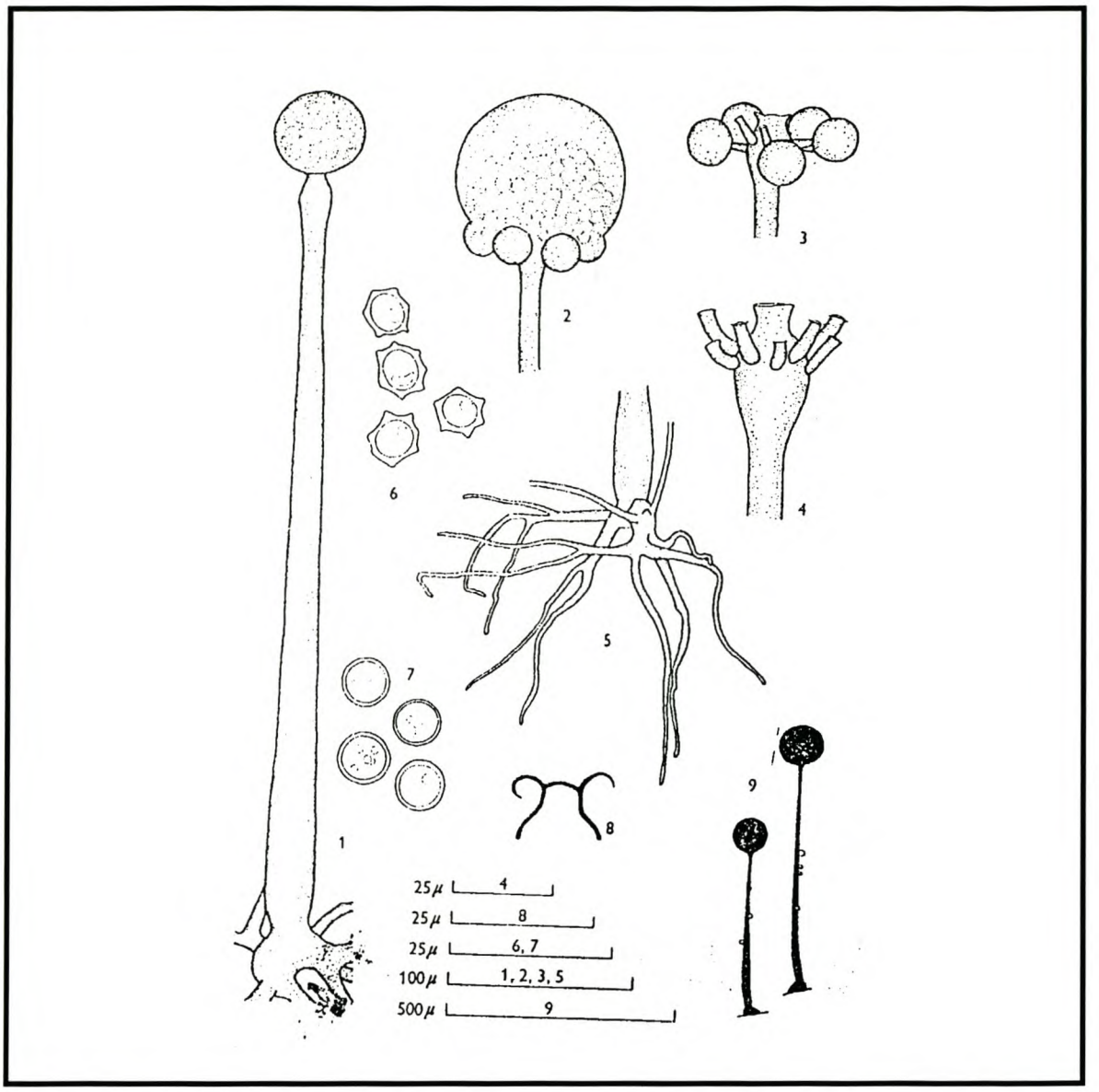

Figure 1.7. Morphological features of *Mortierella capitata* March., a typical member of *Mortierella* Section *Actinomortierella*. 1 - 4. Primary sporangium and the development of secondary sporangia. 5. Base of sporangiophore. 6 - 7. Sporangiospores dried and swollen. 8. Inflated region at the tip of the sporangiophore with remnants of the primary sporangium. 9. Sporangiophores with primary and secondary sporangia of which the walls had merged to produce a single watery structure containing the sporangiospores (Zycha *et al.*, 1969).

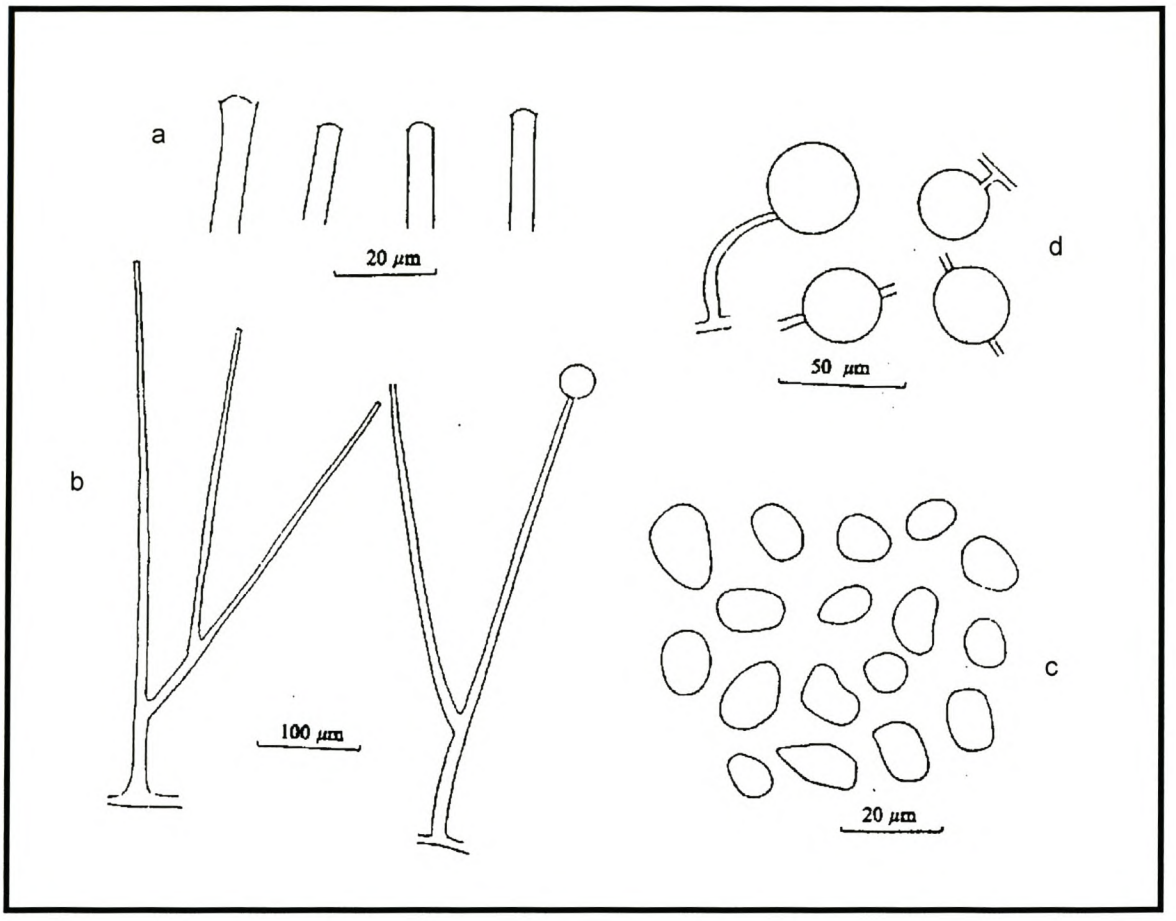

Figure 1.8. Morphological features of *Mortierella gemmifera* M. '*Ellis*', a member of *Mortierella* subgenus *Mortierella* Section *Hygrophila* illustrating (a) the tips of sporangiophores, (b) sporangiophores, (c) sporangiospores and (d) chlamydospores (Fa-jun, 1992).

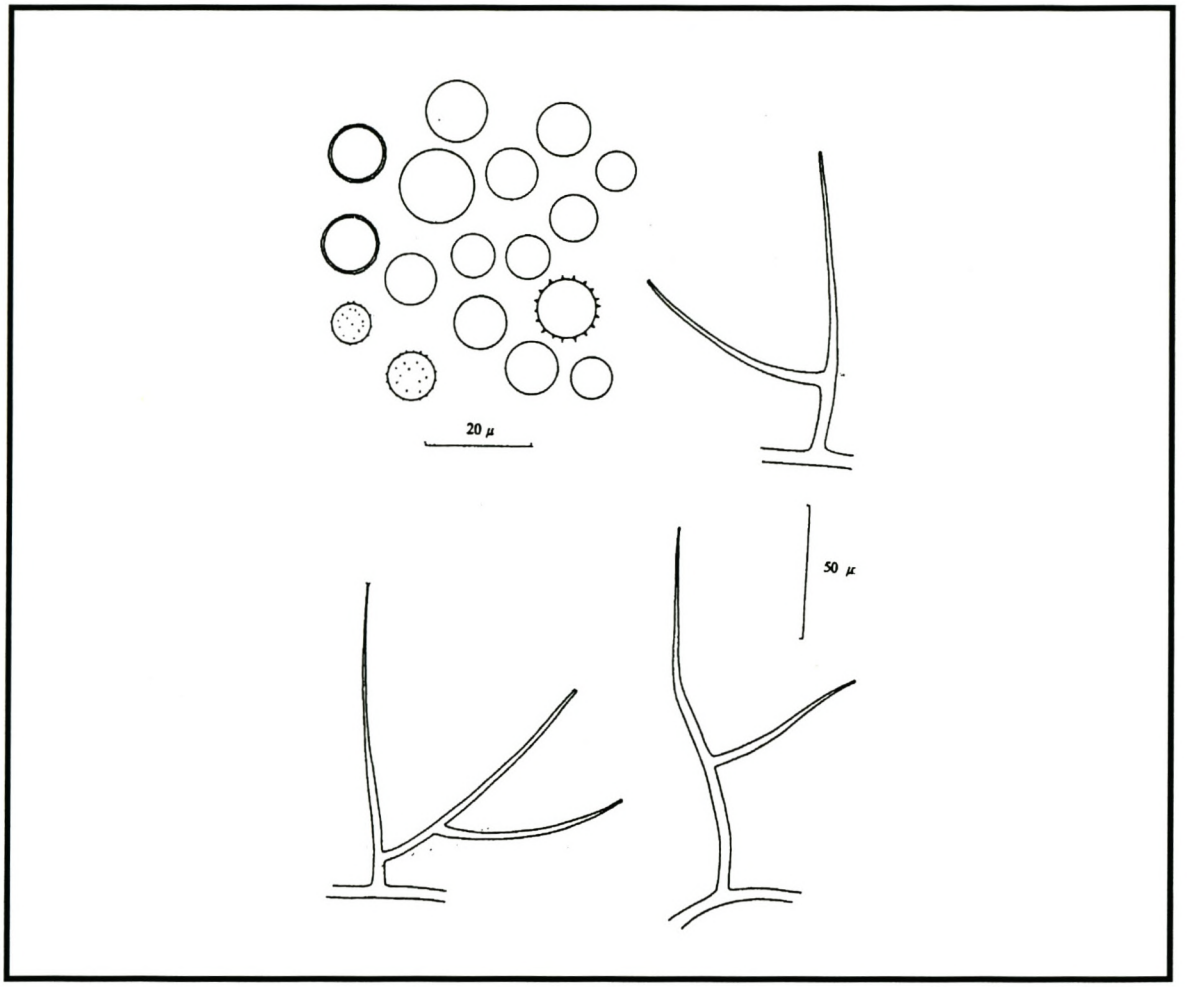

Figure 1.9. Morphology of *Mortierella humilus* Linnem. a member of *Mortierella* subgenus *Mortierella* Section *Stylospora*. Single spored sporangia and sporangiophores are illustrated (Fa-jun, 1992).

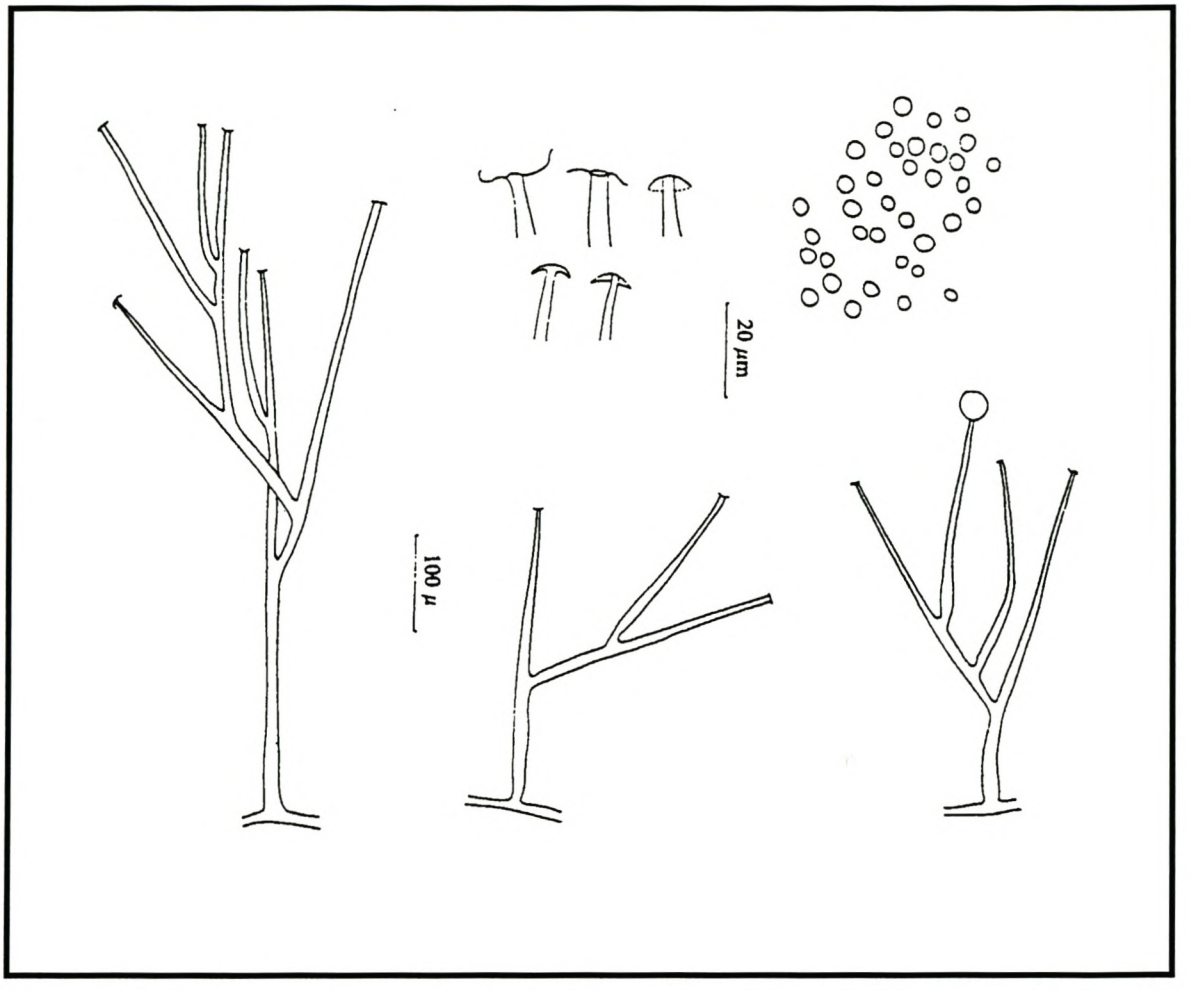

Figure 1.10. Morphological features of *Mortierella parvispora* Linnem., a member of *Mortierella* subgenus *Mortierella* Section *Spinosa*. The morphology of the sporangiophores, containing minute columellae and sporangiospores are illustrated (Fa-jun, 1992).

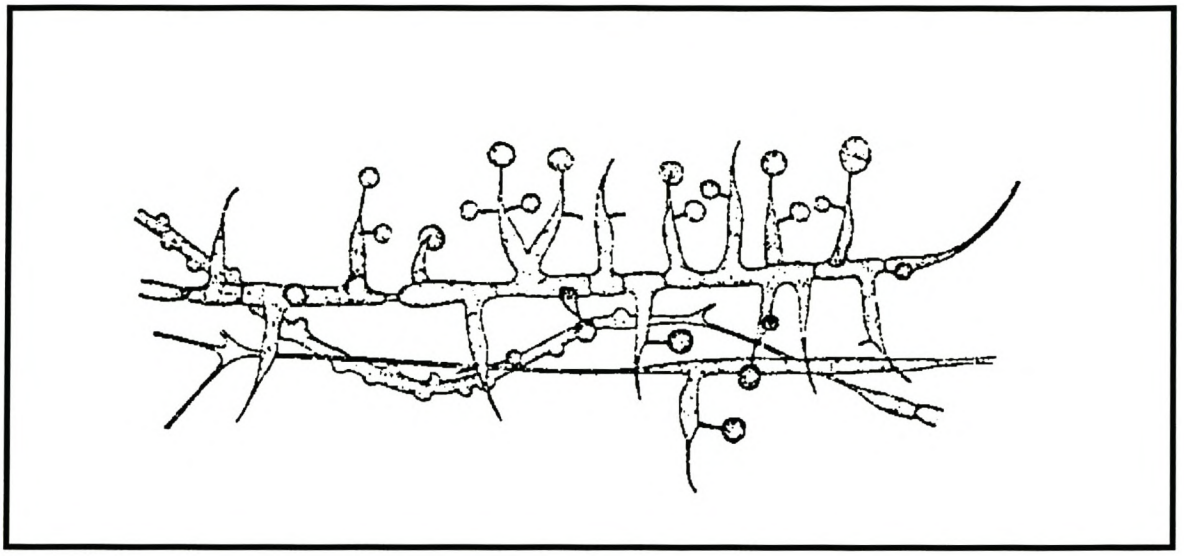

Figure 1.11. The morphology of sporangiophores belonging to *Mortierella bisporalis* (Thaxt.) Björling, a member of *Mortierella* subgenus *Mortierella* Section *Haplosporangium* (Zycha *et al.*, 1969).

for identification, such as morphology of reproductive structures and temperature range of growth (Schipper, 1978), may sometimes be unstable and overlapping. Consequently, DNA-based molecular techniques, such as comparison of the base composition of nuclear DNA, reassociation of nuclear DNA, restriction pattern analyses of the mitochondrial DNA and sequence analyses of the ribosome RNA have been explored by some fungal taxonomist to differentiate between taxa (Zhou *et al.*, 1991). Although currently not generally used in the taxonomy of the Mucorales, some of these techniques show potential to identify mucoralean fungi relatively fast and accurately (Voigt *et al.*, 1999).

A comprehensive DNA sequence database was developed that includes all medically important Zygomycetes including the Mucorales (Voigt *et al.*, 1999). This database was constructed from the nuclear small-subunit (18S) ribosomal DNA and domains D1 and D2 of the nuclear large-subunit (28S) ribosomal DNA. Parsimony analysis of the aligned 18S and 28S DNA sequences was used to investigate phylogenetic relationships among 42 isolates representing different zygomycetous fungi including mucoralean species. The results indicated that except for *Mortierella* subgenus *Mortierella* and the species *Echinosporangium transversale*, the order Mucorales comprises a monophyletic group within the Zygomycetes. Interestingly, these findings support the results of cellular fatty acid and sterol analyses, as well as morphological studies which indicate that *Mortierella* subgenus *Mortierella* occupies a rather isolated taxonomic position within the Mucorales (Weete & Gandhi, 1999; See also "1.2.4. Characteristics of the genus *Mortierella*"). Voigt *et al.* (1999) used aligned 28S sequences to design 13 taxon-specific polymerase chain reaction (PCR) primer pairs for those zygomycetous and mucoralean taxa most commonly implicated in infections. They found that these primers have potential to be used in a PCR assay for the rapid identification of the etiological agents of mucormycoses and entomophthoromycoses. However, Voigt *et al.* (1999) found that the ribosomal DNA -based database they used was not robust enough to resolve closely related species within the genera *Mucor, Rhizopus* and *Cunninghamella*. Consequently, they proposed that the sequences of

intron-containing nuclear genes should be investigated in an attempt to resolve species within these genera.

In an attempt to delimitate species on the basis of genome structure, contour clamped homogenous electric field (CHEF) gel electrophoresis was used to obtain electrophoretic karyotypes from nine *Mucor* strains representing the following species: *M*ucor *bainieri*, *Mucor circinelloides*, *Mucor mucedo*, *Mucor plumbeus* and *Mucor racemosus* (Nagy *et al.*, 2000). High variability was found in the chromosomal banding patterns. The sizes of the DNA in the *Mucor* chromosomes were approximately between 2.5 and 8.7Mb, whereas the total genome sizes were calculated to be between 30.0 and 44.7Mb (Nagy *et al.*, 2000).

Studies on the guanine and cytosine (GC) contents of mucoralean fungal DNA have shown that there may be a relation between GC content and the taxonomic position of a fungus (Zhou *et al.*, 1991). Analyses of the GC content of 60 mucoralean isolates belonging to 40 species, 16 genera and 6 families revealed that the GC content is relatively constant among certain species. The 16 genera were grouped into 3 distinct groups according to their average GC contents. *Gongronella*, *Haplosporangium*, *Mortierella* and *Syncephalastrum* belonged to the group with the highest value (43.9 - 48.8 %). *Cunninghamella* belonged to the group with the lowest value (28.8%), while genera such as *Absidia*, *Mucor* and *Rhizopus* gave GC values in between (38.9 - 43.8%). Interestingly, *Syncephalastrum racemosum* was considered to be the only species in the genus *Syncephalastrum* until *Syncephalastrum monosporum* was described. *Syncephalastrum monosporum* differs from *S. racemosum* in that the merosporangia are monosporous instead of multisporous. GC analyses of the DNA of *S. racemosum* and *S. monosporum* revealed that their GC values are relatively close, supporting the inclusion of both species in a single genus (Zhou *et al.*, 1991).

A useful tool in studying phylogenetic relationships among species in a particular genus is comparison of the variable internal transcribed spacer (ITS) regions, within the ribosomal DNA (Hseu *et al.*, 1996; de Koker, 2000). This

method has been used with success to study phylogenetic relationships within the Dikaryomycota, but according to our knowledge no studies have been conducted on mucoralean fungi. The role of ITS regions in studying phylogenetic relationships are known but, what are ITS regions and what are the role of these sequences in the cell?

1.3.1. Fungal internal transcribed spacer (ITS) regions

To understand the function of ITS regions, it is first necessary to discuss the location of these regions within the ribosomal RNA repeats (White *et al.*, 1990). Ribosomal DNA genes are tandem repeatedly multigene families, which contain genic and nongenic, or spacer regions. The rDNA repetitive units have highly conserved DNA sequences as well as more variable sequence regions (Kasuga *et al.*, 1993). The repetitive units each contain a copy of the 18S- (nuclear small-subunit), 5.8S- and 28S-like rDNA (nuclear large-subunit) and an internal transcribed spacer (ITS) and an intergenic spacer (IGS) that consists of an externally transcribed spacer (ETS) and a non-transcribed spacer (NTS) (O' Donnell, 1992). The 5.8S gene is flanked by an ITS at each side which separates the 5.8S rDNA from the 18S and 28S genes (White *et al.*, 1990; Fig. 1.12).

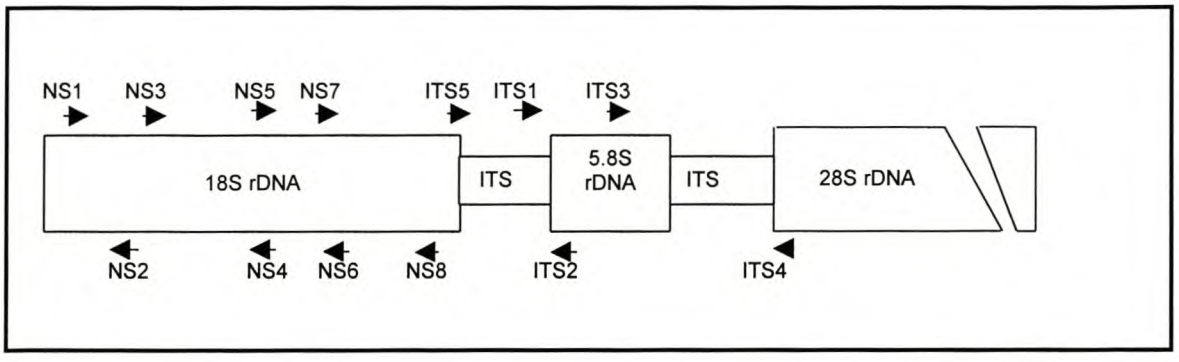

Figure 1.12. Structure of a nuclear rDNA repetitive unit. Adapted from White *et al.* (1990). Primers NS1-NS8 and ITS1-ITS5 are used for amplifying nuclear rDNA. All the odd numbered primers are 5' primers and the even numbered primers are 3' primers.

ITS regions are known to play an important role in the processing and maturation of rRNA (Lalev & Nazar, 1998). In eukaryotes rDNA maturation requires the accurate removal of two external (5' ETS and 3' ETS) and two internal (ITS1 and ITS2) transcribed spacers (Melekhovets *et al.*, 1994; Good *et al.*, 1997). rRNA processing is proposed to be a cleavage pathway in which many fast and independent steps take place. During these steps the ETS regions seem to be removed, after which the small and large subunit sequences are separated by a cleavage within the ITS1. These sequences are then processed and trimmed to form the mature ribosomal subunits (Good *et al.*, 1997).

By using targeted mutagenesis and *in vivo* expression of "tagged" rRNA it was found that there are specific regions in the higher order structure of internal transcribed spacer sequences (ITS1 and ITS2), which are essential for intragenic processing (Melekhovets *et al.*, 1994). Similarly, a study on *Schizosaccharomyces pombe* was done to analyse the effects of mutations in the ITS region by using an efficiently expressed rDNA plasmid. The results revealed that processing of distant external transcribed spacers (ETS) is inhibited if substitution of ITS regions take place. Furthermore it was found that deletion of the ITS2 region prevents the maturation of the large subunit as well as the small subunit rRNA. This is an indication that rRNA processing is a co-operative process, which is sensitive to widely, distributed structural domains in the precursor molecule (Good *et al.*, 1997).

By using the polymerase chain reaction (PCR) and DNA primers (Fig. 1.12) specific for conserved 18S and 28S elements, the ITS region can be amplified and used to produce characteristic DNA fragments from yeasts and fungi (Kasuga, *et al.*, 1993). Variation may occur in the ITS region and the IGS of the nuclear rRNA repeat units of species within a genus, making it useful for analysing phylogenetic relationships (White *et al.*, 1990). Nucleotide sequence analyses of the ITS2 region of the pathogenic yeast species *Candida albicans* revealed that this species can be distinguished from strains of *Candida parapsilosis* and *Candida tropicalis* on the basis of the ITS sequences (Lott, *et al.*, 1993). Similarly Klittich *et al.* (1997) found that differences may exists in the

ITS regions of two morphologically similar filamentous fungal species, *Fusarium thapsinum* (teleomorph: *Gibberella thapsina*) and *Fusarium moniliforme* (teleomorph: *Gibberella fujikuroi* mating population A). A similar study on 86 *Fusarium sambucinum* (teleomorph: *Giberella pulicaris*) strains isolated from different geographical regions and substrates, however revealed that ITS sequence analyses can not always be used to characterise *Fusarium* species (O'Donnell, 1992).

It is generally known that the amplified DNA fragment from different fungal species originating from different geographical areas may vary in base sequence and in length (White *et al.*, 1990; Kasuga *et al.*, 1993). Phylogenetic analyses of the ITS sequences of isolates within an anastomosis group 4 of *Rhizoctonia solani* suggest that the isolates can be divided into three clusters that correlate with habitat and virulence (Boysen *et al.*, 1996). This raises the question whether fungi belonging to different but closely related taxa originating from the same habitat, will group together when the ITS regions of different isolates are compared.

1.4. Purpose of study

With the above as background the purpose of this study was first to determine the diversity of psychrotolerant mucoralean fungi present in vineyard soil and pristine Mountain Fynbos (Low & Rebelo, 1996) soil from the same geographical region in the Western Cape, South Africa (Chapter 2). Secondly, the phylogenetic relationship between different psychrotolerant isolates of *Mortierella* subgenus *Mortierella* was determined through comparison of ITS regions, within ribosomal RNA repeats (Chapter 3).

1. 5. References

Alexander, M. (1961). *Introduction to soil microbiology*. New York: John Wiley & Sons, Inc.

Alexopoulus, C.J. & Mims, C.W. (1979). *Introductory Mycology*. Third Edition. New York: John Wiley & Sons, Inc.

Allsopp, N., Olivier, D.L. & Mitchell, D.T. (1987). Fungal populations associated with root systems of proteaceous seedlings at a lowland fynbos site in South Africa. *South African Journal of Botany* **53,** 365 – 369.

Amano, N., Shinmen, Y., Kawashimao, K.A.H. & Amachi, T. (1992). Chemotaxonomic significance of fatty acid composition in the genus *Mortierella* (Zygomycetes, Mortierellaceae). *Mycotaxon* **XLIV(2),** 257 – 265.

Bartschi, C., Berthier, J., Guiguettaz, C. & Valla, G. (1991). A selective medium for the isolation and enumeration of *Mucor* species. *Mycological Res*earch **95,** 373 – 374.

Benjamin, R.K. (1979). Zygomycetes and their spores. In: B. Kendrick (Ed.) *The whole fungus* (pp. 573 – 621). National museum of Natural Sciences, Canada.

Benny, G.L. (1995). Observations on Thamnidiaceae VII. Two new species of *Helicostylum* and a new genus *Kirkia*. *Mycologia* **87,** 253 – 264.

Benny, G.L. & Benjamin, R.K. (1975). Observations on Thamnidiaceae (Mucorales). New taxa, new combinations, and notes on selected species. *Aliso* **8,** 301 – 351.

Benny, G.L. & Benjamin, R.K. (1993). Observations on Thamnidiaceae (Mucorales). VI. Two new species of *Dichotomocladium* and the zygospores of *D. hesseltinei* (Chaetocladiaceae). *Mycologia* **85,** 660 – 671.

Bokhary, H.A. & Parvez, S. (1991). Soil mycology from wild desert truffle habitats in northern Saudi Arabia. *Arid Environments* **23,** 379 – 388.

Botha, A. & du Preez, J.C. (1999). *Mucor.* In: R. Robinson, C. Batt & P. Patel (Eds.) *Encyclopedia of Food Microbiology* (pp. 1493 - 1500). Academic Press Ltd., London.

Botha, A., Kock, J.L.F., Roux, C., Coetzee, D.J. & Botes,P.J. (1995). An isolation medium for gamma-linolenic acid producing mucoralean fungi. *Systematic and Applied Microbiology* **18,** 448 – 454.

Botha, A., Paul, I., Roux, C., Kock, J.L.F., Coetzee, D.J., Strauss, T. & Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* **75,** 253 – 256.

Botha, A., Roux, C., Botha, A.J., Kock, J.L.F. & Pohl, C.H. (1997a). Notes on the physiology and morphology of *Thamnostylum piriforme* isolated for the first time in South Africa. *South African Journal of Botany* **63,** 104 – 108.

Botha, A., Strauss, T., Kock, J.L.F., Pohl, C.H. & Coetzee, D.J. (1997b). Carbon source utilisation and γ-linolenic acid production by mucoralean fungi. *Systematic and Applied Microbiology* **20,** 165 - 170.

Boysen, M., Borja, M., del Moral, C., Salazar, O. & Rubio, V. (1996). Identification at strain level of *Rhizoctonia solani* AG4 isolates by direct sequence of asymmetric PCR products of the ITS regions. *Current Genetics* **29,** 174 - 181.

Brooks, F.T. & Hansford, C.G. (1923). Mold growth on cold-store meat. *Transactions British Mycological Society* **8,** 113 - 142.

Carreiro, M.M. & Koske, R.E. (1992). Room temperature isolations can bias against selection of low temperature microfungi in temperate forest soils. *Mycologia* **84,** 886 - 900.

Cruz, P.M., Christen, P. & Farres, A. (1993). Medium optimisation by a fractional factorial design for lipase production by *Rhizopus delemar*. *Journal of Fermentation and Bioengineering* **76,** 94 – 97.

de Koker, T.H. (2000). The genetic and enzymatic characterization of South African strains of *Phanerochaete chrysosporium*. Ph.D. dissertation. Department of Microbiology, Faculty of Science, University of Stellenbosch, South Africa.

De Ruiter, G.A., Van Bruggen-Van Der Lugt, A.W. & Rombouts, F.M. (1993). Approaches to the classification of the *Mortierella isabellina* group: antigenic to extracellular polysaccharides. *Mycological Research* **97(6),** 690 – 696.

Díaz-Minguez, J.M. & López-Matas, M.A. (1999). Complementary mating types of *Mucor circinelloides* show electrophoretic karyotype heterogeneity. *Current Genetics* **36,** 383 - 389.

Domsch, K.H., Gams, W. & Anderson, T-H. (1980). *Compendium of soil fungi* (pp. 431 - 460). Academic Press, London.

Eicker, A. (1969). Microfungi from surface soil of forest communities in Zululand. *Transactions British Mycological Society* **53(3),** 381 - 392.

Eicker, A. (1970). Ecological observations on soil fungi. *South African Journal of Science* **66,** 327 – 334.

Eicker, A. (1974). The mycoflora of an alkaline soil of the Open-Savannah of the Transvaal. *Transactions British Mycological Society* **63,** 281 - 288.

Eicker, A., Theron, G.K. & Grobbelaar, N. (1982). 'n Mikrobiologiese studie van 'kaal kolle' in die Giribesvlakte van Kaokoland, S.W.A.-Namibië. *South African Journal of Botany* **1,** 69 – 74.

Eroshin, V.K., Dedyukhina, E.G., Christyakova, T.I., Zhelifonova, V.P. & Bothast, R.J. (1996). Studies on arachidonic acid production by *Mortierella* fungi: A microbial method for selecting arachidonic acid producers. *Microbiology* **65(1),** 26 – 31.

Evans, H.C. & Samson, R.A. (1977). *Sporodinella umbellata*, an entomofungus of the Mucorales from cocoa farms in Ecuador. *Canadian Journal of Botany* **55,** 2981 – 2984.

Fa-jun, C. (1992). *Mortierella* species in China. *Mycosystema* **5,** 23 – 64.

Gams, W. (1977). A key to the species of *Mortierella*. *Persoonia* **9,** 381 – 391.

Good, L., Intine, R.V.A. & Nazar, R.N. (1997). Interdependence in the processing of ribosomal RNAs in *Schizosaccharomyces pombe*. *Journal of Molecular Biology* **273,** 782 - 788.

Guiraud, P., Steinman, R., Seigle-Murandi, F. & Sage, L. (1995). Mycoflora of soil around the Dead Sea II – Deuteromycetes (except *Aspergillus* and *Penicillium*). *Systematic and Applied Microbiology* **18,** 318 – 322.

Hansson, L. & Dostalek, M. (1988). Effect of culture conditions on mycelial growth and production of linolenic acid by the fungus *Mortierella ramanniana*. *Applied Microbiology and Biotechnology* **28,** 240 - 246.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. & Pegler, D.N. (1995). *Dictionary of the fungi*. Eight edition. University Press, Cambridge.

Hayes, D.G. & Gulari, E. (1992). Formation of polyol-fatty acid esters by lipases in reverse micellar medium. *Biotechnology and Bioengineering* **40,** 110 – 118.

Hering, L., Bisping, B. & Rehm, H.J. (1991). Patterns and formation of fatty acids at Tempe fermentation by several strains of *Rhizopus sp.* *Fat Science Technology* **93,** 819 - 830.

Hesseltine, C.W. (1965). A millennium of fungi, food and fermentation. *Mycologia* **57,** 1 - 148.

Hesseltine, C.W. & Anderson, P. (1957). Two genera of molds with low temperature growth requirements. *Bulletin Torrey Botanical Club* **84,** 31-45.

Hesseltine, C. W. & Ellis, J.J. (1973). Mucorales. In: G.C. Ainsworth, F.K. Sparrow & A.S. Sussman (Eds.) *The Fungi*, **vol. 4B** (pp. 187-216). Academic Press, London.

Hseu, R.-S., Wang, H.-H., Wang, H.-F. & Moncalvo, J.-M. (1996). Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Applied and Environmental Microbiology* **62,** 1354 - 1363.

Jannasch, H.W. (1998). Death sentence for a misnomer. *ASM News* **64,** 185 – 185.

Kasuga, T., Woods, C., Woodward, S. & Mitchelson, K. (1993). *Heterobasidion annosum* 5.8s ribosomal DNA and internal transcribed spacer sequence: rapid identification of European intersterility groups by ribosomal DNA restriction polymorphism. *Current Genetics* **24,** 433 – 436.

Kendrick, A. & Ratledge, C. (1992). Lipids of selected moulds grown for production of n-3 and n-6 polysaturated fatty acids. *Lipids* **27(1),** 15 – 20.

Klittich, C.J.R., Leslie, J.F., Nelson, P.E. & Marasas, W.F.O. (1997). *Fusarium thapsinum* (*Gibberella thapsina*): A new species in section *Liseola* from sorghum. *Mycologia* **89,** 643 – 652.

Kolte, S.J. (1985). Disease of Annual Edible Oilseed Crops vol. 3, Sunflower, Safflower and Nigerseed Diseases. CRC Press Inc., Florida.

Lalev, A.I. & Nazar, R.N. (1998). Conserved core structure in the internal transcribed spacer 1 of the *Schizosaccharomyces pombe* precursor ribosomal RNA. *Journal of Molecular Biology* **284,** 1341 – 1351.

Lott, T.J., Randall, J., Kuykendall, R.J. & Reiss, E. (1993). Nucleotide sequence analysis of the 5.8S rDNA and adjacent ITS2 region of *Candida albicans* and related species. Yeast **9,** 1199 - 1206.

Low, A.B. & Rebelo, A.G. (1996). *Vegetation of South Africa, Lesotho and Swaziland.* Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Manocha, M.S. & Campbel, C.D. (1978). The effect of growth temperature on the fatty acid composition of *Thamnidium elegans* Link. *Canadian Journal of Microbiol*ogy **24,** 670 – 674.

Marshall, D.H., Brownstein, S., Jackson, W.B., Mintsioulis, G., Gilberg, S.M. & al-Zeerah, B.F. (1997). Post-traumatic corneal mucormycosis caused by *Absidia corymbifera*. *Opthalmology* **104(7),** 1107 - 1111.

Melekhovets, Y.F., Good, L., Elela, S.A. & Nazar, R.N. (1994). Intragenic processing in yeast rRNA is dependant on the 3' external transciber. *Journal of Molecular Biology* **239,** 170 - 180.

Michailides, T.J., Morgan, D.P., Spotts, R.A., Beglinger, C. & Odiet, P.A. (1992). Role of nitidulid beetles and vinegar flies in the sexual cycle of *Mucor piriformis* in tree fruit orchards. *Mycologia* **84,** 488 - 496.

Nagy, A., Palagyi, Z., Vastag, M., Ferenczy, L. & Vagvolgyi, C. (2000). Electrophoretic karyotypes of some related *Mucor* species. *Antonie Van Leeuwenhoek* **78(1),** 33 – 37.

O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* **22,** 213 - 220.

Opperman, L. & Wehner, F.C. (1994). Survey of fungi associated with grass roots in virgin soils on the Springbok Flats. *South African Journal of Botany* **60(1),** 67 – 72.

Paul, I. (1999). An isolation procedure for arachidonic acid producing mucoralean fungi. M.Sc. Thesis, Department of Microbiology and Biochemistry, Faculty of Science, University of the Orange Free State, Bloemfontein, South Africa.

Pohl, C.H. (1999). Lipid metabolism in *Mucor genevensis* and related species. Ph.D. dissertation. Department of Microbiology and Biochemistry. Faculty of Science. University of the Orange Free State, Bloemfontein, South Africa.

Ratledge, C. (1989). Biotechnology of fats. In: C. Ratledge & S.G. Wilkinson (Eds.) *Microbial lipids*, **vol. 2** (pp. 567 - 668). Academic Press, London.

Ross, I.K. (1979). *Biology of the fungi.* McGraw-Hill, Inc., USA.

Roux, C. & Van Warmelo, K.T. (1997). A survey of the mycobiota of a natural Karoo pasture. *Bothalia* **27(2),** 167 - 183.

Schipper, M.A.A. (1978). On certain species of *Mucor* with a key to all accepted species. *Studies in Mycology* **17,** 1 – 52.

Seabi, B.O., Botha, A., Viljoen, B.C. & Roux, C. (1999). Nitrogen utilisation and growth at reduced water activity by mucoralean fungi present in soil. *South African Journal of Botany* **65(5&6),** 407 - 413.

Spotts, R.A. & Cervantes, L.A. (1986). Populations of *Mucor piriformis* in soil of pear orchards in the Hood River valley of Oregon. *Plant Disease* **70,** 935 – 937.

Steinman, R., Guiraud, P., Sage, L., Seigle-Murandi, F. & Lafond, J-L. (1995). Mycroflora of soil around the Dead Sea, I - Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. *Sytematic and Applied Microbiology* **18,** 310 - 317.

Strauss, T. (1997). The isolation of gamma-linolenic acid producing mucoralean fungi. M.Sc. Thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Streekstra, H. (1997). On the safety of *Mortierella alpina* for the production of food ingredients, such as arachidonic acid. *Journal of Biotechnology* **56,** 153 - 165.

Talaro, K. & Talaro, A. (1993). Environmental and applied microbiology. In: *Foundations in Microbiology* (pp. 711 – 761). Wm.C. Brown Publishers, USA.

Tsuchiura, O.S. & Sakura, T.Y. (1988). Method for the preparation of a fungal body and a lipid rich in γ-linolenic acid therefrom. *U.S. Patent number* 4,783,408.

Voigt, K., Cigelnik, E. & O' Donnell, K. (1999). Phylogeny and PCR identification of clinically important Zygomycetes based on nuclear ribosomal-DNA sequence data. *Journal of Clinical Microbiology* **37(12),** 3957 - 3964.

Warcup, J.H. (1950). The soil-plate method for isolation of fungi from soil. *Nature* **166,** 117 – 118.

Warcup, J.H. (1951). The ecology of soil fungi. *Transactions British Mycological Society* **34,** 376 – 399.

Weete, J.D. & Ghandi, S.R. (1999). Sterols and fatty acids of the Mortierellaceae: Taxonomic applications. *Mycologia* **91(4),** 642 – 649.

Weiss, J.D. (1983). *Oilseed Crops.* Longman, London.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.) *PCR Protocols* (pp. 315 - 322). San Diego, Academic Press.

Zabel, D.D. (1997). Mycotic infection with mucormycosis. *Delaware Medical Journal* **69(9),** 459 - 464.

Zhou, Z-w., Huang, H. & Zheng, R-y. (1991). Base composition of DNA and classification of the Mucorales. *Mycosystema* **4,** 1 - 14.

Zycha, H., Siepmann, R. & Linnemann, G. (1969). Mucorales, Eine besschreibung aller gattungen und arten dieser pilzgruppe. Verlag Von J. Cramer, Lehre.

CHAPTER 2

EXPLORING THE SPECIES DIVERSITY OF PSYCHROTOLERANT MUCORALEAN FUNGI IN SOUTHERN AFRICAN SOILS

2.1. Introduction

Mucoralean fungi are mostly saprotrophs that are frequently encountered in soil habitats, although they may also occur on dung and decaying fruit (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Bokhary & Parvez, 1991; Brock *et al.*, 1994). These fungi were isolated from soil originating from a wide diversity of geographical areas in southern Africa and genera such as *Absidia, Actinomucor, Circinella, Cunninghamella, Gongronella, Mortierella, Mucor, Rhizomucor, Rhizopus* and *Zygorhynchus* were recorded (Eicker *et al.*, 1982; Allsopp *et al.*, 1987; Opperman & Wehner, 1994; Botha *et al.*, 1995; Roux & Van Warmelo, 1997).

Although most of the above-mentioned isolations were performed at *circa* 25°C, it is known that certain mucoralean species are psychrotolerant and can also be isolated at temperatures as low as 0°C (Carreiro & Koske, 1992). Using the soil plate technique of Warcup (1950), it was found that the majority (92.4%) of the fungal isolates obtained at 5°C in mid-winter from pristine Alti Mountain Grassland (Low & Rebello, 1996), were representatives of *Mortierella* subgenus *Mortierella*, while 6% were *Mucor* isolates (Botha *et al.*, 1999). This vegetation type, occuring on the Drakensberg above an altitude of 1850 m, is a short dense grassveld, varying from sweet to mixed and dominated by *Themeda triandra*. This was the only record of psychrotolerant soil fungi in pristine vegetation types in South Africa. Nothing is known about the diversity of psychrotolerant mucoralean soil fungi associated with other vegetation types.

Therefore, the aim of this study was to determine the psychrotolerant mucoralean fungi in soil from a vineyard and from pristine Mountain Fynbos (Low & Rebello 1996), using similar enumeration and isolation procedures as was employed by Botha *et al.* (1999). The diversity and physiology of these mucoralean fungi originating from the different soils were subsequently compared by using Shannon's diversity index (Magurran, 1988) and the radial growth rate at low temperatures of *Mortierella* subgenus *Mortierella* isolates.

2.2. Materials and Methods

2.2.1. Sampling

The two sampling sites, each comprised an area of $10m^2$, are situated in the Jonkershoek mountain (pristine Mountain Fynbos, S 33° 58' 20"; E 18° 55' 10") and on the Lanzerac wine estate (vineyard, S 33°56'10"; E 18°54' 0") near Stellenbosch, South Africa. The minimum daily temperature for the coldest months of the year, in this cool temperate Mediterranean climatic region with a dry summer (Schulze, 1947), ranges from 6.1°C to 6.9°C. Samples were taken in late summer (2000-02-15), autumn (2000-04-15) and mid-winter (2000-07-15). Surface litter at the sampling sites was first scraped away to reduce contamination from this habitat. Samples of the soil up to a depth of 5 cm (*circa* 900 g), comprising nine subsamples, were taken at random over the area of each site. These subsamples were thoroughly mixed in the laboratory to produce a composite sample. Soil plates were prepared from the composite samples.

2.2.2. Characteristics of soil samples

The soil where the fynbos soil samples were taken from, has been classified as sandy loamy soil of the Oakleaf form derived from a mixture of granite and quartzite (Fry, 1987; Soil Classification Working Group, 1991). The vineyard soil is a clayey loamy soil of the Clovelly form derived from quartzite. The main physical and chemical properties of the soils are listed in Table 2.1.

2.2.3. Enumeration of fungi

The soil plate technique of Warcup (1950) and MYP-ps agar as isolation medium (Carreiro & Koske, 1992) were used to determine the psychrotolerant

Table 2.1. Characteristics of the soil at sampling sites.

	Pristine Mountain Fynbos	Vineyard
Physical Characteristics		
[1]Texture		
Stone % (Particle diameter > 2.0 mm)	23.4	32.5
Rough sand % (Particle diameter 0.5 - 2.0 mm)	16.6	11.3
Medium sand % (Particle diameter 0.2 - 0.5 mm)	28.0	13.8
Fine sand % (Particle diameter 0.02 - 0.2 mm)	32.9	17.1
Silt % (Particle diameter 0.002 - 0.02 mm)	16.4	20.8
Clay % (Particle diameter < 0.002 mm)	6.1	37.0
[2]Soil moisture content (%) at sampling times		
2000-02-15, 11:00	10.14 ± 0.44	14.77 ± 0.44
2000-04-15, 11:00	11.75 ± 0.84	13.37 ± 0.79
2000-07-15, 11:00	15.43 ± 0.08	17.01 ± 0.05

[3] Mean monthly soil temperature up to a depth of 5cm, for the months the samples were taken in.		
February	22.56 ± 3.05	
April	16.94 ± 3.56	
July	10.66 ± 1.66	
Chemical Characteristics		
[4]Organic carbon %	3.50	1.65
[5]Total nitrogen %	0.17	0.09
[6]Ammonium (ppm)	14.00	9.28
[7]Nitrate and nitrite (ppm)	0.40	0.20
[8]Phosphorous (ppm)	4.00	14.00
[9]Copper (ppm)	0.06	0.12
[10]Zink (ppm)	0.50	0.60
[11]Manganese (ppm)	5.50	4.40
[12]Boron (ppm)	0.15	0.31

[13]Exchangeable cations		
Calsium (cmol/kg)	1.43	4.76
Magnesium (cmol/kg)	0.57	0.54
Potassium (cmol/kg)	0.31	0.38
Sodium (cmol/kg)	0.05	0.03
[14]pH of a suspension containing 1 part soil and 2.5 parts 1M KCl. (2000-07-15)	4.40	6.40
[15]pH of a soil suspension in water prepared on the sampling date*		
2000-02-15	6.06 ± 0.05	6.39 ± 0.11
2000-04-15	5.64 ± 0.37	6.02 ± 0.12
2000-07-15	6.28 ± 0.02	6.94 ± 0.14

[1]Determined by Bemlab CC using the hydrometer method (Van der Watt, 1966).

[2]The soil moisture content of the soil samples were determined in triplicate by drying the soil in an electric oven at 105°C for 12h (Eicker, 1970).

[3]Data provided for the top 5 cm of soil by a weather station situated in the Jonkershoek valley and owned by the Division for Water-, Environment and Forest Technology, CSIR, Stellenbosch.

[4]Determined by Bemlab CC using the Walkley-Black method (Nelson & Sommers, 1982).

[5]Determined by Bemlab CC through digestion in a LECO FP-528 nitrogen analyser.

[6-7]Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

[8]Determined in a Bray-2 extract by Bemlab CC (Thomas & Peaslee, 1973).

[9-11]Determined in a di-ammonium EDTA extract by Bemlab CC, Somerset West, S.A. according to the methods of Beyers and Coetzer (1971).

[12]Determined in a hot water extract by Bemlab CC according to the methods of the Fertilizer Society of South Africa (1974).

[13]Determined in a 1 M ammonium acetate extract by Bemlab CC according to the methods of Doll and Lucas (1973).

[14]Determined by Bemlab CC according to the method of McClean (1982).

[15]Determined in triplicate according to the method of Spotts and Cervantes (1986).

fungal diversity present in the two soil types. The MYP-ps medium consisted of malt extract (Difco), 7.0g; peptone (Oxoid), 1.0g; yeast extract (Difco), 0.5g; penicillin G, 0.5g; streptomycin sulphate, 0.5g; agar, 16.0g and dH_2O, 1l. Soil plates were prepared by transferring 0.001g soil to a sterile Petri dish, which was then thoroughly mixed with cooled molten agar medium (MYP-ps). The plates were incubated at 4°C for 10 days and developing colonies were enumerated and isolated. These isolates were transferred to 2% (w/w) malt extract agar (MEA) and further purified by successive subculturing at 25°C before identification.

2.2.4. Identification of fungi

The isolates were identified on MEA or on potato-carrot agar (PCA) according to the keys and descriptions of Gams (1976, 1977), Schipper (1978), Domsch *et al.* (1980) and Sutton (1980).

2.2.5. Diversity of psychrotolerant mucoralean species

The diversity of the psychrotolerant mucoralean species in the soil samples of the present study as well as the diversity of psychrotolerant mucoralean species from pristine Alti Mountain Grassland soil (Botha *et al.*, 1999) were estimated using Shannon's diversity index (Magurran, 1988). The Student's t-test was used to compare the diversities of the fungal species in the different soil samples. The following formulas were used in the calculations:

1. $H' = -\sum p_i \ln p_i$

   (H' = Shannon's diversity index; p_i = proportional abundance of each species)

2. $$\text{Var } H' = \frac{\sum p_i(\ln p_i)^2 - (\sum p_i \ln p_i)^2}{N} - \frac{S-1}{2N^2}$$

(Var H' = The variance in diversity; N =Total number of colony-forming units; S = Number of species)

3. $$t = \frac{H'_1 - H'_2}{(\text{Var } H'_1 + \text{Var } H'_2)^{1/2}}$$

(t = Value for t; H'_1 = Shannon's index for the one sample; H'_2 = Shannon's index for the other sample)

4. $$df = \frac{(\text{Var } H'_1 + \text{Var } H'_2)^2}{[(\text{Var } H'_1)^2 / N_1] + [(\text{Var } H'_2)^2 / N_2]}$$

(*df* = degrees of freedom)

2.2.6. Radial growth of psychrotolerant *Mortierella* species

The *Mortierella* subgenus *Mortierella* strains isolated during this study and the *Mortierella* strains isolated from Alti Mountain Grassland soil (Botha *et al.*, 1999) were compared on the basis of radial growth rate at low temperatures. Square blocks (2.5 × 2.5 mm) of two-week old hyphal growth on MEA of each fungal isolate, was inoculated on MEA plates and incubated in triplicate at 4°C and 8°C. The ability to grow at 25°C on MEA was also determined in order to confirm that the isolates were psychrotolerant. Radial growth of developing colonies was measured periodically (Appendix I) and differences in colony diameter after a specific period at a specific temperature was visualized in a series of graphs (Figures 2.1 to 2.6).

2.3. Results and Discussion

2.3.1. Diversity of mucoralean species in soil samples

The psychrotolerant mucoralean isolates obtained in this study from fynbos and vineyard soil as well as those obtained by Botha *et al.* (1999) from Alti Mountain

Grassland soil are listed in Tables 2.2 and 2.3. The results that were obtained when these fungi were enumerated in the fynbos and vineyard soil samples in late summer, autumn and mid-winter, are depicted in Tables 2.4, 2.5 and 2.6 respectively. In late summer (Table 2.4), seven species were found in the fynbos soil; *Mortiella alpina, Mortierella ramanniana, Mortierella selenospora, Mucor circinelloides, Mucor guilliermondii, Mucor racemosus* and *Mucor zonatus*. However, only *Mortierella wolfii* and *Rhizopus oryzae* were recorded in the vineyard soil. Consequently, a significant difference (Student's t-test, $P< 0.05$) was detected between the Shannon's diversity indices of the mucoralean species in the two soil samples. This difference in diversity of the fungal species may be attributed to differences in the chemical and physical composition of the soil (Table 2.1). Since it is known that anthropogenic activities may change the composition of fungal communities in pristine environments (Kerry, 1990), the difference in the diversity of mucoralean species may also have been as a result of agricultural practices in the vineyard.

Similar to those from late summer, the dominant mucoralean species enumerated in autumn in the fynbos soil still was *Mucor circinelloides* (Table 2.4 and 2.5), however, only five other mucoralean species were found in this habitat; *Mortiella alpina, Mortierella ramanniana,* a *Mortierella* species with no distinctive features except producing smooth chlamydospores, *Mucor plumbeus* and *Mucor racemosus.* As was found in late summer (Table 2.4) the dominant mucoralean species enumerated in the vineyard soil in autumn was *Rhizopus oryzae* (Table 2.5). The other species found in the vineyard soil were *Mortierella ramanniana, Mucor circinelloides* and *Mucor racemosus.* As was found when the soil fungi were enumerated in late summer (Table 2.4), the diversity of psychrotolerant mucoralean species enumerated in the fynbos soil was significantly (Student's t-test, $P< 0.05$) higher than in the vineyard soil.

In winter however, no significant difference was detected between the Shannon's diversity indices of mucoralean species in the soil samples taken from the two habitats (Table 2.6). Five mucoralean species were detected in each sample and the dominant species in both samples belonged to *Mortierella*

Table 2.2. List of psychrotolerant mucoralean isolates originating from fynbos and vineyard soil.

Species	Isolate no.
Mortierella alpina Peyronel	F4g
	F5f
	FM22
	FM23
	FM33
	wj12
	wj17
	wj18
	wj19
	wj22
	wj23
Mortierella elongata Linnem.	fj14
Mortierella ramanniana Linnem.	F4j
	F5j
	WM35
	FM11
	FM35
Mortierella sarnyensis Milko	fj1
	fj3
Mortierella selenospora W. Gams	F5d
	fj2
Mortierella wolfii Mehrota & Baijal	W4c
Mortierella sp[1]	wj1
	wj2
	wj8
	wj9
	wj11
	wj13
	wj28
	fj4
	fj8
	fj12
	fj13

Table 2.2. Continued.

Species	Isolate no.
Mortierella sp[2]	wj10
	wj21
	fj6
	fj9
Mucor circinelloides	F6h
Tiegh.	F6l
	F4e
	F6e
	WM23
	WM25
	FM14
	FM16a
	FM21
	FM25
	FM31
	wj20
Mucor guilliermondii	F6d
Nadson & Philippov	F6j
Mucor plumbeus	FM10
Bonord.	FM32
Mucor racemosus	F6c
Fres.	WM24
	FM24
	FM26
	FM34
Mucor zonatus	F4h
Milko	
Rhizopus oryzae	w4a
Went & Prinsen Geerl.	w4d
	WM13
	WM22
	WM36
	wj14
	wj16
	wj29

♦Isolates designated by a "F" number (isolated in February), "FM" number (isolated in April) and "fj" number (isolated in July) were obtained from fynbos soil while those designated by a "W" number (isolated in February), "WM" number (isolated in April) and "wj" number (isolated in July) were obtained from vineyard soil.

[1]Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, as well as the presence of smooth chlamydospores, however no other distinguishing characteristics were noted.

[2]Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, however no other distinguishing characteristics were noted.

Table 2.3. List of psychrotolerant mucoralean isolates, originating from Alti Mountain Grassland soil (Botha *et al.*, 1999).

Species	Isolate no.
Mortierella alpina Peyronel	4j
	5d
	5i
	4k
	5c
	2k
	5h
	10c
	2h
	4f
	2c
	4h
	9a
	4r
Mortierella angusta (Linnem.) Linnem.	10g
Mortierella antarctica Linnem.	4a
	4q
	4g
	10L
	9g
	9o
Mortierella basiparvispora W. Gams & Ginsberg	5a
	10d
	9i
	4p
	9j
Mortierella camargensis W. Gams & R. Moreau	2i
Mortierella clonocystis W. Gams	10e
Mortierella elongata Linnem.	5j
Mortierella epicladia W. Gams & van Emden	2L
	10i
	2g
	9f
Mortierella gamsii Milko	4d
	4e
	4s

Table 2.3. Continued.

Species	Isolate no.
Mortierella globalpina W. Gams & Veenbaas-Rijks	9d
Mortierella horticola Linnem.	10f 4L
Mortierella minutissima Tiegh.	4o
Mortierella parazychae W. Gams	5b
Mortierella parvispora Linnem.	9c
Mortierella sarnyensis Milko	9e 4n
Mortierella selenospora W. Gams	2d
Mortierella verticillata Linnem.	2b
Mortierella zychae Linnem.	9q
Mortierella sp*	9n 10j 9k 2e 2a 10m 10a 5g 9m 5e
Mortierella sp*	5f 2j 9h 10h
Mucor sp.	

*Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, as well as the presence of smooth chlamydospores, however no other distinguishing characteristics were noted.

**Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, however no other distinguishing characteristics were noted.

Table 2.4. Psychrotolerant fungi enumerated as colony-forming units per g soil (cfu/g) in the soil samples taken during February 2000.

TAXA	Fynbos			Vineyard		
	cfu/g	p_i	$p_i \ln p_i$	cfu/g	p_i	$p_i \ln p_i$
Higher fungi						
TOTAL	8001	0	0	3000	0	0
Mucorales						
Mortierella subgenus *Micromucor*						
Mortierella ramanniana	667	0.154	-0.288	0	0	0
Mortierella subgenus *Mortierella*						
Mortierella alpina	667	0.154	-0.288	0	0	0
Mortierella selenospora	333	0.077	-0.197	0	0	0
Mortierella wolfii	0	0	0	333	0.250	-0.346
Mucor						
Mucor circinelloides	1333	0.308	-0.362	0	0	0
Mucor guilliermondii	667	0.154	-0.288	0	0	0
Mucor racemosus	333	0.077	-0.197	0	0	0
Mucor zonatus	333	0.077	-0.197	0	0	0
Rhizopus						
Rhizopus oryzae	0	0	0	1000	0.750	-0.216
TOTAL	4333	1.000	-1.817	1333	1.000	-0.562
% Mucorales	35			31		
H'			1.817			0.562

p_i: The proportional abundance of each mucoralean species; % Mucorales: Percentage of total fungi enumerated at 4°C that were representatives of the Mucorales. H': Shannon's diversity index of mucoralean species enumerated on the isolation medium.

Table 2.5. Psychrotolerant fungi enumerated as colony-forming units per g soil (cfu/g) in the soil samples taken during April 2000.

TAXA	Fynbos			Vineyard		
	cfu/g	p_i	$p_i \ln p_i$	cfu/g	p_i	$p_i \ln p_i$
Higher fungi						
TOTAL	6666			3000		
Mucorales						
Mortierella subgenus *Micromucor*						
Mortierella ramanniana	667	0.133	-0.269	333	0.125	-0.260
Mortierella subgenus *Mortierella*						
Mortierella alpina	333	0.067	-0.180	0	0	0
Mortierella sp[1]	667	0.133	-0.269	0	0	0
Mucor						
Mucor circinelloides	1667	0.333	-0.367	667	0.250	-0.347
Mucor plumbeus	667	0.133	-0.269			
Mucor racemosus	1000	0.200	-0.322	333	0.125	-0.260
Rhizopus						
Rhizopus oryzae				1333	0.500	-0.347
TOTAL	5001		-1.676	2666	1.000	-1.214
% Mucorales	43			47		
H'			1.676			1.214

p_i: The proportional abundance of each mucoralean species; % Mucorales: Percentage of total fungi enumerated at 4°C that were representatives of the Mucorales. H': Shannon's diversity index of mucoralean species enumerated on the isolation medium.

[1]Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, as well as the presence of smooth chlamydospores, however no distinguishing characteristics were noted.

Table 2.6. Psychrotolerant fungi enumerated as colony-forming units per g soil (cfu/g) in the soil samples taken during July 2000.

TAXA	Fynbos			Vineyard		
	cfu/g	p_i	$p_i \ln p_i$	cfu/g	p_i	$p_i \ln p_i$
Higher fungi						
TOTAL	1333	0	0	3334	0	0
Mucorales						
Mortierella subgenus *Mortierella*						
Mortierella alpina	0	0	0	2000	0.300	-0.361
Mortierella elongata	333	0.100	-0.230	0	0	0
Mortierella sarnyensis	667	0.200	-0.322	0	0	0
Mortierella selenospora	333	0.100	-0.230	0	0	0
Mortierella sp[1]	1333	0.400	-0.366	2333	0.350	-0.367
Mortierella sp[2]	667	0.200	-0.322	667	0.100	-0.230
Mucor						
Mucor circinelloides	0	0	0	333	0.050	-0.150
Rhizopus						
Rhizopus oryzae	0	0	0	1333	0.200	-0.322
TOTAL	3333	1.000	-1.470	6666	1.000	-1.430
% Mucorales	72			67		
H'			1.470			1.430

p_i : The proportional abundance of each mucoralean species; % Mucorales: Percentage of total fungi enumerated at 4°C that were representatives of the Mucorales. H': Shannon's diversity index of mucoralean species enumerated on the isolation medium.

[1]Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, as well as the presence of smooth chlamydospores, however no distinguishing characteristics were noted.

[2]Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, however no distinguishing characteristics were noted.

subgenus *Mortierella*. The only other mucoralean taxa that were found, were *Mucor circinelloides* and *Rhizopus oryzae*, both occurred in the vineyard soil.

It was found that low incubation temperatures (0°C) are selective for soil fungi belonging to *Mortierella* subgenus *Mortierella* (Carreiro & Koske, 1992; Botha *et al.*, 1999). Botha *et al.* (1999), found that the majority (*circa* 92%) of detectable psychrotolerant mucoralean soil fungi present in pristine Alti Mountain Grassland (Low & Rebello, 1996) during mid-winter, were representatives of *Mortierella* subgenus *Mortierella.* In addition, it is known that mucoralean fungi occur more frequently in moist environments such as forest litter than in the soil of arid desert regions (Eicker, 1969; Steiman *et al.,* 1995). It is also known that one of the most common mucoralean soil fungi, *Mortierella alpina,* which belongs to *Mortierella* subgenus *Mortierella*, occurs preferentially in soils that are very humid (Domsch *et al.*, 1980).

The results of the present study is in accordance with the above mentioned observations of other workers. Not only did the percentage mucoralean fungi on the plates increased as the project proceeded from summer to winter, but the numbers of detectable *Mortierella* subgenus *Mortierella* also increased (Tables 2.4, 2.5 and 2.6). Interestingly, a concurrent increase in soil moisture content at the sampling time and a decrease in the mean monthly soil temperature was noted (Table 2.1). The diversity of mucoralean species obtained during winter in fynbos and vineyard soil (Table 2.6), was significantly less (Student's t-test, $P < 0.05$) than the diversity of these species in Alti Mountain Grassland soil (Table 2.7).

2.3.2. Radial growth of psychrotolerant *Mortierella* species

The above-mentioned *Mortierella* subgenus *Mortierella* isolates, obtained from the soil at 4°C, were all psychrotolerant and able to grow at 25°C (Appendix I). To determine if these isolates and those obtained from Alti Mountain Grassland soil (Botha *et al.*, 1999) differ in low temperature growth rate, the colony

Table 2.7. Psychrotolerant fungi enumerated as colony-forming units per g soil (cfu/g) in Alti Mountain Grassland soil during mid-winter (Data obtained from Botha *et al.*, 1999).

TAXA	cfu/g	p_i	$p_i \ln p_i$
Higher fungi			
TOTAL	40		
Mucorales			
Mortierella subgenus *Mortierella*			
Mortierella alpina	560	0.212	-0.329
Mortierella angusta	40	0.015	-0.064
Mortierella antartica	240	0.092	-0.218
Mortierella basiparvispora	200	0.076	-0.195
Mortierella camargensis	40	0.015	-0.064
Mortierella clonocystis	40	0.015	-0.064
Mortierella elongata	40	0.015	-0.064
Mortierella epicladia	160	0.061	-0.170
Mortierella gamsii	120	0.044	-0.141
Mortierella globalpina	40	0.015	-0.064
Mortierella horticola	80	0.030	-0.106
Mortierella minutissima	40	0.015	-0.064
Mortierella parazychae	40	0.015	-0.064
Mortierella parvispora	40	0.015	-0.064
Mortierella sarnyensis	80	0.030	-0.106
Mortierella selenospora	40	0.015	-0.064
Mortierella verticillata	40	0.015	-0.064
Mortierella zychae	40	0.015	-0.064
Mortierella sp*	160	0.061	-0.170
Mortierella sp**	400	0.152	-0.285
Mucor			
Mucor sp.	160	0.061	-0.170

TOTAL	2640	0.983	-2.596
% Mucorales	98		
H'			2.596

p_i : The proportional abundance of each mucoralean species; % Mucorales: Percentage of total fungi enumerated at 5°C that were representatives of the Mucorales. H': Shannon's diversity index of mucoralean species enumerated on the isolation medium.

*Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, as well as the presence of smooth chlamydospores, however no distinguishing characteristics were noted.

**Identifiable as *Mortierella* subgenus *Mortierella* due to colony odour and habit, however no distinguishing characteristics were noted.

diameter of each isolate was noted after a specific incubation time at 4°C and 8°C (Appendix I).

The results indicate that not only did seasonal changes occur in the dominance of taxa within *Mortierella* subgenus *Mortierella* (Tables 2.4, 2.5 and 2.6), but that the isolates obtained from the soil in different seasons also differed in the ability to grow at low temperatures (Figures 2.1, 2.2, 2.3 and 2.4). The percentage of isolates that had reached greater colony diameters (36-45 mm) after 8 days of incubation at 4°C, was higher for the isolates obtained in the cold wet month of July than for those obtained in the warmer dryer month of February (Figures 2.1 and 2.2). Similar results were obtained with the radial growth experiments conducted at 8°C (Figures 2.3 and 2.4).

The *Mortierella* subgenus *Mortierella* isolates obtained in winter from fynbos and vineyard soil, showed less variation in low temperature growth rate than the isolates of this taxon obtained in winter from Alti Mountain Grassland soil (Figures 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6). This variation corresponds to the greater number of *Mortierella* subgenus *Mortierella* species (20) found in the grassland soil (Botha *et al.*, 1999). Only seven species of this subgenus could be detected during the present study in the fynbos and vineyard soil samples (Tables 2.2, 2.4, 2.5 and 2.6).

2.4. Conclusions

The results indicate that the pristine Mountain Fynbos and vineyard soil were significantly different in terms of diversity of psychrotolerant mucoralean species occurring in them during the dryer warmer months of February and April. This difference in diversity diminished in the cold wet month of July. In this month *Mortierella* subgenus *Mortierella* was found to be the dominant taxon in both the fynbos and vineyard soil. A greater percentage *Mortierella* subgenus *Mortierella* isolates obtained in mid-winter, seem to be better adapted to grow at low temperatures than those obtained in the warmer dry periods.

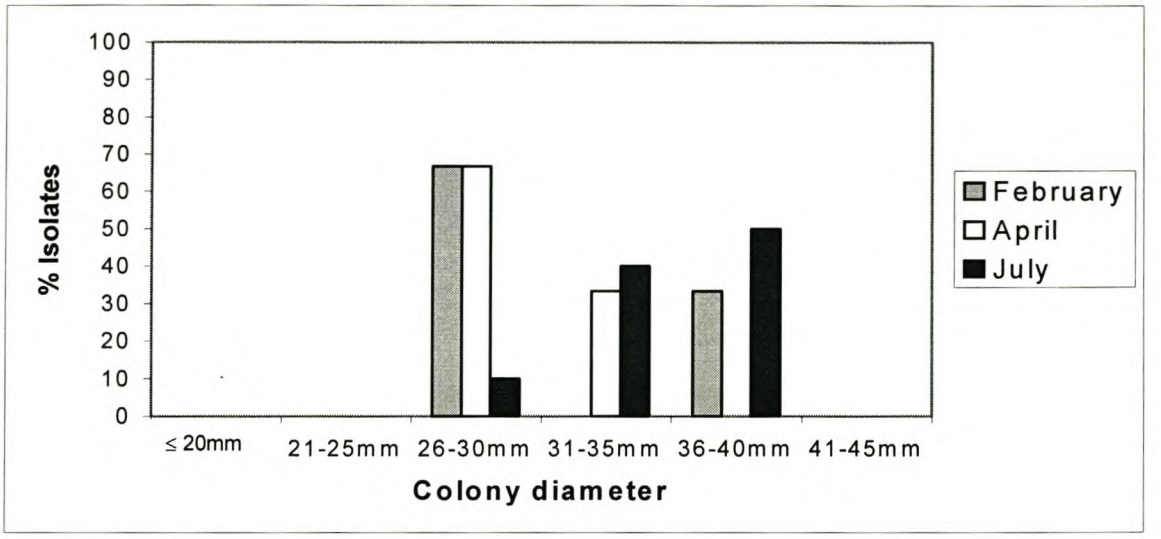

Figure 2.1. Percentage of *Mortierella* subgenus *Mortierella* isolates obtained during each analysis (in February, April and July) from fynbos soil that had reached the indicated colony diameters after 8 days of incubation at 4°C.

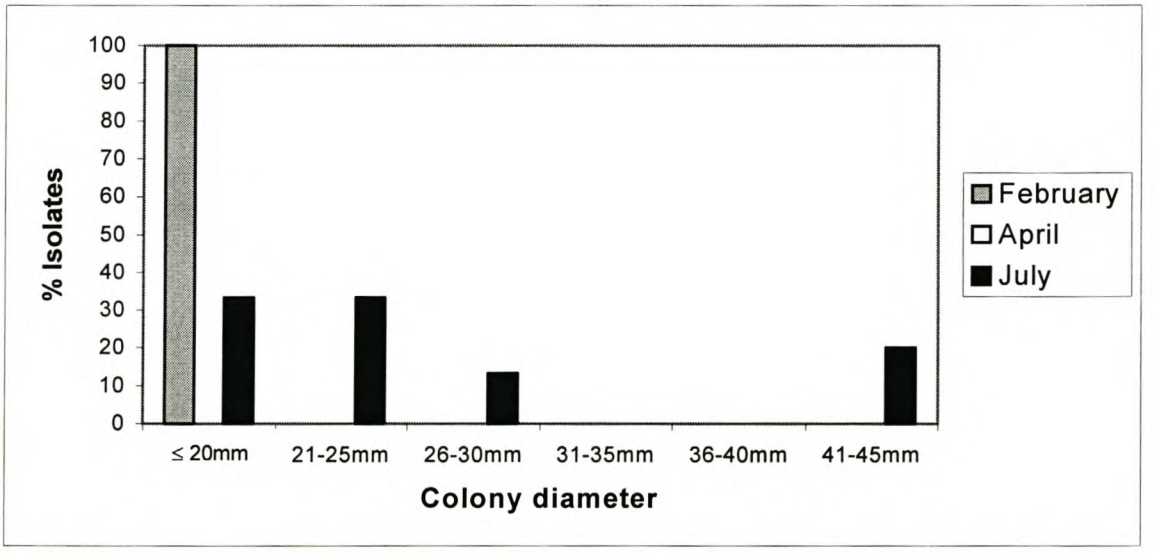

Figure 2.2. Percentage of *Mortierella* subgenus *Mortierella* isolates obtained during each analysis (in February, April and July) from vineyard soil that had reached the indicated colony diameters after 8 days of incubation at 4°C.

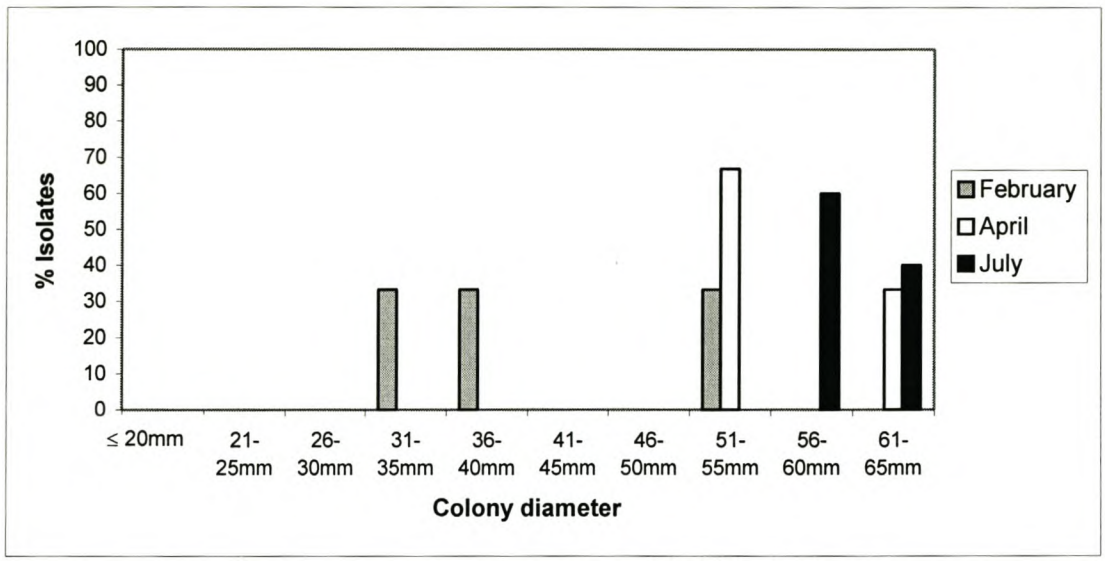

Figure 2.3. Percentage of *Mortierella* subgenus *Mortierella* isolates obtained during each analysis (in February, April and July) from fynbos soil that had reached the indicated colony diameters after 8 days of incubation at 8°C.

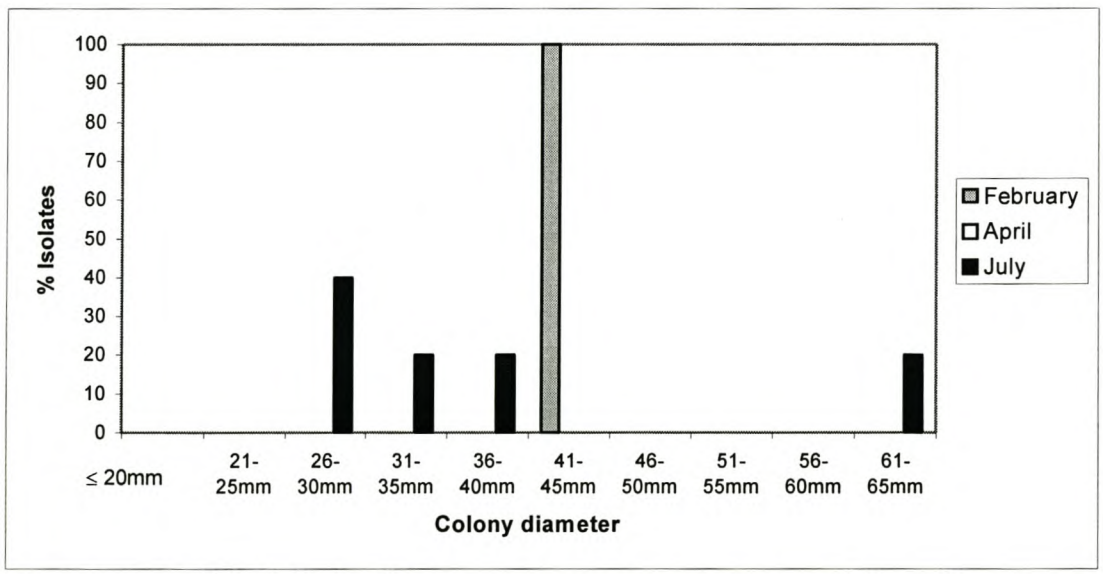

Figure 2.4. Percentage of *Mortierella* subgenus *Mortierella* isolates obtained during each analysis (in February, April and July) from vineyard soil that had reached the indicated colony diameters after 8 days of incubation at 8°C.

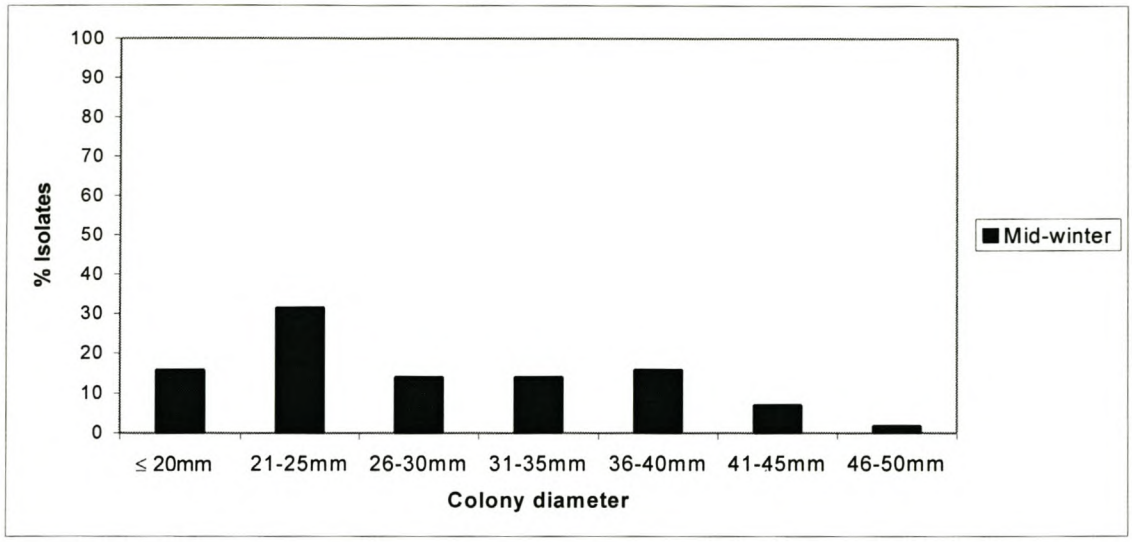

Figure 2.5. Percentage of *Mortierella* subgenus *Mortierella* isolates obtained from Alti Mountain Grassland soil during mid-Winter (Botha *et al.*, 1999), that had reached the indicated colony diameters after 8 days of incubation at 4°C.

Figure 2.6. Percentage of *Mortierella* subgenus *Mortierella* isolates, obtained from Alti Mountain Grassland soil during mid-Winter (Botha *et al.*, 1999), that had reached the indicated colony diameters after 8 days of incubation at 8°C.

In winter, the diversity of psychrotolerant mucoralean species in pristine Mountain Fynbos and vineyard soil was significantly lower than the diversity of these fungi in Alti Mountain Grassland. Also, the grassland isolates representing *Mortierella* subgenus *Mortierella,* showed greater variation in low temperature growth rate than the isolates of this taxon obtained in winter from the fynbos and vineyard soil. However, the numbers of *Mortierella* subgenus *Mortierella* in the fynbos and vineyard soil (i.e. 3333 cfu/g and 5000 cfu/g respectively), were notably higher than the 2480 cfu/g obtained for these fungi in the grassland (Tables 2.6 and 2.7). If these numbers are an indication of biomass, an inverse relation exists between biomass and species diversity, that suggests these elevated numbers of *Mortierella* subgenus *Mortierella* in the fynbos and vineyard soil reflects the success of a relatively few well-adapted or opportunistic species (Atlas, 1984). It must however be emphasized that this difference in estimated diversity between the fynbos and vineyard isolates, as well as the grassland isolates, may also have been as a result of differences in the amount of soil used per Petri dish in the preparation of the soil plates. We used 0.001g and Botha *et al.* (1999) used 0.005 g.

In this chapter, the diversity of psychrotolerant mucoralean fungal species in soils originating from pristine Mountain Fynbos, a vineyard and from pristine Alti Mountain Grassland was described. Also, the ability to grow at low temperature by psychrotolerant *Mortierella* subgenus *Mortierella* isolates originating from these soils was compared to each other. In the next chapter the molecular diversity of these isolates, originating from different habitats will be investigated.

2.5. References

Atlas, R.M. (1984). Use of microbial diversity measurements to assess environmental stress. In: M.J. Klug & C.A. Reddy (Eds.) *Current perspectives in microbial ecology* (pp. 540-545). American Society for Microbiology, Washington, D.C.

Allsopp, N., Olivier, D.L. & Mitchell, D.T. (1987). Fungal populations associated with root systems of proteaceous seedlings at a lowland fynbos site in South Africa. *South African Journal of Botany* **53,** 365 – 369.

Beyers, C.P. de L. & Coetzer, F.J. (1971). Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. *Agrochemophysica* **3,** 49 - 54.

Bokhary, H.A. & Parvez, S. (1991). Soil mycology from wild desert truffle habitats in northern Saudi Arabia. *Arid Environments* **23,** 379 – 388.

Botha, A., Kock, J.L.F., Roux, C., Coetzee, D.J. & Botes, P.J. (1995). An isolation medium for gamma-linolenic acid producing mucoralean fungi. *Systematic and Applied Microbiology* **18,** 448 - 454.

Botha, A., Paul, I., Roux, C., Kock, J.L.F., Coetzee, D.J., Strauss, T. & Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* **75,** 253 – 256.

Bremner, J.M. (1965). Inorganic forms of nitrogen. In: C.A. Black (Ed.) *Methods of soil analysis*. Part 2. American Society of Agronomy, Madison, Wisconsin.

Brock, T.D., Madigan, M.T., Martinko, J.M. & Parker, J. (1994). *Biology of microorganisms*. Seventh edition. Prentice Hall, New Jersey.

Carreiro, M.M. & Koske, R.E. (1992). Room temperature isolations can bias against selection of low temperature microfungi in temperate forest soils. *Mycologia* **84,** 886 - 900.

Doll, E.C. & Lucas, R.E. (1973). Testing soils for potassium, calcium and magnesium. In: L.M. Walsh & J.D. Beatons (Eds.) *Soil testing and plant analysis* (pp. 133-152). Soil Science Society of America, Madison, Wisconsin.

Domsch, K.H., Gams , W. & Anderson, T-H. (1980). *Compendium of soil fungi* (pp. 431 – 460). Academic Press, London.

Eicker, A. (1969). Microfungi from surface soil of forest communities in Zululand. *Transactions British Mycological Society* **53,** 381 – 392.

Eicker, A. (1970). Ecological observations on soil fungi. *South African Journal of Science* **66,** 327 - 334.

Eicker, A., Theron, G.K. & Grobbelaar, N. (1982). 'n Mikrobiologiese studie van 'kaal kolle' in die Giribesvlakte van Kaokoland, S.W.A.-Namibië. *South African Journal of Botany* **1,** 69 – 74.

Fertilizer Society of South Africa (1974). *Manual of soil analysis methods.* FSSA Publiction No. 37.

Fry, M.St.L. (1987). A detailed characterisation of soils under different fynbos-climate-geology combinations in the southwestern Cape. M.Sc. Thesis. University of Stellenbosch, Stellenbosch, South Africa.

Gams, W. (1976). Some new noteworthy species of *Mortierella*. *Persoonia* **9,** 111 – 140.

Gams, W. (1977). A key to the species of *Mortierella*. *Persoonia* **9,** 381 – 391.

Hesseltine, C.W. & Ellis, J.J. (1973). Mucorales. In G.C. Ainsworth, F.K. Sparrow & A.S. Sussman (Eds.) *The fungi*, vol 4B (pp. 187-216). Academic Press, London.

Kerry, E. (1990). Microorganisms colonizing plants and soil subjected to different degrees of human activity, including petroleum contamination, in the Vestfold Hills and MacRobertson Land, Antartica. *Polar Biology* **10,** 423-430.

Low, A.B. & Rebelo, A.G. (1996). Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Magurran, A.E. (1988). *Ecological Diversity and Its Measurements*. Princeton University Press, Princeton, New Jersey.

McClean, E.O. (1982). Soil pH and lime requirement. In: A.L. Page (Ed.) *Methods of soil analysis*. Part 2 (p. 199). American Society of Agronomy, Madison, Wisconsin.

Nelson, D.W. & Sommers, L.E. (1982). Total carbon, organic carbon and organic matter. In: A.L. Page (Ed.) *Methods of soil analysis*. Part 2 (pp. 570 - 571). American Society of Agronomy, Madison, Wisconsin.

Opperman, L. & Wehner, F.C. (1994). Survey of fungi associated with grass roots in virgin soils on the Springbok Flats. *South African Journal of Bot*any **60,** 67 - 72.

Roux, C. & Van Warmelo, K.T. (1997). A survey of the mycobiota of a natural Karoo pasture. *Bothalia* **27(2),** 167-183.

Schipper, M.A.A. (1978). On certain species of *Mucor* with a key to all accepted species. *Studies in Mycology* **17,** 1 – 52.

Schulze, B.R. (1947). The climates of South Africa according to the classifications of Köppen and Thornthwaite. *Die Suid Arikaanse Aardrykskundige Tydskrif* **29,** 32 - 42.

Soil classification working group (1991). Soil classification. A taxonomic system for South Africa. Memoirs on the Agricultural Natural Resources of South Africa No. 15. Department of Agricultural Development, Pretoria, South Africa.

Spotts, R.A. & Cervantes, L.A. (1986). Populations of *Mucor piriformis* in soil of pear orchards in the Hood River valley of Oregon. *Plant Disease* **70,** 935 – 937.

Steinman, R., Guiraud, P., Saga, L., Seigle-Murandi, F. & Lafond, J. (1995). Mycroflora of soil around the Dead Sea I – Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. *Systematic and Applied Microbiology* **18,** 310 – 317.

Sutton, B.C. (1980). *The Coelomycetes.* Commonwealth Mycological Institute. Kew, England.

Thomas, G.W. & Peaslee, D.E. (1973). Testing for soil phosphorus. In: L. Walsh & J. Beaton (Eds.) *Soil testing and plant analysis* (pp. 15 - 122). Soil Science Society of America, Madison, Wisconsin.

Van Der Watt, H.V.H. (1966). Improved tables and a simplified procedure for soil particle size analysis by the hydrometer method. *South African Journal of Agricultural Science* **9,** 911 - 916.

Warcup, J.H. (1950). The soil-plate method for isolation of fungi from soil. *Nature* **166,** 117-118.

CHAPTER 3

INVESTIGATING MOLECULAR DIVERSITY OF PSYCHROTOLERANT *MORTIERELLA* STRAINS USING ITS1 SEQUENCE ANALYSES

3.1. Introduction

Many phylogenetic studies of fungi were based on comparisons of the nucleotide sequences from the ribosomal gene cluster (White *et al.*, 1990; Kasuga *et al.*, 1993; Wilmotte *et al.*, 1993). Ribosomal DNA (rDNA) genes are tandem repeatedly multigene families, which contain genic and nongenic, or spacer regions (Mitchell *et al.*, 1995). The repetitive units each contain a copy of three structural regions coding for the 5.8S, 18S, and 28S ribosomal RNA genes, as well as two internal transcribed spacers (ITS1 and ITS2) and an intergenic spacer (IGS). The latter consists of an externally transcribed spacer (ETS) and a non-transcribed spacer (NTS). It is generally accepted that the nucleotide sequences of the structural genes are most conserved and it was found that the analyses of these sequences could be used to study distantly related fungi (White *et al.*, 1990; Berbee & Taylor, 1992; Wilmotte *et al.*, 1993; Mitchell *et al.*, 1995).

ITS and IGS regions are much more variable and it was suggested that sequence analyses of these regions may be used to separate species within a genus (White *et al.*, 1990). For example, it was found that the pathogenic yeast species *Candida albicans* can be distinguished from strains of *Candida parapsilosis* and *Candida tropicalis* on the basis of the nucleotide sequence of the ITS2 region (Lott *et al.*, 1993). Similarly, differences exist in the ITS regions of two morphologically similar filamentous fungal species, *Fusarium thapsinum* (teleomorph: *Gibberella thapsina*) and *Fusarium moniliforme* (teleomorph: *Gibberella fujikuroi* mating population A) (Klittich *et al.*, 1997). Members of the two species were found to be reproductively isolated and can also be distinguished by other characters such as mycotoxins produced, isozyme polymorphism, electrophoretic karyotype and benomyl sensitivity.

However, it was found that ITS sequence analyses might not always be used to characterise *Fusarium* species (O' Donnell, 1992). Three divergent ITS types were discovered within *Fusarium sambucinum* (teleomorph: *Gibberella pulicaris*), after 86 isolates of this species, originating from a wide diversity of geographical regions and substrates, were analysed. Similarly, it was found

that clinical isolates of the yeast species *Candida parapsilosis* could be grouped in three separate clusters on the basis of significant differences in the sequence of the ITS1 region (Lin *et al.*, 1995). This phenomenon was also observed in another yeast species, *Zygosaccharomyces bailii,* when the nucleotide sequences of ITS1 and ITS2 regions of different strains were analysed (James *et al.*, 1996). However, no such interstrain sequence variation was observed for either *Zygosaccharomyces bisporus* or *Zygosaccharomyces rouxii.*

The findings above on the intraspecific variation of ITS regions are not surprising since it is generally accepted that these regions are non-coding and that they are less subject to evolutionary constrains due to functional loss. (Valente *et al.*, 1999). Interestingly, it was found that three intersterility groups within the phytopathogenic fungal species, *Heterobasidion annosum*, which are prevalent on three different host conifers, differ in the sequences of the ITS regions (Kasuga *et al.*, 1993). These results are significant, since it may indicate that among closely related taxa, the sequences of ITS regions correlate with the particular ecological habitat of a fungus.

With the above as background it was decided to explore this possible correlation between ecological habitat and ITS sequence in the fungal domain using a group of closely related fungi originating from similar habitats. Consequently, 45 psychrotolerant *Mortierella* subgenus *Mortierella* isolates originating from three different soil habitats was compared on the basis ITS 1 nucleotide sequence composition and radial growth rate at 4°C.

3.2. Materials and Methods

3.2.1. Fungal cultures

All the fungal strains analysed in this study are psychrotolerant representatives of *Mortierella* subgenus *Mortierella* isolated in mid-winter from specific soil

habitats. The strains listed in Table 3.1 were isolated from pristine Alti Mountain Grassland soil (Botha *et al.*, 1999), while those in Table 3.2 were isolated from pristine Mountain Fynbos and vineyard soil (Chapter 2). A reference strain, *Mortierella alpina* CBS 527.72, was included in the experiments (Table 3.2). The strains were maintained on 2% (w/v) malt extract agar at 22°C.

3.2.2. Culture conditions for DNA isolation

The fungal cultures were inoculated into 100ml malt extract broth in 1litre flasks and incubated for 5 days at 22°C. The mycelia were harvested by filtering it through miracloth (Calbiochem) and quick freezing it in liquid nitrogen. The harvested mycelia were stored overnight at -80°C.

3.2.3. DNA isolation, internal transcribed spacer (ITS) region amplification and sequencing

Genomic DNA was isolated by using the method of Raeder and Broda (1985). The ITS region was amplified by the polymerase chain reaction (PCR) using universal ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') oligonucleotide primers as described by White *et al.* (1990). The PCR reactions were performed by using the Expand^{TM} High Fidelity DNA Polymerase from Roche in a Perkin-Elmer 2400 thermal cycler. The conditions under which the PCR reactions were performed are as follow: denaturation for 2 min at 95°C and 30 s at 94°C, followed by 35 cycles of annealing for 30 s at 55°C, elongation for 1 min at 72°C, denaturation for 1 min at 94°C followed by a final elongation step of 5 min at 72°C. The PCR products were purified by column chromatography (Nucleospin^{R}, Seperations) and sequenced using a Perkin Elmer ABI PRISM^{tm} 377 DNA sequencer. The data from the forward and reverse sequences were compared and aligned by using the BLAST program of the National Center for Biotechnology Information (NCBI) and a phylogenetic tree of the ITS 1 region was constructed by using the

Multiple Sequence Alignment tool in the PC-based DNAMAN (version 4.1) software from Lynnon Biosoft.

3.2.4. Radial growth rate of psychrotolerant *Mortierella* species at 4°C

Square blocks (2.5 × 2.5 mm) of two-week old hyphal growth on malt extract agar (MEA) of each fungal isolate, was inoculated in triplicate on MEA plates and incubated at 4°C as explained in Chapter 2. Radial growth of developing colonies was measured after 8 days of incubation (Tables 3.1 and 3.2; Appendix I).

3.3. Results and Discussion

Phylogenetic analyses of the ITS1 sequences of the fungal isolates revealed the presence of two separate clusters, each of which consisted of two subgroups (Figure 3.1). The ITS1 sequences did not correlate with morphological classification of the isolates into different sections as proposed by Gams (1977). Representatives of *Mortierella* subgenus *Mortierella* section *Alpina* occurred in all the subgroups, while three of the subgroups (II, III and IV) contained representatives of the section *Hygrophila* (Figure 3.1). Two subgroups (II and III) contained representatives of the section *Stylospora*. Subgroup II also contained representatives of sections *Simplex* and *Spinosa*, while representatives of section *Schmuckeri* occurred in Subgroup III.

Interestingly, it was found that accept for subgroup IV and the reference strain occurring in subgroup II, all the subgroups contained isolates originating from a single soil habitat (Figure 3.1). In addition, the mean radial growth rate at 4°C of all the isolates in cluster B (consisting of subgroups III and IV) were higher than the mean radial growth rate at this temperature than the isolates of cluster A (consisting of subgroups I and II). The isolates from the grassland clustered in

Table 3.1. Isolates originating from Alti Mountain Grassland soil (Botha *et al.*, 1999) that were used in the experimentation.

Species	Isolate no.	#Colony diameter in mm at 4°C
Mortierella alpina Peyronel	4r	19±1
Mortierella angusta (Linnem.) Linnem.	10g	18±2
Mortierella antarctica Linnem.	4g	38±1
Mortierella basiparvispora W. Gams & Ginsberg	9i	38±0
Mortierella camargensis W. Gams & R. Moreau	2i	38±3
Mortierella clonocystis W. Gams	10e	15±2
Mortierella elongata Linnem.	5j	30±6
Mortierella epicladia W. Gams & van Emden	2L	25±3
Mortierella gamsii Milko	4s	37±1
Mortierella globalpina W. Gams & Veenbaas-Rijks	9d	23±1
Mortierella horticola Linnem.	4L	33±2
Mortierella minutissima Tiegh.	4o	30±1

Means and standard deviations of colony diameters obtained after 8 days on MEA at 4°C.

Table 3.1. Continued.

Species	Isolate no.	#Colony diameter in mm at 4°C
Mortierella parazychae W. Gams	5b	36±1
Mortierella parvispora Linnem.	9c	16±3
Mortierella sarnyensis Milko	9e	40±3
Mortierella selenospora W. Gams	2d	34±1
Mortierella verticillata Linnem.	2b	23±1
Mortierella zychae Linnem.	9q	44±1
Mortierella sp*	9k	47±1
Mortierella sp*	10h	39±1

Means and standard deviations of colony diameters obtained after 8 days on MEA at 4°C.

* Identifiable as *Mortierella* due to colony odour and habit, however no other distinguishing characteristics were noted.

** Identifiable as *Mortierella* due to colony odour and habit and the presence of smooth chlamydospores, however no other distinguishing characteristics were noted.

Table 3.2. Isolates originating from fynbos and vineyard soil that were used in the experimentation.

Species	▽Isolate no.	#Colony diameter in mm at 4°C
Mortierella alpina	wj12	41±1
Peyronel	wj17	21±1
	wj18	23±0
	wj19	24±1
	wj22	19±0
	wj23	26±1
Mortierella elongata	fj14	39±2
Linnem.		
Mortierella sarnyensis	fj1	34±2
Milko	fj3	35±2
Mortierella selenospora	fj2	37±2
W. Gams		
Mortierella sp[1]	fj4	30±4
	fj8	38±1
	fj12	37±2
	fj13	34±2
	wj1	24±0
	wj2	26±2
	wj8	41±1
	wj9	21±1
	wj11	20±1
	wj13	41±2
	wj28	20±1

▽ Isolates designated by a "fj" number were obtained from fynbos soil while those designated by a "wj" number were obtained from vineyard soil.
Means and standard deviations of colony diameters obtained after 8 days on MEA at 4°C.
[1] Identifiable as *Mortierella* due to colony odour and habit, however no other distinguishing characteristics were noted.

Table 3.2. Continued.

Species	Isolate no.	#Colony diameter in mm at 4°C
Mortierella sp[2]	fj6	37±0
	fj9	34±1
	wj10	20±1
	wj21	20±1
** ***Mortierella alpina*** Peyronel	CBS 527.72	

[▽] Isolates designated by a "fj" number were obtained from fynbos soil while those designated by a "wj" number were obtained from vineyard soil.
[#] Means and standard deviations obtained after 8 days on MEA at 4°C.
[2] Identifiable as *Mortierella* due to colony odour and habit and the presence of smooth chlamydospores, however no distinguishing characteristics were noted.
** References strain obtained from the Centraalbureau voor Schimmelcultures (CBS), the Netherlands.

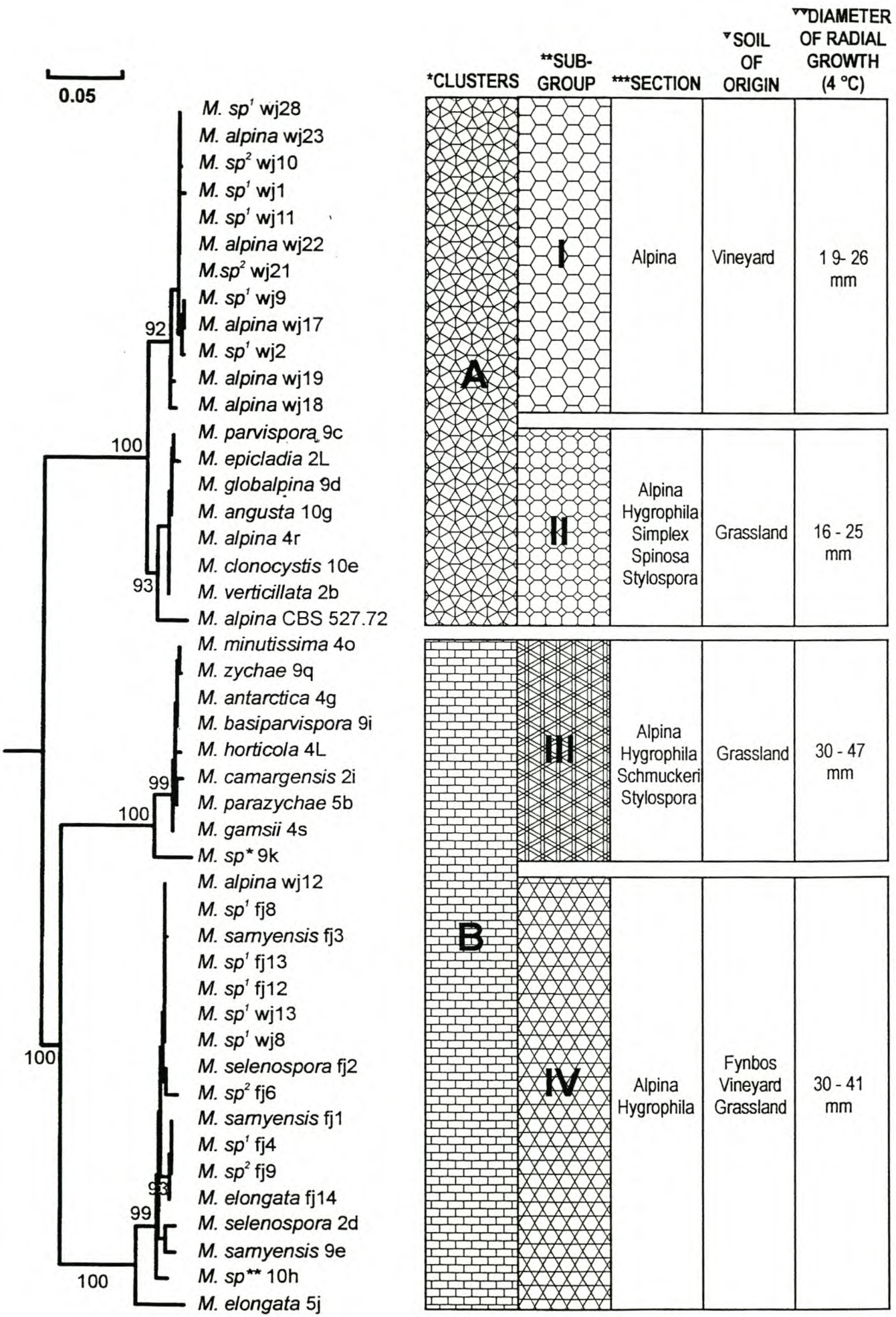

Figure 3.1. Phylogenetic tree based on ITS1 sequence data of *Mortierella* strains. Bootstrap values above 90 (expressed as percentages) based on 100 attempts are indicated at branch points. *Column highlights the two major clusters that formed. **Column highlights the four subgroups that formed. ***The sections of *Mortierella* subgenus *Mortierella* (Gams, 1977) in which the isolates of each subgroup that formed distinguishable characteristics, was classified. $^{\nabla}$Indicates the soil from which the isolates of each subgroup originated. $^{\nabla\nabla}$Range of colony diameters reached by the isolates of each subgroup after 8 days on MEA at 4°C.

three subgroups (II, III and IV), those from the vineyard in two (I and IV) and those from the fynbos in one (IV).

Considering only the pristine habitats, since the vineyard soil may contain strains that were introduced by man, the following becomes evident: Not only does the grassland soil contain more psychrotolerant *Mortierella* subgenus *Mortierella* species than the fynbos soil (Chapter 2; Tables 2.1 and 2.2), but the isolates from the grassland show a greater variation in ITS1 sequence composition and ability to grow at low temperatures than the fynbos isolates (Figure 3.1). These results, together with the fact that the fynbos isolates clustered in one subgroup (IV), which is characterised by a high growth rate at low temperatures, indicate that the environmental-physiological stress on *Mortierella* subgenus *Mortierella* is higher in the fynbos soil than in the grassland. This phenomenon of decreasing diversity in natural microbial communities as stress increases is well known (Atlas, 1984). The stress on the *Mortierella* subgenus *Mortierella* populations in fynbos soil may be as a result of lower nutrient conditions in this habitat than in the grassland. The organic matter content of the fynbos soil was *circa* 6 % (w/w), calculated using the method of Baldock and Skjemstad (1999), while the grassland soil, from which the isolates used in this study originated from, had an organic matter content of *circa* 40 % (w/w) (Botha *et al.*, 1999).

3.4. Conclusions

In literature, evidence exists that the fungal ITS 1 region plays a critical role in RNA processing, ribosome maturation and hence growth (Lavel & Nazar, 1998). In this study it was found that the ITS 1 sequence of a psychrotolerant strain of *Mortierella* subgenus *Mortierella* may indicate the original habitat and ability to grow at low temperatures. This correlation of the ITS sequence with the ecological habitat has also been observed in other fungal groups.

It was found that ITS sequence data correlates with the separation of isolates of the autoecious host-specific rust fungus *Puccinia carduorum* into two host groups (Berthier *et al.*, 1996). Similarly, phylogenetic analyses based on ITS sequences suggested that strains within an anastomosis group 4 of the soil-borne phytopathogenic species, *Rhizoctonia solani*, can be divided into three clusters that correlate with habitat and virulence (Boysen *et al.*, 1996). In addition, ITS sequence analyses of the causal agent of Dutch elm disease, *Ophiostoma ulmi*, revealed that ITS sequence composition correlates with the subdivision of this species into aggressive and non-aggressive isolates (Jeng *et al.*, 1996). More recently a new aggressive *Phytophthora* pathogen of alder trees in Europe was described containing a unique ITS sequence with additive nucleotide bases (Brasier *et al.*, 1999).

Considering the above findings on phylogenetically diverse fungal taxa, together with the results obtained on psychrotolerant representatives of *Mortierella* subgenus *Mortierella*, it seems that the ITS sequence may be indicative of the ecological habitat of a fungus. Therefor ITS sequence data in future may be of value in the determination of the original source of fungal contamination in agriculture, industry and medicine.

3.5. References

Atlas, R.M. (1984). Use of microbial diversity measurements to assess environmental stress. In: M.J. Klug & C.A. Reddy (Eds.) *Current perspectives in microbial ecology* (pp.540 - 545). American Society for Microbiology, Washington, D.C.

Baldock, J.A. & Skjemstad, J.0. (1999). Soil organic carbon/Soil organic matter. In: K.I. Perverill, L.A. Sparrow and D.J. Reuter (Eds.) *Soil analysis. An interpretation manual.* CSIRO Publishing, Collingwood, Australia.

Berbee, M.L. & Taylor, J.W. (1992). Detecting morphological convergence in true fungi, using 18S rRNA gene sequence data. *Biosystems* **28,** 117-125.

Berthier, Y.T., Bruckart, W.L., Chaboudez, P. & Luster, D.G. (1996). Polymorphic restriction patterns of ribosomal internal transcribed spacers in the biocontrol fungus *Puccinia carduorum* correlate with weed host origin. *Applied and Environmental Microbiology* **62,** 3037 - 3041.

Botha, A., Paul, I., Roux, C., Kock, J.L.F., Coetzee, D.J., Strauss, T. & Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* **75,** 253 – 256.

Boysen, M., Borja, M., del Moral, C., Salazar, O. & Rubio, V. (1996). Identification at strain level of *Rhizoctonia solani* AG4 isolates by direct sequence of asymetric PCR products of the ITS regions. *Current Genetics* **29,** 174 -181.

Brasier, C.M., Cooke, D.E.L. & Duncan, J.M. (1999). Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences USA* **96**, 5878 - 5883.

Gams, W. (1977). A key to the species of *Mortierella*. *Persoonia* **9,** 381 – 391

James, S.A., Collins, M.D. & Roberts, I.N. (1996). Use of a rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulaspora*. *International Journal of Systematic Bacteriology* **46**, 189 - 194.

Jeng, R., Hintz, W.E., Bowden, C.G., Horgen, P.A. & Hubbes, M. (1996). A comparison of the nucleotide sequence of the cerato-ulmin gene and the rDNA ITS between aggressive and non-aggressive isolates of *Ophiostoma ulmi sensu lato*, the causal agent of Dutch elm disease. *Current Genetics* **29**, 168 - 173.

Kasuga, T., Woods, C., Woodward, S. & Mitchelson, K. (1993). *Heterobasidion annosum* 5.8s ribosomal DNA and internal transcribed spacer sequence: rapid identification of European intersterility groups by ribosomal DNA restriction polymorphism. *Current Genetics* **24,** 433 – 436.

Klittich, C.J.R., Leslie, J.F., Nelson, P.E. & Marasas, W.F.O. (1997). *Fusarium thapsinum* (*Gibberella thapsina*): A new species in section *Liseola* from sorghum. *Mycologia* **89**, 643 - 652.

Lalev, A.I. & Nazar, R.N. (1998). Conserved core structure in the internal transcribed spacer 1 of the *Schizosaccharomyces pombe* precursor ribosomal RNA. *Journal of Molecular Biol*ogy **284,** 1341-1351.

Lin, D., Wu, L.C., Rinaldi, M.G. & Lehmann P.F. (1995). Three distinct genotypes within *Candida parapsilosis* from clinical sources. *Journal of Clinical Microbiology* **33,** 1815 -1821.

Lott, T.J., Randall, J., Kuykendall, R.J. & Reiss, E. (1993). Nucleotide sequence analysis of the 5.8S rDNA and adjacent ITS2 region of *Candida albicans* and related species. *Yeast* **9,** 1199 - 1206.

Mitchell, J.I., Roberts, P.J. & Moss, S.T. (1995). Sequence or structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. *Mycologist* **9,** 67 – 75.

O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genettics* **22,** 213 - 220.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiol*ogy **1,** 17 - 20.

Valente, P., Ramos, J.P. & Leoncini, O. (1999). Sequencing as a tool in yeast molecular taxonomy. *Canadian Journal of Microbiol*ogy **45,** 949-958.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innes, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.) *PCR Protocols* (pp.315 - 322). San Diego, Academic Press.

Wilmotte, A., Van de Peer, Y., Goris, A., Chapelle, S., De Baere, R., Nelissen, B., Neefs, J-M., Hennebert, G.L. & De Wachter, R. (1993). Evolutionary relationships among higher fungi inferred from small ribosomal subunit RNA sequence analysis. *Systematic and Applied Microbiol*ogy **16,** 436 – 444.