Comparative Enzyme Studies of *Microsporum canis* and *Microsporum cookei* in Relation to their Pathogenicity and Diversity.

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ABSTRACT

Infections by dermatophytes can be contracted from animals, humans, soil or contaminated fomites. In the genus *Microsporum*, some species e.g. *M. canis* are commonly associated with cats and dogs which act as an important reservoir for human infections. Others, e.g. *M. cookei* are nonpathogenic and found in the soil. The present studies have investigated the incidence of these ecologically contrasting species on cats, dogs and in the soil, their enzyme expression, and enzyme types as identified by proteinase inhibitors, gelatin/SDS-PAGE and multilocus enzyme electrophoresis, and have led to an investigation of their phenotypic variation. The primary aim was to attempt to detect differences in enzyme production which might be related to mechanisms of pathogenicity of *M. canis*.

Isolation procedures employed were the hairbrush technique for small animals and the keratin-baiting technique for soil with samples being cultured on SDA containing antibiotics. Soil samples revealed 19 fungal genera, three being of keratinolytic fungi, representing 50% of total isolations. *Trichophyton* species were the most common (39% samples) but *M. cookei* was isolated from 6.8%.

Fungi isolated from cats and dogs represented 20 genera, with the predominant isolates being keratinolytic fungi (51.9% of total samples). Cats were the major carriers of keratinolytic fungi (*Chrysosporium*, *Microsporum* and *Trichophyton*). *M. canis* was frequently isolated (18.5% of cats) and its distribution had a seasonal variation, with a peak appearing in May-June.

All isolates of *M. canis* were of the "-" mating type. *M. cookei* isolates were of both the "+" and "-" mating types, but "+" types were predominant.

Biochemical assays showed that *M. canis* produced higher proteinase and keratinase activities in shake cultures than in stationary cultures. Elastase activity was greater in stationary cultures. *M. cookei*'s proteinase and keratinase activities were lower but again greater in shake cultures. There was no detectable keratinase activity in stationary cultures of *M. cookei*, and no significant difference in elastinolytic activity

between shake and stationary cultures. Growth in shake culture produces the "pseudo-parasitic" morphology which mimics that found in infection, therefore, the differing enzyme expression of the two *Microsporum* species may be a reflection of their differing ecological roles.

Characterisation of the enzymes with chemical inhibitors revealed that *M. canis* and *M. cookei* produced serine proteinases, but only *M. canis* produced cysteine and probably aspartic and metallo-proteinases. The serine and cysteine proteinases are considered likely to be of particular significance in the pathogenesis of *M. canis* infections.

Using substrate copolymerised gel electrophoresis (gelatin\SDS-PAGE), shake and stationary cultures were again compared for enzyme expression. Among the six different M_r proteinases (122 KDa, 64 KDa, 62 KDa, 44 KDa, 36 KDa, and 28 KDa) expressed by *M. canis*, three (122 KDa, 62 KDa and 28 KDa) were found to be more highly expressed in shake cultures. In contrast, *M. cookei* isolates expressed seven different proteinases (67 KDa, 64 KDa, 63 KDa, 62 KDa, 54 KDa, 52 KDa, and 42 KDa), of which two (67 KDa, 64 KDa) were expressed only in stationary cultures and one (52 KDa) although expressed in shake cultures was more highly expressed in stationary cultures. Possibly the high and low M_r proteinases expressed by *M. canis* are more important in its pathogenicity than the middle range proteinases also detected in *M. cookei*.

Multilocus enzyme electrophoresis using starch gels and examining eight enzymes, showed *M. canis* to be phenotypically more diverse than *M. cookei* as measured by the normalised Shannon-Wiener diversity statistic. *M. canis* showed a substantial within population variability (84.9%) by geographical region, with a moderate level (21.7%) of interpopulation differentiation. Cluster analysis confirmed this diversity and also revealed a possible grouping of isolates from clinical infections, and based on the accumulated data of these studies, EST phenotype 9 although present in a few carrier isolates was commonly associated with isolates from clinical cases and perhaps deserves further investigation.

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LIST OF ABBREVIATIONS

3A9EC = 3-amino-9-ethylcarbazole
BPB = Bromophenol blue
CAT = Catalase
DDW = Double distilled water
DFM = Dimethyl formamide
DW = Distilled water
E-64 = L-trans-epoxysuccinyl leucylamido (4-guanidino)-butane
EC = Enzyme Commission
EST = Esterase
Fast blue B = o-dianisidine dihydrochloride
G6P = Glucose-6-phosphate dehydrogenase
G6PDH = Glucose-6-phosphate dehydrogenase
GPI = Glucose-6-phosphate isomerase
IAA = Iodoacetic acid
ITM = Prince Leopold Institute for Tropical Medicine
IUBNC = International Union of Biochemistry
LAP = Leucine aminopeptidase
MDH = Malate Dehydrogenase
M_r = Molecular weight
MTT = Methyl thiazolyl tetrazolium
NAD = β-Nicotinamide adenine dinucleotide
NADP = Nicotinamide adenine dinucleotide phosphate
NBT = Nitro blue tetrazolium
PAGE = Polyacrylamide gel electrophoresis
*p*CMB = *p*-Chloromecuric acid
α_1-P = α-1-proteinase
PEPS = Pepstatin
PEP = Peptidase
PER = Peroxidase
PMS = Phenazine methosulphate
PMSF = phenylmethylsulfonyl fluoride

LIST OF ABBREVIATIONS Contd.

PT = 1,10-phenanthroline
SDS = Sodium dodecyl sulfate
SDW = Sterile distilled water
SGE = Starch gel electrophoresis
SPCA = Society for the Prevention of Cruelty to Animals
TCA = Trichloroacetic acid
TEMED = Tetramethyl ethylenediamine
TRIS = Tris[hydroxymethyl]aminomethane

PREFACE

Medical mycology can be said to have originated with the demonstration by Agostino Bassi in 1835 of the relationship between a disease of silkworms known as muscardine and its causal agent, a fungus, *Beauvaria bassiana* (Utz, 1981). This disease had threatened to destroy the silk industry in France and Italy (Ajello, 1977). But after Bassi's discoveries, other early work was concentrated on the superficial fungal diseases of man.

Robert Remak in 1837 observed spores and hyphae in crusts recovered from a child suffering from favus and later published accounts of the fungus in hair shafts obtained from the patient (Howard, 1983). He also successfully reproduced the disease by self-inoculation. In 1845, he cultivated and named the aetiological agent, *Achorion schöenleinii*, during his work in his mentor Professor Schöenlein's clinic. The fungus is now more commonly known as *Trichophyton schöenleinii*.

Although Remak was the first to associate a microorganism with human disease, the studies of David Gruby made a greater contribution to medical mycology (Ajello, 1974; Howard, 1983). Between 1841 and 1844, Gruby released several publications which described the main pathogens causing ringworm and also independently described the fungal nature of favus (Wilson and Plunkett, 1974). In 1843, he published an account of scalp ringworm caused by a fungus which he named *Microsporum audouini* (on the basis of the *in vivo* growth pattern), in honour of a colleague, Victor Audouin. His work also included studies of endothrix trichophytosis and thrush, a fungal disease of the mouth caused by *Candida albicans*.

Another who contributed a great deal to the development of our knowledge of the ringworm fungi or dermatophytes was Raymond Sabouraud. Sabouraud, in 1892 started issuing numerous reports which culminated in the publication of "Les Teignes" (Sabouraud, 1910). Sabouraud was able, through the techniques of pure culture, which he introduced into medical mycology, to demonstrate the plurality of dermatophytes. He placed these fungi into four genera: *Achorion*,

Epidermophyton, *Microsporum* and *Trichophyton* (Sabouraud, 1910, cited by Ajello, 1968; Seelinger, 1988).

Sabouraud had realised the complex manner in which these fungi grow in culture, with successive cultures taken from the parent stock often showing variation. This capacity for variation can render dermatophytes very difficult to identify and resulted in great difficulties in devising a uniform, internationally accepted classification (Ajello, 1962).

The following years saw incomplete and inaccurate reporting because diagnosis was not based on sound mycological techniques but on small variations in clinical appearance of lesions or slight differences in colonial morphology. The natural history of the fungus was unknown or ignored (Wilson and Plunkett, 1974; Howard, 1983). Due to numerous misleading reports, several hundred "new species" were described and named as human pathogens. Dodge (1935) in his monograph even described 118 dermatophyte species, placed in 9 genera (Baxter and Rush-Munro, 1980b). This confusion, which hindered clinicians in classifying human disease on a mycological basis, forced them to adopt a clinical-anatomical or topographical categorisation.

In 1934, Emmons developed and outlined in extensive detail a strict botanical classification based on accepted rules of nomenclature and using fungal morphology *in vitro*, which avoided classification systems based on clinical appearance. He placed the dermatophyte species into three genera, *Epidermophyton*, *Microsporum* and *Trichophyton*, embracing 18 species (Emmons, 1934). This was generally well accepted by mycologists and clinicians alike (Ainsworth, 1986).

The dermatophytes can be included in an ecological group known as the keratinophilic fungi i.e. fungi with an affinity for keratin. Such fungi may merely use keratin as a surface for growth. In other cases simple mechanical penetration of the substrate may be achieved. But some including the dermatophytes and a number of dermatophyte-like fungi have a marked ability to enzymatically digest keratin and can be termed keratinolytic fungi. The keratinolytic fungi comprise the dermatophytes and certain other fungi such as *Chrysosporium* spp..

The use of Vanbreuseghem's (1952) hair-baiting technique has enabled the detection and isolation of soil-borne (geophilic) non-pathogenic, keratinolytic fungi. Notable amongst these are *Trichophyton (Keratinomyces) ajelloi* (Vanbreuseghem, 1952) Ajello, 1968, *T. terrestre* Durie and Frey, 1957 and *M. cookei* Ajello, 1959. All these geophilic species, with regard to morphology, sexual behaviour (Campbell, 1988) and antigenicity (Mackenzie, 1988) are dermatophytic. The only factor differentiating them from true dermatophytes is their inability to cause disease in man and animals.

The dermatophytes in the broadest sense can be divided into three ecological groups, zoophiles (mammalian and avian hosts); anthropophiles (human hosts) and geophiles, which for the most part degrade keratinous material, e.g. skin, hair, hooves, horns, feathers, in the soil. Of the zoophiles at least ten species are recognised, three of which are of real importance to man, namely; *Microsporum canis*, *Trichophyton mentagrophytes* (and its varieties) and *T. verrucosum.* Of the three, *Microsporum canis* is the most important epizoonotic fungal pathogen, causing a severe public health problem on a world-wide scale.

Besides its impact on human health, there are also social and economic considerations as a result of its infections. For example, ringworm of the scalp is (wrongly) considered to be a social stigma (English, 1972). In New Zealand, the most susceptible age of infection is up to 15 years but the impact of loss of school days on school-going children has not been documented (Mycoses Newsletter, CDCNZ, 1992). In addition to human suffering, the economic cost of medical consultation and drugs for treatment is not known.

M. canis is responsible for a polymorphism of scalp and skin infections in both humans and animals. Its ability to produce enzymes has been implicated in the pathogenesis of skin infections of the host organisms, in countering host defense mechanisms and for providing its nutritional needs. It has been suggested that extracellular enzymes involved in pathogenesis include keratinases, proteinases, elastases, peptidases, aminopeptidases, catalases and peroxidases (Ernst, 1989).

In contrast to *M. canis*, the soil inhabiting dermatophyte, *M. cookei* is of little medical importance. It is morphologically rather similar to *M. canis*

and also has a world-wide distribution. Although it may be isolated from animals, clinical disease is not observed (Rees, 1967). Even though tinea corporis caused by *M. cookei* has been reported in humans (Frey, 1971), the fungus must be considered a non-pathogen.

Accepted therapeutic measures alone do not appear to have altered materially the frequency and course of *M. canis* infection in most communities. An increase in our knowledge of the biological and biochemical properties of a pathogen compared to a nonpathogen could suggest ways of controlling and treating infections. The relatively small number of effective antifungal agents reflects to a large extent the fact that many aspects of fungal physiology and virulence are not well understood (Ernst, 1989). The determination of relative sizes and numbers of, for example, proteinases expressed could suggest possible mechanisms of pathogenicity involving certain of the enzymes and therefore ways of making drugs and/or vaccines for controlling dermatophyte pathogens.

The main purpose of the work to be reported in this thesis was to study and compare aspects of the ecology and biochemical variability of *M. canis*, a pathogen and *M. cookei*, a nonpathogen and to investigate certain enzymes produced by these fungi e.g. keratinases, proteinases and elastases which have been implicated in the pathogenesis of disease. Furthermore, genetic studies of these species are practically non existent. The genetics of the group is not well characterised and there is a lack of suitable methods and scoreable markers for assaying variability in natural populations. Thus an investigation of enzyme marker systems, especially for enzymes implicated in pathogenicity, could be useful in determining phenotypic or genetic relationships among strains.

CHAPTER 1

INTRODUCTION - LITERATURE REVIEW

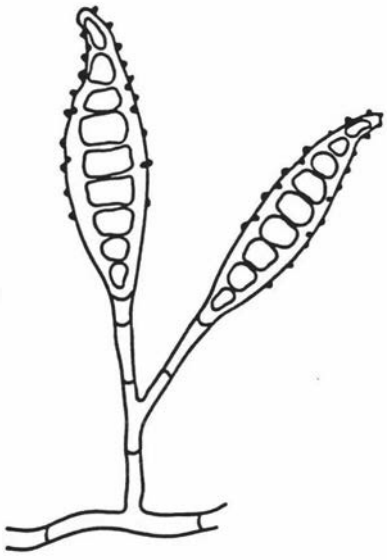
1.1 DERMATOPHYTES.

In culture dermatophyte morphology, for purposes of nomenclature, can be divided into two states on the basis of stage in the life cycle, the anamorphic and the teleomorphic states. The anamorph is the state where asexual or somatic reproduction occurs and has a distinct morphology. The teleomorph, on the other hand, is the sexually reproductive ("perfect") state, morphologically (and/or karyologically) differentiated from the anamorph (De Vroey and Hennebert, 1982). A workable classification of dermatophytes is best based on the macroscopic and microscopic morphology of the asexual state in culture and the Emmons (1934) classification system emphasises these characteristics.

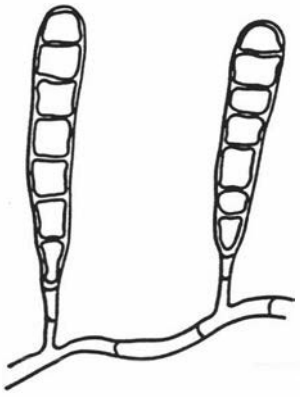
1.1.1 The Anamorphic States - Classification and Identification.

The microscopic appearance of the various species shows a range of vegetative structures and spores (conidia), viz., large multiseptate macroconidia, single-celled microconidia with typical arrangement on hyphae, chlamydospores, spirals, antler-shaped hyphae (chandeliers), nodular organs, pectinate organs and racquet hyphae (Emmons, 1934; Ajello, 1966) (Fig.1-1).

Fig.1-1: Principal microscopic structures used in identifying dematophyte species.

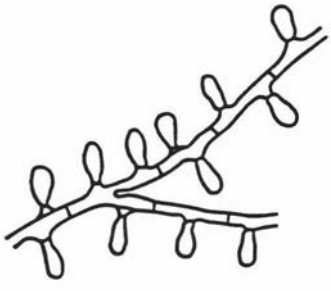


macroconidia of
Microsporum

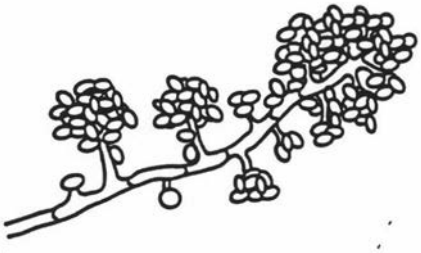


macroconidia of
Trichophyton

macroconidia of
Epidermophyton



microconidia along sides
of vegetative hyphae



microconidia in grape-like
bunches (en grappe)

In addition, some physiological characteristics based on nutritional requirements (Stockdale, 1953a, 1953b; Georg and Camp, 1957a; Philpot, 1977b; Ajello, 1966), such as vitamin deficiency can be used to help to identify some dermatophytes.

Most dermatophyte colonies develop forms and pigmentation which can allow a presumptive identification of that dermatophyte species. Colony appearance of a fungus depends on the medium used but for comparative purposes, Sabouraud dextrose agar (SDA) medium is conventionally used to obtain colonies which can be compared to others reported in the literature (Ajello, 1966).

Ajello (1966) lists five important colony characteristics to look for in presumptive identification of a dermatophyte culture when it is one to three weeks old:

1. rate of growth
2. general topography (flat, heaped, regularly or irregularly folded)
3. texture (yeast-like, glabrous, powdery, granular, velvety or cottony)
4. surface pigmentation
5. reverse pigmentation

Based on the above criteria, particularly on differences in conidial morphology, dermatophyte species can be classified into three genera within the Fungi Imperfecti (or Deuteromycotina) namely; *Epidermophyton*, *Microsporum*, and *Trichophyton* (Emmons, 1934). The studies of Cole and Samson (1979) have shown that the ontogeny of the holothallic conidia of *Microsporum* and *Trichophyton* is essentially the same. Their only difference is the macroconidial cell-wall thickness and presence of echinulations in *Microsporum* species which are absent in *Trichophyton* species (Ajello,l977; McGinnis, 1980).

However, there has been some controversy in the broad classification of some dermatophytes. Benedek (1948) felt that the genus *Achorion* should have been retained by Emmons (1934) as was *Epidermophyton*

simply because of its "established usage" and that Emmon's proposal cannot be considered a natural classification. The system proposed by Vanbreuseghem (1977) and Vanbreuseghem *et al.*, (1978), distinguished the genera *Epidermophyton*, *Microsporum*, *Trichophyton*, *Microides* and *Keratinomyces*. Ajello (1982) rejected Vanbreuseghem's genus *Microides* based on the similarity in morphology of *M. interdigitalis* to *T. mentagrophytes*. He proposed that *M. interdigitalis* should be considered as a variety of *T. mentagrophytes*, to be known as *T. mentagrophytes var. interdigitale*. (Emmons (1934) considered *T. interdigitale* to be a synonym of *T. mentagrophytes*).

One particularly controversial genus is *Keratinomyces*. This genus, with its species *K. ajelloi*, was established by Vanbreuseghem (1952b) but was modified by Ajello in 1968 to *T. ajelloi* due to the earlier inadequate treatment of the genus by not providing essential facts that differentiate *Keratinomyces* from the genus *Trichophyton*. An important reason for the transfer was the observation that *K. ajelloi* apparently produces microconidia (Georg *et al.*, 1959b). The transfer was further supported by the fact that *K. ajelloi* produced cleistothecia of the genus *Arthroderma* (discussed under Section 1.1.4), in which the sexual states of *Trichophyton* were classified (Dawson and Gentles, 1961).

However, antigenic studies have shown that *T. ajelloi* has little similarity to *Trichophyton* species, emphasing the need to retain its original designation. Additionally *K. ajelloi* has thick smooth cell walls while *Trichophyton* species have thin walls (Vanbreuseghem, 1952b; Vanbreuseghem, 1977; Vanbreuseghem *et al.*, 1978; Mackenzie, 1988).

There are also disagreements of the species concept in certain groups, for example, Vanbreuseghem *et al.*, (1978) separated *Microsporum langeroni* (Vanbreuseghem, 1951) and *M. rivalieri* (Vanbreuseghem, 1963a) from the classic *M. audouinii* Gruby, 1843, a move which has not been favoured by several American and European workers.

The principal classification systems of dermatophytes, compared to that of Sabouraud is presented below in Table 1-1.

Table 1-1: Classification of dermatophytes.

Sabouraud	Vanbreuseghem *et al.*	Emmons, Ajello *et al.*
1. *Epidermophyton* Lang, 1879	*Epidermophyton*	*Epidermophyton*
2. *Microsporum* Gruby, 1843	*Microsporum* (+*Achorion gypseum*)	*Microsporum* (+*A. gypseum*)
3. *Achorion* Remak, 1845	*Trichophyton* (-*A. gypseum*)	*Trichophyton* (-*A. gypseum*)
4. *Trichophyton* Malmsten, 1845		
.endothrix	*Trichophyton*	*Trichophyton*
.ectothrix megaspore	*Trichophyton*	*Trichophyton*
.ectothrix microides	*Microides*,	*Trichophyton*
5. Unknown	*Keratinomyces*	*Trichophyton*

(- = excluding *A. gypseum*; + = including *A. gypseum*)
Modified from Vanbreuseghem *et al.*, 1978

Besides the traditional mycological criteria of identification, other techniques applied more recently have been based on analysis of serological antigens (Philpot, 1978b), comparison of DNA base compositions (Davidson *et al.*, 1980; Davidson and Mackenzie, 1984), fatty acid composition (Jones and Noble, 1981) and enzyme isoelectric focusing (Jeffries, 1982; Jeffries *et al.*, 1984). Such studies and others have supported the differentiation of morphologically and physiologically similar species e.g. *M. canis*, *M. equinum* and *M. distortum* and *T. kuryangei* and *T. megninii* (Jeffries, 1982; Jeffries *et al.*, 1984). Radiolabelled DNA hybridization techniques employed by Davidson and Mackenzie (1984) as a taxonomic tool, supported the present

mycological grouping of dermatophytes based on the limited number of species examined.

Additional recent developments include the use of electrophoretic protein patterns (discussed under Section 1.8.2) used in the study of *M. canis* and reported to be able to distinguish between genetically similar strains of *M. canis* (Tucker and Noble, 1992). Also, Mochizuki *et al.*, (1990) used mitochondrial DNA restriction analysis to investigate the taxonomical relationship between *T. interdigitale* (*T. mentagrophytes var. interdigitale*) and other members of the *T. mentagrophytes* complex (discussed under Section 1.1.4).

1.1.2 Current Concepts of the Anamorphic State.

On the basis of anamorph morphology, 2 species of *Epidermophyton*, approximately 18 species of *Microsporum* (Table 1-2) and 25 species of *Trichophyton* (Table 1-3) are considered valid members of these genera

The main features distinguishing the three dermatophyte genera are:

Epidermophyton
The genus is characterised by large macroconidia which are thin-walled, multicellular, club-shaped and clustered in bunches. Microconidia are not produced. The genus' features are based on *E.floccosum* (Emmons, 1934).

Microsporum
The genus produces both micro- and macroconidia. Macroconidia are multiseptate, with a thin or thick echinulate cell wall (Krempl-Lamprecht, 1986), spindle shaped and may be numerous or scarce. However, the essential distinguishing feature of this genus is the echinulations of the macroconidial cell wall. The thickness of the cell wall and shape varies depending on the species. Microconidia are pyriform, about 2-3µm. The type species is *M.audouinii* Gruby, 1843.

Table 1-2: The principal members of the genera *Epidermophyton* and *Microsporum*.

Epidermophyton Sabouraud,1910	
E. floccosum (Harz,1870) Langeron & Milochevitch, 1930	*M. fulvum* Uriburu, 1909
E. stockdaleae Prochacki & Englehardt-Zasada, 1974	*M. gallinae* (Megnin, 1881) Grigorakis, 1929
Microsporum Gruby, 1843	*M. gypseum* (Bodin,1907) Guiart & Grigorakis,1928
M. amazonicum Moraes, Borelli & Feo, 1967	*M. magellanicum* Coretta & Piontelli, 1977
M. audouinii Gruby, 1843	*M. nanum* Fuentes, 1956
M. boullardii Dominik & Majchrowicz, 1965	*M. persicolor* (Sabouraud 1910) Guiart & Grigorakis, 1928
M. canis Bodin, 1902	*M. praecox* Rivalieri, 1954
M. cookei Ajello, 1959	*M. racemosum* Borelli,1965
M. equinum (Bodin,1898) Guegen, 1904	*M. ripariae* Hubalek & Rush-Munro, 1973
M. distortum Di Menna & Marples, 1954	*M. vanbreuseghemii* Georg, Ajello, Friedman & Brinkman, 1962
M. ferrugineum Ota, 1921	

Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1978), Howard (1983) and Rippon (1985)

Trichophyton

This genus produces smooth walled macroconidia and microconidia. Macroconidia are thin walled and cigar-shaped. Microconidia may be pyriform 2-3µm or irregular in form. Some species rarely produce macroconidia. The type species is *T.tonsurans* Malmsten, 1845.

Table 1-3: Members of the genus *Trichophyton*+ (Malmsten, 1845).

T. ajelloi (Vanbreuseghem, 1952) Ajello, 1968	*T. phaseoliforme* Borelli & Feo, 1966
T. concentricum Blanchard, 1895	*T. rubrum* (Castellani, 1910) Sabouraud, 1911
T. equinum (Matruchot & Dassonvile, 1898) Gedoelst, 1902	*T. schöenleinii* (Lebert, 1845) Langeroni & Milochevitch, 1930
T. flavesens Padhye & Carmichael 1971	*T. simii* (Pinoy, 1912) Stockdale, Mackenzie & Austwick, 1965
T. georgiae Varsavsky & Ajello 1964	*T. soudanense* Joyeux, 1912
T. gloriae Ajello, 1967a	*T. terrestre* Durie & Frey, 1957
T. gourvilii Catanei, 1933	*T. tonsurans* Malmsten, 1845
T. longifusus (Florian & Galgoczy, 1964) Ajello, 1968	*T. vanbreuseghemii* Rioux, Jarry & Juminer, 1964
T. mariatti Ajello & Cheng, 1967	*T. verrucosum* Bodin, 1902
T. megninii Blanchard, 1896	*T. violaceum* Bodin, 1902
T. mentagrophytes (Robin, 1853) Blanchard, 1896	*T. yaoundei* Cochet & Doby-Dubois, 1957
var. interdigitale Priestley, 1917	
var. erinacei Smith & Marples, 1963	
var. quinckeanum (Zopf,-?) Macleod & Muende, 1940	

+ Various authors differ in their treatment of certain of the species.
Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1978), Howard (1983) and Rippon (1985)

1.1.3 *M. canis* and *M. cookei*.

The species selected for the present comparative investigations were the two most frequently encountered *Microsporum* species, *M. canis*, a very common zoophilic and pathogenic dermatophyte and *M. cookei*, a common geophilic, non-pathogenic, dermatophyte.

Colonies on SDA and Microscopic Characteristics.

M. canis grows fairly rapidly, producing a cottony fluffy mycelium with bright yellow pigment in the peripheral growth (Fig.1-2). The reverse is golden yellow (Fig.1-3), which is very characteristic for *M. canis*.

Macroconidia are many, large and thick walled, spindle-shaped terminating in a distinct knob and echinulated (Fig.1-4), 8-20x40-150µm, with 2-15 septa. Microconidia normally scarce, clavate, sessile or borne on short pedicels laterally on the hyphae.

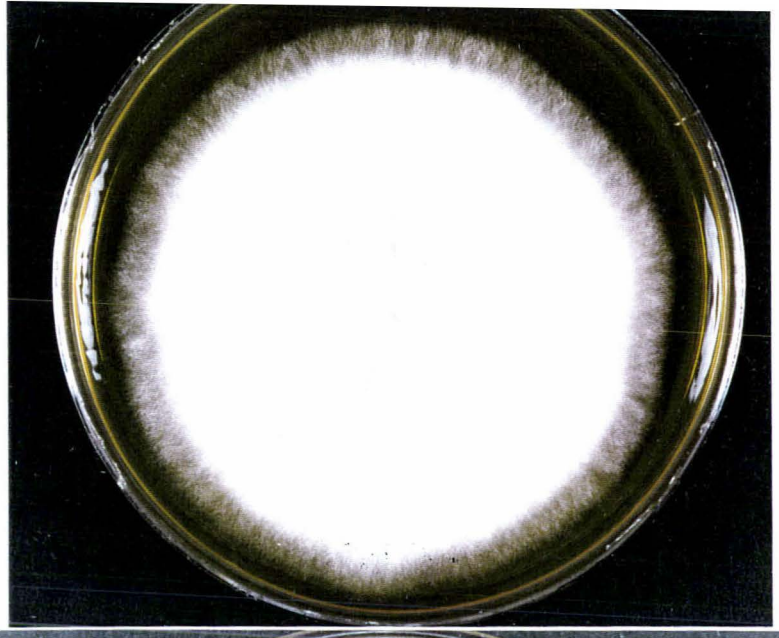
M. cookei grows rapidly and is flat and spreading, with a powdery surface which is buff or dark tan (Fig.1-5). The reverse is deep red-brown (Fig.1-6).

Macroconidia are oval to ellipsoidal, thick walled and echinulated, 10-15x31-50µm with 2-8 septa. Microconidia are obvate and produced abundantly (Fig.1-7).

Plate 1-1: Colony of *M. canis* on SDA with fluffy growth after 2 weeks at 25°C.

Plate 1-2: Reverse with golden yellow pigmentation.

Plate 1-3: *M. canis* Spindle shaped, echinulated and pointed macroconidia, and microconidia (magnification x400).



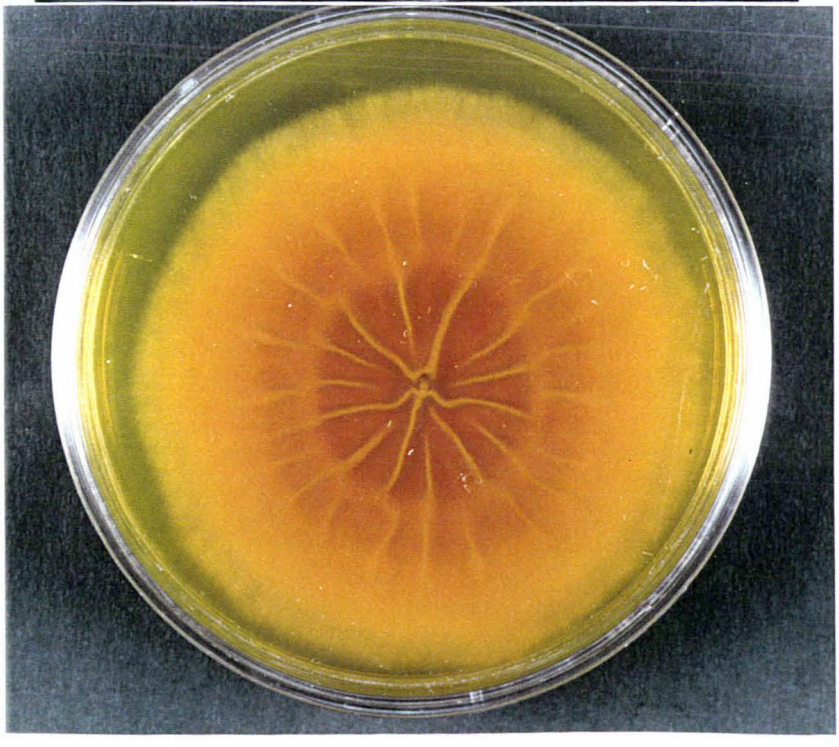
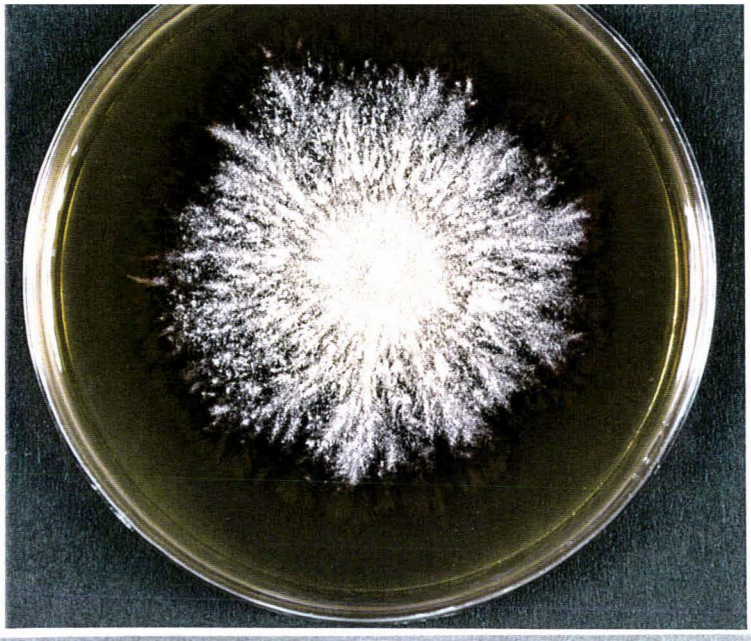
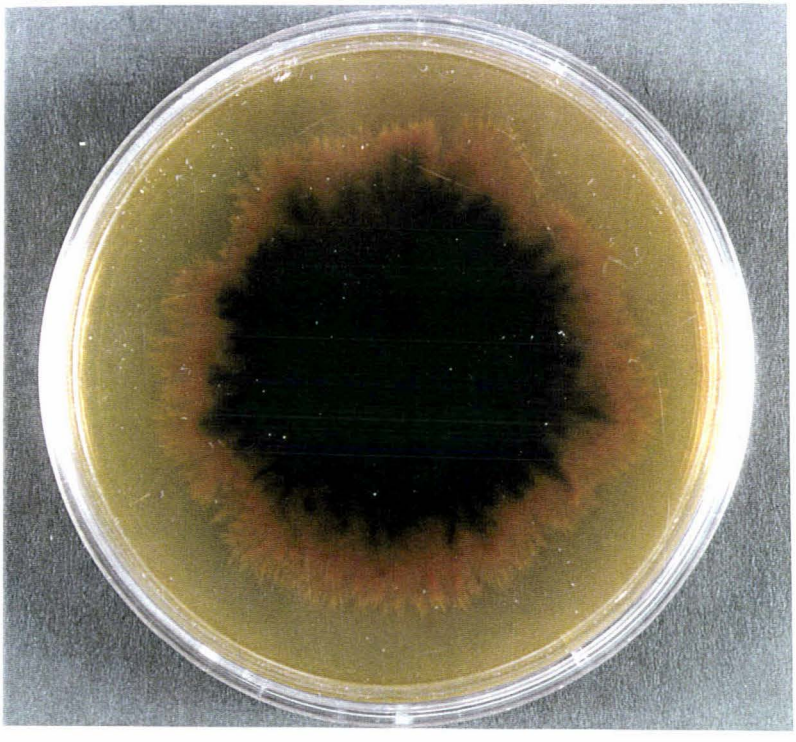


Plate 1-4: Colony of *M. cookei* on SDA with granular texture after two weeks at 25°C.

Plate 1-5: Reverse with deep red-brown pigmentation.

Plate 1-6: *M. cookei* Spindle shaped, echinulated and less pointed macroconidia and numerous microconidia (magnification x400).





1.1.4 The Teleomorphic States.

The existence of a sexual phase in the growth cycle of dermatophytes was first reported by Nannizzi in 1927. He described the perfect stage of a strain of *Microsporum* (*Sabouraudites*) *gypseum* (Bodin) Guiart and Grigorakis, 1928, when he obtained cleistothecia with ascospores by cultivating *M. gypseum* on soil baited with feathers (Griffin, 1960b; de Vries, 1962). He named the fungus *Gymnoascus gypseus* (Griffin, 1960c; Howard, 1983). His work was discredited by Langeron and Milochevitch (1930) and other workers in the field on the grounds that the soil and feathers used were not sterilised. However, Nannizzi's work was later confirmed by Griffin (1960a, 1960c) and Stockdale (1961) and at the sametime Dawson and Gentles (1961) demonstrated the existence of a sexual stage of *Trichophyton terrestre* Durie and Frey, which they named *Arthroderma quadrifidum*.

Since then the perfect states of a number of dermatophytes have been discovered and this has resulted in some changes to the classification of these fungi (Ajello, 1974, 1977; Vanbreuseghem, 1977; Takashio, 1979). All are members of the subdivision *Ascomycotina* which includes all fungi that after nuclear fusion and chromosomal reduction, proceed to form asci and ascospores. They belong to the Class *Plectomycetes*, Order *Onygenales* of Alexopoulos and Mims, 1979.

The production of asci occurs inside an ascocarp (cleistothecium or gymnothecium, as proposed by Padhye and Carmichael, 1971). The wall (peridium) of the cleistothecium is composed of loosely interwoven, thin-walled, light coloured hyphae, which is characteristic of the family *Gymnoascaceae*.

In the genus *Arthroderma* Berkeley, 1860 the outer cells of the peridial hyphae are short and markedly swollen at each end, appearing constricted in the middle. The swelling may be symmetrical or mostly on the outward side of the curved hyphae. The swellings are thick-walled and markedly spiny, while the short intervening constricted portion is thin walled, smooth and inconspicuous (Padhye and Carmichael, 1971).

> The second genus *Nannizzia* (Stockdale, 1961) is characterised by the peridial hyphae being branched in a verticillate manner and composed of thick-walled, aseptate, hyaline cells with one or more symmetrical constrictions. There are numerous free ends and various appendages - ring, straight or loosely coiled hyphae and spiral hyphae (Stockdale, 1961).

It has been proposed that the two genera should be unified into one genus: *Arthroderma* Berkeley, 1860 because the morphological differences are not significant to warrant a new genus (Vanbreuseghem *et al.*, 1978; Weitman *et al.*, 1986). But Stockdale (1961) had argued that the branching pattern of *Arthroderma* is consistently dichotomous and never verticillate while *Nannizzia* is commonly verticillate, sometimes dichotomous and rarely uncinate. Stockdale (1961) considers these differences of generic significance while McGinnis (1980) and Weitzman *et al.* (1986) regard them as of little significance. Another important difference which McGinnis (1980) and Weitman *et al.* (1986) overlook is the fact that cross-mating between the two genera has never been reported to occur.

Mating studies have led to the discovery of the perfect states of a number of dermatophyte species. Some of the anamorphic states have been found to represent a complex of species, e.g. the *M. gypseum-fulvum* complex, the *T. terrestre* complex and the *T. mentagrophytes* complex.

The *M. gypseum-fulvum* complex represents three sexual dermatophyte species, namely, *Nannizzia incurvata* (Stockdale, 1961), *N. gypsea* and *N. fulva* (Stockdale, 1963).

Similarly, the *T. terrestre* complex represents three sexual states, *Arthroderma quadrifidum*, *A. insingulare*, and *A. lenticularum* (Padhye and Carmichael, 1968, 1972). Both *A. quadrifidum* and *A. insingulare* have been isolated from soil, animal hair and feathers as *T. terrestre* (Marples and Smith, 1962b; Marples, 1965; Beneke and Rogers, 1980).

The *T. mentagrophytes* complex represents two sexual dermatophyte species, *Arthroderma vanbreuseghemii* (Takashio, 1973b) and *A.*

benhamiae (Ajello and Cheng, 1967). *T. mentagrophytes var. interdigitale*, one of the most prevalent species and of great public health importance, is an imperfect species resembling morphologically the conidial state of *A. vanbreuseghemii*. Mitochondrial DNA restriction enzyme mapping has shown that the restriction profiles of strains of *T. mentagrophytes var. interdigitale* and *A. vanbreuseghemii* are identical (Mochizuki, *et al.*, 1990), reinforcing a linkage of the two species, as postulated from its morphology (Watanabe and Hironaga, 1981). *T. mentagrophytes var. interdigitale* is therefore considered a member of the *T. mentagrophytes* complex.

Takashio (1973a, 1973b, 1974, 1977, 1979) and Vanbreuseghem and Takashio (1981) have suggested that the species *A. benhamiae* can be divided into two races, one Americano-European and one African, with two varieties, *var. caviae* and *var. erinacei* respectively. The variety *erinacei* has two mating types: the "+" corresponding to *T. erinacei* (Padhye and Carmichael, 1968) or *T. mentagrophytes var. erinacei* (Smith and Marples, 1963). The "-" species was isolated from the African hedgehog (Collinge *et al.*, 1974). *A. benhamiae var. caviae* has only the "-" mating type.

To date, 11 conidial species of *Trichophyton* (*Arthroderma*, Table 1-4) and 10 species of *Microsporum* (*Nannizzia*, Table 1-5) are known to reproduce sexually (De Vroey, personal communication).

Table 1-4: *Trichophyton* species with a known teleomorphic state.

Teleomorph	Anamorph
Arthroderma Berkeley, 1860	*Trichophyton* Malmsten, 1845
A. curreyi Berkeley, 1860	Not named
A. tuberculatum Kuehn, 1960	Not named
A. benhamiae Ajello & Cheng, 1967	*T. mentagrophytes var. mentagrophytes*
A. ciferrii Varsavsky & Ajello, 1964	*T. georgiae*
A. flavescens Rees, 1967	*T. flavescens*
A. gertleri Böhme, 1967	*T. vanbreuseghemii*
A. gloriae Ajello, 1967a	*T. gloriae*
A. insingulare Padhye & Carmichael, 1972	*T. terrestre*
A. lenticularum Pore, Tsao & Plunkett, 1965	*T. terrestre*
A. quadrifidum Dawson & Gentles, 1961	*T. terrestre*
A. simii Stockdale, Mackenzie & Austwick, 1965	*T. simii*
A. uncinatum Dawson & Gentles, 1961	*T. (K.) ajelloi*
A. vanbreuseghemii Takashio, 1973	*T. mentagrophytes var. interdigitale* (some)

Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1978), Howard (1983) and De Vroey (personal communication).

Table 1-5: *Microsporum* species with a known teleomorphic state.

Teleomorph	Anamorph
Nannizzia Stockdale, 1961	*Microsporum* Gruby, 1843
N. borellii Padhye & Ajello, 1975	*M. amazonicum*
N. cajetani Ajello, 1961	*M. cookei*
N. fulva Stockdale, 1963	*M. fulvum*
N. grubyia Georg, Ajello, Friedman & Brinkman, 1962	*M. vanbreuseghemii*
N. gypsea Stockdale, 1963	*M. gypseum*
N. incurvata Stockdale, 1961	*M. gypseum*
N. obtusa Dawson & Gentles, 1961	*M. nanum*
N. otae Hasegawa & Usui, 1975	*M. canis*
N. persicolor Stockdale, 1967	*M. persicolor*
N. racemosa Rush-Munro, Smith & Borelli, 1970	*M. racemosum*
N. corniculata Takashio & De Vroey, 1982	*M. boullardii*
N. cookiella De Clercq, 1983	not named

Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1978), Howard (1983), De Vroey (personal communication).

1.1.4.1 Techniques for Mating Studies.

All dermatophyte species with a known sexual stage are heterothallic. Matings of compatible strains of the same dermatophyte species are able to produce ascocarps containing ascospores which usually develop in approximately one month. The appropriate in vitro techniques have made the identification of dermatophytes more accurate and reliable (Rebell and Taplin, 1978).

In the early studies on sexual reproduction in dermatophytes the substrate used was either sterilised or unsterilised soil with keratin, such as hair or feathers sprinkled on top (Stockdale, 1981). Dawson *et al.* (1964) studying species of *Arthroderma* and *Nannizzia*, found that sterilised soil was unsatisfactory, while unsterilised soil sprinkled with horse mane or tail hair is very satisfactory in stimulating sexual reproduction among the dermatophytes. Human hair was found to be a poor keratin bait in mating studies.

Although one can obtain cleistothecia of dermatophytes on soil, it is not a satisfactory medium for mating studies because the keratin becomes colonised by hyphae, making it difficult to observe the reaction between the two colonies. Mating is inhibited on media containing high concentrations of nutrients supporting good vegetative growth (Davidson and Unestam, 1974). De Vroey (1964) devised a niger-seed medium which supported cleistothecium formation by *M. gypseum.* Weitzman and Silva-Hunter (1967a) formulated an oatmeal agar with salts and with or without tomato paste, of the following composition: $MgSO_4.7H_2O$ 1g, KH_2PO_4 0.5g, $NaNO_3$ 1g, Baby oatmeal 10g, agar 10g in 1000ml distilled water. Takashio (1969, 1970, 1973a) used a diluted Sabouraud's dextrose agar with salts: dextrose 2g, neopeptone (or peptone) 1g, $MgSO_4.7H_2O$ 1g, KH_2PO_4 1g, agar 20g in 1000ml distilled water. Another medium used for mating studies is that of Takashio *et al.* (1976) composed of ground *Guizzotia abyssinica* seeds 7.5g, $MgSO_4.7H_2O$ 1g, KH_2PO_4 1g, agar 20g in 1000ml distilled water.

The first observation of "sexual stimulation" as opposed to true mating between different dermatophyte species was made following the discovery of *A. simii*, the perfect state of *T. simii* (Stockdale *et al.*, 1968).

A mating type can be revealed by the proliferation of white fluffy hyphae, often with the formation of ascocarp initials, which occur when a different dermatophyte species of unknown mating type makes contact with *A. simii* of the opposite mating type (Rebell and Taplin, 1978). This technique has significantly increased our ability to determine mating types.

Difficulties in obtaining perfect states may be due to the predominance or existence of only one mating type, (the only exception is *A. curreyi* Berkeley (1860) in which only the sexual form is isolated). Alternatively, the tester strains ("+" and "-") may be in an unfavourable state, since reactivity decreases after maintenance on conventional media. The mating of opposite strains must be carried out on a medium favourable for mating. Other environmental factors such as temperature must also be taken into account for each species (Dawson *et al.*, 1964).

Once fertile cleistothecia have been obtained, single ascospore culturing can be performed especially for further mating studies and for taxonomical purposes (Weitzman *et al*, 1964a, 1964b; Takashio, 1981). Dermatophyte colonies started from single spores produce a uniform and consistent appearance which is important for identification (Ajello, 1966) and the technique can also be used to rejuvenate degenerating sexual strains (Takashio, 1981).

To obtain ascospores from an ascus, the standard technique has been the use of a dissecting De Fonbrune micromanipulator (Weitzman, 1964a, 1964b; Rebell and Taplin, 1978). Another technique is a manual method introduced by Weitzman (1964a) which employs a fine needle to isolate a mature cleistothecium, after first rolling it on a 4% solid agar surface to remove conidia, soil particles or hair depending on the medium used for mating. The cleaned cleistothecium can be transferred to a second agar plate where it is crushed in a loopful of sterile water. Mature ascospores as determined by microscopic examination are subcultured before or after germination (Rebell and Taplin, 1978).

1.2 ECOLOGICAL GROUPINGS AND SOURCES OF INFECTION.

The dermatophytes have been divided by Georg (1959) into three ecological groups: geophiles, zoophiles and anthropophiles. Probably some of these fungal pathogens in evolving from their natural habitat in the soil, have developed host specificity, resulting in these three groups. Individual dermatophytes differ considerably in their host range and importance as agents of disease in man and animals. The differences in host specificity has been attributed to the differences in keratin of the hosts (Rippon, 1982).

Geophiles are primarily soil-inhabiting and only rarely encountered as agents of ringworm, with the exception of *M. gypseum.*

Zoophiles are essentially animal pathogens, although they may cause infection in humans.

Anthropophiles are restricted to man, very rarely infecting animals.

1.2.1 Geophiles

Geophiles exist as saprophytes in the soil and have the ability to competitively colonise keratinous substrates successfully. Their distribution appears to relate to the distribution of available keratin (Marples, 1965; Mantovani, 1978; De Vroey, 1984). But the distribution is also influenced by the pH of the soil and generally they prefer a near neutral pH (Böhme and Ziegler, 1969).

A few geophiles do have the additional capacity to cause ringworm in some species of animals, including man. These dermatophytes are generally contracted directly from soil containing a high number of spores and are only rarely transmitted from man to man or lower animals to man (Ajello, 1974; De Vroey, 1984). For example, *M. nanum*, which causes ringworm in animals, especially pigs, is mainly associated with surroundings having pigs (Baxter, 1969; De Vroey, 1984; Connole, 1990). The proof for its geophilic existence was provided by observation of macroconidia in soil by Ajello *et al.* (1964), since it is well known that these spores are not formed on infected animals.

But the principle virulent geophilic dermatophytes are members of the *M. gypseum-fulvum* complex. This complex has been well documented as a pathogen in man and animals. According to Georg (1960), soil isolates of *M. gypseum* compared to animal isolates have a low pathogenicity and only very virulent strains are able to establish infection. Alternatively, strains of low infectivity may increase in virulence after "passage" through a host of low resistance. Of the three dermatophyte strains, pathogenicity studies with laboratory animals have shown *N. fulva* to be the least pathogenic, while no notable differences have been observed with *N. gypsea* and *N. incurvata* (Weitzman *et al.*, 1967b; Gordon *et al.*, 1967). The distribution of the *M. gypseum-fulvum* complex is world-wide (Ajello, 1953; Londero and Ramos, 1985; Durie and Frey, 1962; Marples, 1965).

The *T. terrestre* complex is considered to be non-pathogenic (Howard, 1983), although human infections by *T. terrestre* have been reported (Marples and Smith, 1960) and experimental animal infections have also been successfully induced (Bakerspigel, 1974b).

Other geophilic dermatophytes include *M. cookei* and *T. ajelloi* which are non-pathogenic. *M. cookei* is a geophile with a global distribution, often isolated from soil and also from rodents and other animals not showing any clinical symptoms of ringworm (Ajello, 1959). Human infections by *M. cookei* have rarely been reported (Lundell, 1965; Frey, 1971).

T. ajelloi is commonly found in colder climates but is sporadic in hot climates (Marples, 1965), possibly because higher temperature inhibits its growth. The fungus has been found to be more often associated with acid soils than with alkaline soils (Marples, 1965).

Other less common geophilic dermatophytes are included in Table 1-6 and their distribution and pathogenicity noted in Table 1-7.

Table 1-6: Geophilic dermatophytes.

Microsporum amazonicum	*Trichophyton ajelloi*
M. boullardii	*T. georgiae*
M. cookei	*T. gloriae*
M. fulvum	*T. longifusus*
M. gypseum	*T. phaseoliforme*
M. nanum	*T. simii*
M. persicolor	*T. terrestre*
M. racemosum	*T. vanbreuseghemii*
M. ripariae	
M. vanbreuseghemii	

Table 1-7: Geographical distribution of geophilic dermatophytes and host preference.

Species	Geographical distribution	Pathogenicity
M. amazonicum	Brazil	none
M. ripariae	Central Europe	bird (*Riparia riparia*) guinea pigs and humans
M. vanbreuseghemii	World wide?	Malabar squirrel, dog and humans
T. vanbreuseghemii	World wide	humans
M. boullardii	Africa	none
T. georgiae	World wide	none
T. gloriae	North America	none
T. longifusus	Europe	none
T. phaseoliforme	World wide?	none

Infective propagules originating from saprobic sources can be transmitted directly or indirectly, and are referred to as saprobic-parasitic (S-P) infections by De Vroey, 1984, 1985. This mode of infection is common for *M. gypseum*, where the source of most infections in man and animals is the soil (Ajello *et al.*, 1966). In children facial ringworm by *M. gypseum* can follow recreational exposure to soil-born propagules of this fungus (De Vroey, 1985). Occupational exposure is illustrated by reported cases in gardeners (De Vroey, 1985) and small epidemics observed in, for example, cucumber growers (Alsop and Prior, 1961; Gentles, 1962). The macro- and microconidia, ascospores and other propagules are produced during the saprophytic growth of dermatophytes on keratin in soil or other biotopes (e.g. birds nests in the case of *M. ripariae*) (De Vroey, 1984) and it is these which form the potential inoculum.

1.2.2 Zoophiles

Zoophilic species are basically animal pathogens, often with a single preferred animal host or very limited host range, outside which they are found in exceptional circumstances (English, 1972). Zoophilic dermatophytes rarely grow actively as saprophytes but survive in a dormant state on contaminated materials of animal origin.

M. canis, *T. verrucosum* and *T. mentagrophytes* are common agents of ringworm in animals but are also frequently associated with human infection. The amount of literature on human infections due to the three dermatophytes is enough evidence of their human affinity. Of the three, *M. canis* is the best documented (e.g. Marples, 1956; Klokke and de Vries, 1963; Gentles *et al.*, 1965; Baxter, 1973). This is mainly because it causes a lot of scalp ringworm in children (English, 1972). *M. canis* commonly infects pet animals and especially cats and dogs which shed infective particles into the domestic environment and contact with this results in familial infections (De Vroey, 1985). Like other types of ringworm, young children particularly in the age range 5 - 14 years are more susceptible to infection than adults. Similarly, kittens and puppies are more susceptible to ringworm than adult animals (Philpot, 1977a). *M. canis* is also known to cause ringworm in horses, monkeys, apes, chinchillas (Vanbreuseghem *et al.*, 1978).

Another dermatophyte species closely related to *M. canis* is *M. distortum*, known to cause ringworm infections in monkeys, dogs and cats. It has

been reported to occur mainly from New Zealand (di Menna and Marples, 1954; Marples and Smith, 1962a), Australia and the United States (Vanbreuseghem *et al.*, 1978). It is now regarded as a variety of *M. canis*.

T. verrucosum, on the other hand, is a common cause of tinea in cattle. It has also been reported in donkeys, dogs, goats, sheep and horses (Beneke and Rogers, 1980). Close contact by man with infected animals and their fomites leads to contracting the fungus. It is also generally accepted that in countries with cold winters where housing of the animals is required, the incidence of *T. verrucosum* rises in both animals and humans at that time of the year (English, 1972). Cattle breeders and veterinarians, occasionally suffer from tineas due to *T. verrucosum*. *T. verrucosum* is mainly an agent of inflammatory skin and scalp lesions (kerion).

Members of the *T. mentagrophytes* complex (with the exception of *T. mentagrophytes var. interdigitale*) are transmitted from wild rodents and the prevalence of human infections due to this fungus is known to be higher in rural areas where there is a reservoir of rodents e.g. North America and Europe (Georg, 1956; Gentles and O'Sullivan, 1957; Kaben, 1967). *T. mentagrophytes* has occasionally been isolated from the soil (Baxter, 1966; Padhye and Carmichael, 1968) where it can survive for several months.

Other important zoophilic dermatophytes are included in Table 1-8.

Table 1-8: Zoophilic dermatophytes.

Microsporum canis	*Trichophyton equinum*
var. distortum	*var equinum*
M. gallinae	*var autotrophicum*
M. persicolor	*T. flavescens*
M. equinum	*T. mentagrophytes*
	var. erinacei
	var. quinckeanum
	var. mentagrophytes
	T. verrucosum
	var. verrucosum

1.2.3 Anthropophiles

Anthropophilic species are primarily adapted for parasitism of man, but some species occasionally cause ringworm in animals. For example, *T. rubrum* has been reported to have caused an infection in a dog (Kaplan and Gump, 1958; Georg, 1960).

Anthropophilic dermatophytes are mainly associated with community living. Since transmission is man to man, contracting the disease therefore requires human contact. The spread of anthropophiles is more common in communities like schools, barracks, prisons and the family (Philpot, 1977a; Rippon, 1982b). In concentrated communities, the use of facilities such as shower-rooms and common headgear leads to rapid spread of infection.

Four of the *Microsporum* species, according to Vanbreuseghem, can be distinguished from each other on clinical, epidemiological and mycological grounds: *M. audouinii*, *M. langeronii*, *M. rivalierii* and *M. ferrugineum*. *M. langeronii* Vanbreuseghem, 1950, 1963a and 1963b has been separated from the classic *M. audouinii* by its geographic region (restricted to Central Africa) and unlike *M. audouinii* can cause tinea corporis (ringworm of the glabrous skin) and can be inoculated to produce experimental lesions in guinea pigs.

Of the anthropophilic *Trichophyton* species *T. rubrum* is a very common cause of tinea unguium, cruris, and pedis (Marples, 1959; De Vroey, 1985; Rippon, 1982b, 1985). *T. rubrum* very rarely invades hair *in vivo*. The distribution of *T. rubrum* is global, cutting across all populations and ethnic groups (Rippon, 1985; De Vroey, 1985). It is a dermatophyte becoming more prevalent among urban populations, especially in developed countries, due mainly to the "modern" way of life such as the wearing of occlusive shoes, which maintain heat and humidity (Philpot, 1977a). It is also able to adapt to its environment in a way other species can not emulate (English, 1980). In India, *T. rubrum* causes tinea corporis in women and tinea cruris in men due to the sari (worn by women around the waist) and the dhobie (loin cloth) worn by men, both of which are tight-fitting (Rippon, 1982a, 1985).

T. rubrum is also known to cause chronic forms of infections and it has been suggested that the amino acid composition of the perspiration may predispose individuals to chronic infection. Certain amino acids are considered as "inducers" of *T. rubrum* infections (Rippon 1982a). Pushkarenko and Pushkarenko (1969) in their investigations found patients with chronic *T. rubrum* had a higher than normal content of leucine, lysine, asparagine and histidine in their sweat. Rippon and Scherr (1959) were able to induce arthroconidia formation in *T. rubrum* at 32°C and 37°C with a medium containing a high amino acid concentration.

T. mentagrophytes var. interdigitale, a member of the *T. mentagrophytes* complex, is essentially a cause of tinea pedis and tinea cruris, and does not invade hair in vivo (De Vroey, 1988). The infection of the skin of the foot usually originates in the interdigital clefts, sometimes spreading to the soles and dorsum and occasionally the ankles and leg and ultimately to the toenails, resulting in tinea unguium (Baxter and Rush-Munro, 1980).

E. floccosum, the only pathogenic species in this genus is a common cause of tinea pedis and tinea cruris (eczema marginatum of Hebrae) affecting inguinal areas, particularly in males, although some infections do occur in females (Vanbreuseghem *et al.*, 1978; Rippon, 1982; Baxter and Rush-Munro, 1980; Howard, 1983). Sometimes it is also responsible for tinea unguium infections. But *E.floccosum* is not known to invade hair (Vanbreuseghem *et al.*, 1978).

Anthropophilic dermatophytes of limited geographical distribution (apart from *M. audouinii var audouinii*) are included in Table 1-9. Humans are the common host. It should be noted that a number of other species have been described but are of very limited distribution (Tanaka *et al.*, 1992).

Table 1-9: Anthropophilic dermatophytes.

Epidermophyton floccosum	*Trichophyton concentricum*
Microsporum audouinii	*T. gourvilii*
var. langeronii	*T. kuryangei*
var. rivalieri	*T. megninii*
M. ferrugineum	*T. mentagrophytes*
	var. interdigitale
	T. rubrum
	T. schöenleinii
	T. soudanense
	T. tonsurans
	T. violaceum
	T. yaoundei

Anthropophilic dermatophytes are commonly transmitted by infective propagules originating from active lesions to another individual. This mode of transmission has been called the parasitic - parasitic (P-P) mode of infection by De Vroey, 1984, 1985. This transmission can be direct or else indirect through an intermediary item such as hairbrushes, combs, clothes, towels and bedding (Mackenzie, 1961) or even from contaminated furniture or dressing rooms. In addition, transmission can occur from carriage of pathogenic dermatophytes on "normal" human scalps or "healthy" animals (Gentles and O'Sullivan, 1957; Baxter, 1973). In the case of "carriers", "carriage" could be of either or both of propagules derived from infected animals or propagules from the saprobic growth of the fungus (Mariat and Adan-Campos, 1967).

The environmental occurence of dermatophytes is well documented. Indirect transmission through the environment for all types of tinea infections can be acquired by contact with a significant inoculum. Such transmission has often been reported to play a prominent role (Mackenzie, 1961; Uscavage and Kral, 1961; De Vroey, 1984; Midgley and Clayton, 1972). Using various indirect sampling techniques, a number of authors have demonstrated the presence of pathogenic

dermatophytes in the environment (e.g. Gentles' "velvetpad", Mackenzie's "hairbrush" and "gauze pad" methods and Mariat's "carpet-square technique"). Examples of dermatophyte species isolated include *M. audouinii* (Friedman *et al.*, 1960), *T. tonsurans* (Mackenzie, 1961), *M. canis* (Uscavage and Kral, 1961; Smith, 1982) and *T. mentagrophytes* (Alteras and Lehrer, 1980), all of which must have been derived from fomites contaminated from active infections.

1.3 SAMPLING TECHNIQUES FOR DERMATOPHYTES.

1.3.1 The Keratin-baiting Technique.

Fungi from different ecological groups require different sampling methods and isolation techniques (Baxter and Illston, 1977, 1980; Hussein, 1987). Prophetically, Sabouraud (1910) expressed an opinion that the soil may represent a continuous and inexhaustible reservoir of dermatophytes being a natural habitat for their saprophytic life. The early failure to recognise the existence of soil keratinophilic fungi was due to a lack of an isolation technique for these fungi. The introduction of the hairbaiting technique by Vanbreuseghem in 1952 led the way to many other investigations. The technique has firmly established the source of some infections, but only for some dermatophytes, such as those by *M. gypseum* (Gentles, 1962).

Vanbreuseghem's method was essentially that which Nannizzi used when he reported the discovery of a sexual stage of *Microsporum gypseum* in 1927 (Gentles, 1962). The technique has now become a standard method for isolation of keratinophilic fungi from the soil (Durie and Frey, 1957; Ajello, 1959; Marples, 1965; Chmel *et al.*, 1972; Bakerspigel, 1974b; Caretta *et al.*, 1990).

Chmel (1972) considers fungi such as dermatophytes to have started as saprophytes on dead decaying parts of plants or other organic substances. Litter, therefore, was and is the main reservoir for keratinophilic fungi. Gordon (1953) demonstrated by direct visualisation that *M. gypseum* existed in the soil in the form of spores (macroconidia). The same has also been demonstrated for *M. nanum* (Ajello *et al.*, 1964), *M. cookei*, *T. ajelloi* and *T. terrestre* (Usunov, 1966).

Soil litter according to Satchell (1974) is composed of dead plant material unattached to a living plant and of animal "manures" which may be present on the soil surface. This definition includes organic material originating from plants or animals, such as leaves, branches, animal hair, feathers, feacal material or dead tissue.

Since fungal communities exist in different forms in the environment, and because of the wide range of litter components, different methods for sampling and processing of the soil for different fungal groups are used. The aim of an investigation and the type of litter are important in the choice of sampling procedures. In the case of keratinophilic fungi, surface sampling seems to be preferred. Some investigators before collecting the soil removed the top layer and then representative samples were scooped from the lower layers of soil (Chmel *et al.*, 1972; Lee, 1979). Marples (1965) collected the surface soils while Roy *et al.* (1972) collected from the top 10mm layer.

Sub-sampling may be necessary as it may not be possible to process the entire sample. The size of the sub-sample is variable, as there is no recommended standard amount or method. This may further reduce the chances of isolating keratinophilic fungi from the soil. Marples (1965) used soil sub-samples of approximately 50g per Petri dish without replications, but most authors do not give an indication of the quantity of soil used (Orr, 1969; Chmel *et al.*, 1972; Ajello and Alpert, 1972; Chmel *et al.*, 1972; Roy *et al.*, 1972; Carreta, 1990).

The soil is usually processed on the day of collection, but some workers have stored their samples at a temperature of 0-4°C for varying periods. Some have refrigerated their samples at -20°C, arguing that cell multiplication will be slowed down. However, very few studies have been conducted on the effect of low temperatures on the death rate of soil microorganisms (Parkinson *et al.*, 1971). Still other workers have attempted to keep their samples at the same temperature at which they were sampled, but this poses practical difficulties.

At present there is no clear-cut information on a permissible storage time for different soils, but according to Parkinson *et al.* (1971), "most investigators tend to have a practical outlook on this problem". Somerville and Marples (1967) stored unenriched soil samples in plastic bags at laboratory temperature for 11 months before reprocessing. They found that storage alone did not significantly affect recovery rate at least of *M. gypseum.*

Marples (1965) processed soil samples immediately following collection. But Somerville and Marples (1967), investigating an enrichment

technique in which sterilised cowhorn was incorporated with the bulk soil sample, stored their enriched samples for 14 days before processing to isolate keratinophilic fungi. They found that this selectively increased subsequent recovery of *M. gypseum* compared to the controls originating from the same sampling sites that were processed immediately.

Rebell and Taplin (1978) consider the enrichment technique probably favours some keratinophilic fungi more than others and can therefore lead to a false impression of the balance of keratinophilic fungi initially present in the soil.

Bakerspigel (1953) found that many fungi remain viable in sterilised soil for 1 to 4 years. Loam soil was the most satisfactory and was far superior to Sabouraud's agar for storage of some fungi e.g. *E. floccosum*.

While the technique for the isolation of keratinophilic fungi has remained largely the same, there are variations in the use of the baiting material. Chicken feathers, sheep's wool (Baxter, 1969), human hair (Marples, 1965; Ajello *et al.*, 1966), horse hair (Somerville and Marples, 1967; Chmel *et al.*, 1972; Bakerspigel, 1974), hedgehog quill, guinea pig hair (Marples, 1965; Somerville and Marples, 1967) or a mixture of human and horse hair and chicken feathers (Carreta *et al.*, 1990) have all been used. According to Otcenasek (1978), differences in host specificity in spontaneous infections may be due to differences in the affinity of the dermatophytes for the various kinds of keratin. Keratin substances in the feathers of birds and hair of animals are known to be different in their biochemical composition, e.g. in their content of nicotinic acid, cystine, arginine, and tryptophane (Bogaty, 1969). The classical work of Georg and Camp (1957a), expanded by Philpot (1977b) and Shadomy and Philpot (1980) also demonstrated that some dermatophytes of the *Trichophyton* and *Microsporum* genera have specific nutritional requirements. Whether differences in affinity for keratin bait "*in vitro*" is related to preferential stimulation of dermatophytes with a specific nutritional requirement present in the type of hair provided is not known.

1.3.2 The Hairbrush Technique.

Sampling of animal coats which may carry keratinophilic fungi is usually based on a modification of Mackenzie's (1961, 1963) "hairbrush technique". This technique has been used for studying the carrier state of both animals and humans for keratinophilic fungi (Rosenthal and Wapnick, 1963; Connole, 1965; Ive, 1966; Midgley and Clayton, 1972; Yamamoto and Kagawa, 1986).

The animals are brushed over the back, shoulders, sides, hindquarters and legs and the brushes transported to the laboratory in brown paper (Connole, 1965) or plastic bags (Baxter, 1973; Katoh *et al.*, 1990). The brushes are pressed into a suitable medium.

Gentles *et al.* (1965), Baxter (1973) and Katoh *et al.* (1990) have successfully used the technique for isolation of keratinophilic fungi from cats and dogs. After use the brushes are sterilised in 0.1% chlorhexidine ("Hibitane") solution. Connole (1965) immersed the brushes for 30 minutes while Baxter (1973) immersed them for 24 hours before cleaning with soap and running hot water. Any residual hair was then picked off with forceps.

The brushes with hair samples are normally cultured the same day. If storage of samples is necessary, they can be kept at laboratory temperature until the next day.

1.3.3 Sampling of Clinical Material.

If a Wood's lamp (an ultraviolent light which emits light at 360nm) is available, hair may first be examined in a darkened room before sampling. Hair infected by some species of *Microsporum* particularly *M. canis*, even when no clinical symptoms are apparent, emit a greenish yellow fluorescence (Georg, 1959; Vanbreuseghem *et al.*, 1978; Ajello *et al.*, 1966; Baxter and Rush-Munro, 1980; Howard, 1983). However, the Wood's lamp is of no value for hairs parasitised by *Trichophyton* species which do not produce fluorescence, except *T. schöenleinii* infected hairs which may emit a dull greenish fluorescence (Vanbreuseghem *et al.*, 1978; Howard, 1983). *E. floccosum* never invades hair in vivo

(Vabreuseghem *et al.*, 1978), while infected skin scales and nails do not fluoresce. For such cases Wood's lamp is of little value in detecting infection. Medicaments, artificial fibres and natural secretions also can obscure true fluorescence.

However, mycologically positive hair for *M. canis* have been reported in the USA and UK as not producing fluorescence (Baxter and Rush-Munro, 1980). Ajello *et al.* (1966) have also reported hairs from animals infected with *M. gypseum* do not produce fluorescence.

For suspected cases of skin or hair infection, direct microscopy can provide a first indication of infection. It allows direct examination for fungal elements, septate hyphae and spores. To clear the keratin and expose the fungal elements, 10-20% KOH is used (Georg, 1959; Ajello *et al.*, 1966; Vanbreuseghem *et al.*, 1978; Rippon, 1982b), although chlorallactophenol can also be used alone or with a dye. Microscopical examination is made using a low intensity light source to ensure a good contrast between the fungus and the keratinous material.

Fungi from infected materials can then be isolated using SDA containing penicillin/streptomycin or chloramphenicol and actidione incubated in the dark at 25-30°C (Georg, 1959; Ajello *et al.*, 1966). The incubation period generally varies from 1 to 4 weeks on SDA medium.

1.4 COLONISATION OF KERATIN BY DERMATOPHYTES.

1.4.1 Colonisation *in vivo*.

The dermatophyte species within the three genera *Epidermophyton*, *Microsporum* and *Trichophyton* differ in their pathogenicity *in vivo*. While all species invade the stratum corneum of the epidermis and the follicular ostium of hairs, different species vary widely in their capacity to invade hair and nail (Lachapelle and Armijo, 1983). The reasons for this observed tissue specificity are unknown, but are thought to be related to specific nutritional requirements or the enzyme production of individual organisms (Bulmer and Fromtling, 1983).

1.4.1.1 Hair.

Spores on the epidermis germinate and penetrate into the hair follicle, descending first in the root sheath area and then invading the hair proper by pushing between the overlapping cuticle cells to enter the cortex. Mycelial invasion is oriented towards the hair bulb. The invading hyphae do not enter living tissue and the zone of active growth is the newly differentiated, or differentiating keratin. Under certain circumstances, some can even extend and grow in the deeper tissues which are without keratin. According to Pinetti (1962), invasion of deeper tissues is not as a result of active penetration but of passive transportation. But studies by Ajello *et al.*, (1985) of histopathological material suggested pressure exerted by mycelial growth within the hair follicles led to mycelial filaments and hyphal clumps escaping into the dermis.

The type of hair invasion produced by individual species can be determined by direct microscopy. The pattern of invasion can be either endothrix (spores inside the hair e.g. *T. rubrum*) or ectothrix (spores inside and outside the hair e.g. *T. mentagrophytes*, *M. canis*). Further differentiation can be based on the size of the spores ("large" e.g. *T. verrucosum* or "small" e.g. *M. canis*). In "favic-type" hair invasion due to *T. schöenleinii* the infected hair contains branched hyphae and empty spaces appearing as lines of air bubbles where the mycelium has degenerated. Fat droplets are commonly present (Beare *et al.*, 1968).

1.4.1.2 Skin.

The presence of dermatophytes in the horny layer of the epidermis provokes two types of pathological lesions: scales and vesicles. The morphological appearance of dermatophytes is commonly as long septate and branched hyphae, and arthrospores in various numbers. These later lie more or less parallel to the surface of the epidermis. At maturity they round up and appear as chains of beads. The scaling produced is a result of the activity of enzymes produced by the fungi (Cruickshank and Trotter, 1956).

1.4.1.3 Nails.

Commonly dermatophytes invade the nail-bed, which becomes hyperkeratotic, and the inferior part of the nail plate. Dermatophytes tend to be orientated at random in hyponychial keratin, but lie parallel to the surface in the nail plate. Long septate and branched hyphae, with arthrospores, are the main microscopic features found in dermatophytic nail infections.

1.4.2 Colonisation *in vitro*

1.4.2.1 Hair.

Colonisation of hair *in vitro* is quite unlike that occuring *in vivo*. Vanbreuseghem (1952a) and English (1963) experimentally demonstrated *in vitro* the stages by which detached hair is attacked by keratinolytic fungi. The stages have been summarised by Barlow and Chattaway (1955): 1. cuticle lifting 2. cortical erosion 3. perforating organs and 4. colonisation of the medulla.

(a) Cuticle lifting.

The fungal mycelium gives rise to lateral branches which form branched fronds of mycelium, the "eroding mycelium" which forces its way beneath adjacent overlapping scales. At the same time, hyphae grow longitudinally along the hair, producing lateral branches at intervals, which eventually grow round the hair following the edges of the scales.

(b) Cortical erosion.

Here there is a continuation of the fronds produced, to form new fronds of mycelium below the original ones. The new fronds are seen lying at the bottom of sharply marked troughs, deeper than those created by cuticle lifting fronds and are especially characteristic of *T. rubrum*. *M. canis*, unlike some other dermatophytes, produces overwhelmingly thick fronds, forming masses of eroding mycelium many cells in depth.

(c) Perforating organs.

At this stage, a perforating organ (Vanbreuseghem, 1949) made up of a column of short, wide cells, the lowest one cone-shaped, penetrates tangentially and radially into the hair and often projects backwards. This organ is said to arise from one or more hyphae of short, wide cells which arch over the perforating organ and lie along the hair at either side of it, giving the appearance of a handle ("anse" Vanbreuseghem, 1952a).

(d) Colonisation of medulla.

The last phase, the colonisation of the medulla, by longitudinally growing hyphae, takes place by hyphae forming in the hair. Growth is more rapid in the medulla than in the cortex, probably due to the softer keratin in the medulla (Rothman, 1953) and its less compact cellular structure.

Raubitschek (1957, 1961) confirmed Vanbreuseghem's (1952a) observations of the perforating organs of dermatophytes invading hair and thereby obtaining nourishment. Raubitschek (1961), however, argued against production of keratinolytic enzymes by dermatophytes in order to obtain nourishment. He postulated that dermatophytes obtained nourishment from the non-keratinous material, thus destroying the framework of the structure while leaving the keratin molecules intact. But this theory has not been widely accepted.

1.4.2.2 Skin.

In vitro studies on skin invasion by dermatophytes have employed stratum corneum stripped by adhesive tape or cyanoacrylate contact

cement. Stratum corneum comprises dead cells produced during the process of exfoliation and dermatophytes in lesions remain confined to it. Aljabre *et al.* (1992) used the adhesive tape method to strip stratum corneum and found that the pattern of growth of *T. mentagrophytes* on the strips followed a cycle, first the germination of arthroconidia, forming germ tubes and then penetration of the stratum corneum, followed by formation of hyphae. These stages were considered to be important in pathogenicity, the intra- and extracellular location of the dermatophyte impling its ability to utilise components of both the intra- and extracellular environments by some diffusible product.

1.4.2.3 Nails.

Studies by Raubitschek (1957) Raubitschek and Maoz (1957) using nails, demonstrated true invasion of the nail plate in continuous shake culture with *T. tonsurans*, *T. rubrum* and *T. mentagrophytes.* All the dermatophytes produced nail invasions similar to clinical infections, with the formation of hyphae and arthrospores. In a number of places on the nail, single hyphae were observed entering the nail plate through breaks in the surface.

Thus both *in vitro* and *in vivo* studies of the colonisation of keratin indicate that dermatophytic invasion involves the use of both mechanical pressure e.g. the perforating organs *in vitro*, and enzymatic digestion.

1.5 PROTEOLYTIC ENZYMES OF DERMATOPHYTES.

1.5.1 Role In the Colonisation Process.

Although a number of researchers followed Davidson and Gregory (1934) in supposing that dermatophytic hyphae and such modifications as perforating organs invade and penetrate by means of enzymes, early workers experienced considerable difficulty in isolating keratinolytic enzymes from fungal cultures grown on laboratory media (English, 1963). However, it was known that with some fungi causing soft rots in plants, the enzyme pectinase was secreted *in vivo* on host plants and not *in vitro* using laboratory media. It was strongly believed that both perforating organs and eroding mycelium *in vitro* functioned by means of enzymes in addition to mechanical pressure, during both radial and longitudinal penetration. English (1963) observed that in old undisturbed slide cultures, she could not find cells lying free, suggesting that the hair was not merely disintegrated by the mechanical action of the fungus, but digested as well. Biochemical evidence had earlier been provided by Daniels (1953) who had identified accumulated amino acids as degradation products of keratin in broth culture.

Furthermore, in dermatophyte infections of the skin, the fungus remains confined to the stratum corneum, while the pathological changes are produced in the deeper layers of the epidermis and in the dermis (Pillsbury *et al.*, 1956). This suggests that the pathological reactions are mediated by some diffusible product(s) of the fungus or the interaction of the fungus with the stratum corneum (Minocha *et al.*, 1972). That dermatophytes produce a number of enzymes which may be involved in the overall interaction has been demonstrated a number of times.

Tate (1929) using acetone dried enzyme powders, was able to detect protease, catalase, keratinase, lipase, urease, invertase, lactase, maltase and diastase activity in representatives of the main groups of dermatophytes. In 1954, Chattaway *et al.*, carried out some enzyme studies on *M.canis*, using crushed mycelium, and were able to detect oxidase, phosphatase and diastase activity, in part confirming Tate's (1929) observation. It was not until 1963, when Chattaway *et al.*, using a laboratory broth medium, were able to isolate peptidases from

dermatophytes. Later, Rippon (1968) also isolated an extracellular collagenase enzyme from *T. schöenleinii*. This enzyme was demonstrated to have general proteolytic and collagenase activity. Studies by Cruickshank and Trotter (1956) using culture filtrate from *T. mentagrophytes* and *T. rubrum* found the filtrate affected the epidermal-dermal bonds resulting in separation of the epidermis from the dermis. This finding means that proteolytic enzymes diffusing from the fungus loosen the epidermal attachment, resulting in vesicles.

Following the above successes, many researchers have reported isolating enzymes from different dermatophytes using varying types of synthetic media (Yu *et al.*, 1968, 1969; Rippon and Garber, 1969; Minocha *et al.*, 1972; Sanyal *et al.*, 1985; Apodaca and McKerrow, 1989a, 1989b, 1990). The exocellular enzymes have been found to hydrolyse proteins such as azoalbumin, casein, elastin, collagen and keratin.

Self synthesised enzymes may serve fungi in a number of ways. They may enhance survival in tissues by chemically or physically altering the immediate environment, or they may act directly by digesting host proteins, thus providing a source of nutrition. The pathogenic potential of a fungal agent may depend on its ability to produce enzymes. In turn variations in enzymatic potential of a fungus may be responsible for differences in the pathogenic effects of various strains (Waldorf, 1986).

Studies by Rippon and Varadi (1968) found certain strains of *Microsporum* and *Trichophyton* species produced enzymes which were able to solubilise the keratin and related fibrous proteins found in skin, hair, claws and hoof. Keratin, elastin and collagen make up 25% of the body weight of mammals (Wood and Bladon, 1985). The role of enzymes as virulence factors has also been inferred as they are often found in the tissues of infected animals (Rippon, 1968, 1975). It has been suggested that they may play a role of breaking down part of the infected tissue(s).

Other studies by Rippon (1967), Rippon *et al.*, (1971) have suggested that differences in virulence of mating types may be related to differences in their ability to produce extracellular enzymes such as elastase. Experimental results have given conflicting results, so that more studies are needed to test the hypothesis (Kwon-Chung, 1974; Stockdale, 1981).

1.5.2 Laboratory Production of Dermatophytic Enzymes.

Some of the factors which influence enzyme production by a dermatophyte under laboratory conditions include, medium, pH, incubation period and temperature, size of inoculum, type of culture (stationary or agitated) and the strain of the fungus.

1.5.2.1 Media and Incubation.

Mainly synthetic substrates in both the solid (Rippon, 1967; Weitzman *et al.*, 1971; Hopsu-Havu *et al.*, 1972) and the liquid state (Noval and Nickerson, 1959; Weary *et al.*, 1965; Chattaway *et al.*, 1963; O'Sullivan and Mathison, 1971; Asahi *et al.*, 1985; Apodaca and McKerrow, 1989a, 1989b, 1990) have been used to study enzyme production. One problem with enzyme production studies is that there is no standard medium and therefore comparison between studies is difficult and unreliable. However, the most commonly used has been Sabouraud's broth.

In general, enzyme production studies using synthetic media tend to include a carbohydrate source (commonly glucose) and a nitrogen source (e.g. peptone), which can also support the growth of the dermatophyte independently. Some dermatophytes are unable to assimilate some nitrogen compounds from synthetic media without the addition of one or more growth substances (Stockdale, 1953a). Some example medium constituents in addition to Sabouraud's broth that have been employed for studies of exocellular enzymes include $MgSO_4.7H_2O$, KH_2PO_4, K_2HPO_4 and thiamine-HCL with an addition of either elastin or keratin (Meevootisom and Niederpruem, 1979), glucose-peptone broth (Sanyal *et al.*, 1985), horse hair, glucose, $MgSO_4$, thiamine, pyridoxine and inositol (Yu *et al.*, 1968), $MgSO_4$, KH_2PO_4, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$ and unsterilised keratin (Grzywnowicz *et al.*, 1989).

Other factors which will affect enzyme production are temperature and period of incubation. Kotrajaras (1965) incubated cultures at 32°C and assayed for enzyme activity for 4 weeks at 1 week intervals with a skin epidermal separation assay. He found that maximum enzyme production was during the fourth week. Meevootisom and Niederpruem (1979)

incubated *T. rubrum* at 25°C and found maximum enzyme production to be at 3 weeks, using a proteinase activity assay.

But there is very little information on the effect of different temperatures on enzyme production. Most workers use a temperature range of 25-37°C.

1.5.2.2 Stationary and Agitated Cultures.

Studies by Raubitschek (1955) and Evron-Maoz and Raubitschek (1960) demonstrated that the continuous shake culture method produces a constant amount of growth from a given inoculum. This method, which is useful for enzyme studies, makes the fungus produce hyphae, arthrospores and chlamydospores resembling the parasitic life phase of the fungi found in scales, hair and nails (Raubitschek, 1955). This growth type is referred to as "pseudo-parasitic" (Evron-Maoz and Raubitschek, 1960). Stationary culture on the other hand, produces mostly macro- and microconidia as found in the saprophytic phase of dermatophytes. However, most authors studying enzyme production have used either shake or stationary cultures only. Most workers have not provided any reason(s) for the choice of the culture method.

1.5.2.3 Hydrogen ion Concentration (pH).

The dermatophytes are not exacting in their pH requirements, with a pH range of 4-10 being compatible with the growth of most species. The optimum pH is slightly lower than pH 7.0 (Stockdale, 1953a). Most media tend to have the pH adjusted before use. A study by Weary and Canby (1969) using *T. schoenleinii* and *T. rubrum* in broth culture but separated from radioactive wool by a semi-permeable membrane found that the optimum pH for keratinolytic activity was between 7-8.

1.5.2.4 Oxygen and Carbon dioxide Tensions.

The growth of fungi, like other organisms, is influenced by environment. Variations in external conditions can affect rate of growth and can bring about differences in type of growth. Some fungi produce atypical structures resembling yeast when submerged in liquid medium.

According to Smith (1938), in culture flasks with a greater active surface relative to the plugged opening, analyses of air drawn from flasks containing actively growing moulds have shown that there must be a very rapid removal of carbon dioxide and intake of fresh air.

However, little has been done to study such factors as oxygen requirement. But in general dermatophytes grow well under aerobic conditions. Some studies by Giblett and Henry (1950) found the growth of *Microsporum* was inhibited at certain oxygen concentrations, while *M. audouinii* and *M. canis* showed no growth in 40% and 60% carbon dioxide respectively. In addition, the effect of different flask sizes is unknown, although most workers have reported using 250ml flasks (Meevootisom and Niederpruem, 1979; Hellmich and Schauz, 1988; Okeke and Muller, 1991).

1.5.2.5 Fungal Strain.

Rippon (1967) carried out studies on elastase enzyme production using various dermatophyte species. He assayed for elastase activity by demonstrating ability to clear 1% particulate elastin suspended in nutrient agar. He found that of the 18 strains of *T. mentagrophytes* tested, four originating from patients with marked or severe inflammatory infection had elastase activity. Similarly, in further studies with three strains of *M. fulvum* (*N. fulva*), two isolates from patients with inflammatory ringworm had elastase activity. The one strain from a non-inflammatory infection had no elastase activity. He confirmed the observations by assaying cultures grown in tryptose-yeast-glucose broth using an orcein-elastin method.

Later, 21 *N. fulva* isolates including the three from ringworm infections were mated with their opposite mating types. 12 strains of the "-" mating type were found to be elastase negative while nine "+" mating types were positive.

1.6 ENZYME ANALYSIS.

Physico-chemical techniques in use for enzyme work can be divided into (1) conventional chromatography, such as gel filtration and ion-exchange (2) high performance liquid chromatography (HPLC) and (3) electrophoresis. The last named is the one chosen for the present studies.

Use of an electrophoretic method simultaneously exploits differences in molecular size and charge for purposes of fractionation (Chrambach and Rodbard, 1971) and is a widely used technique for characterisation of purified proteins (Scopes, 1987). Earlier electrophoretic techniques using supports such as paper and other cellulose-based materials did not achieve desired protein resolutions, since separation relies on an approximate charge/size ratio of a protein (Scopes, 1987), the use of such supports resulted in some proteins moving together as one peak.

However, specially high resolution was achieved in the method of Ornstein (1964) with disc electrophoresis employing a synthetic gel medium, a cross-linked polyacrylamide, in which a carefully designed system of discontinuous buffers was used (Arcus, 1970). Although disc electrophoresis is still used, it has been largely replaced by slab gels, on which several samples can be run at the same time with direct comparison of mobility (Scopes, 1987; Osterman, 1984; See and Jackowski, 1989). Polyacrylamide gel (PAGE) electrophoresis can also be carried out at 0°C as well as at higher temperatures (Chrambach and Rodbard, 1971), and the electrophoretic method causes no protein damage (Arcus, 1970). The method is therefore applicable to enzymes (Nishihara *et al.*, 1967; Apodaca and McKerrow, 1989a, 1989b and 1990). Thin-slab gel electrophoresis using polyacrylamide has been developed extensively in recent years. The advantage of a thin gel (≤1mm) is that less heat is produced per cm^2 of gel surface for a given applied voltage (Scopes, 1987).

1.6.1 Clean-up Procedures.

A crude enzyme extract from a fungal culture usually contains contaminants such as fungal cells and carbohydrates. So that a clean up

step to remove most of the contaminants is required. Purifying a crude enzyme filtrate improves the activity of the enzyme. The three techniques commonly employed are (1) neutral salt and/or organic solvent concentration (2) centrifugation and (3) ultrafiltration.

Precipitation by addition of neutral salts such as ammonium sulphate is probably the most commonly used for fractionating proteins (Harris and Angal, 1989), although protein precipitation by water miscible solvents such as ethanol or acetone has been used since the early days of protein purification (Scopes, 1987). The principal effect of organic solvents is reduction of water activity (Scopes, 1987). Although these techniques are suitable for many applications, precipitative and solvent extraction procedures are apt to be denaturing because of the phase changes common to these techniques (Blatt, 1971).

1.6.2 Polyacrylamide Gel Electrophoresis.

This involves running the sample in a buffer at a pH where the proteins remain stable in their native form and makes use of differences in charges and size between different proteins (Scopes, 1987; Dunn, 1989). Although the buffer may depend on the nature of the proteins, it is generally in the pH range of 7 - 9, where most proteins are negatively charged and thus move towards the anode (Scopes, 1987) at the bottom of the gel in the thin slab gel apparatus. If basic proteins are being analysed a lower pH can be employed.

Pore size variability is achieved by use of variable concentrations of polyacrylamide according to the size of molecules to be separated (Chrambach and Rodbard, 1971; Scopes, 1987). Two variations are possible: variation in total acrylamide content and variation in cross-linker percentage (N,N^1-methylene bisacrylamide). An increase of either one reduces the pore size, slowing larger molecules. The ratio of cross-linker is 1 part to between 20-50 parts of monomer. Generally, gels of 7-15% are used. The polymerisation process is initiated with freshly prepared ammonium persulphate (1.5-2mM) in the presence of N,N,N^1,N^1-tetramethyl ethylenediamine (0.05-0.1% vol/vol TEMED), as a catalyst. This method is more efficient than the alternative of UV - activated polymerisation with riboflavin (Osterman, 1984; Scopes, 1987; Dunn, 1989).

Electrophoresis can also be carried out in starch gel medium, but this is less popular compared to polyacrylamide. However, it is specially useful when detecting enzymic activity for indexing allelic variation of species after electrophoresis (Selander *et al.*, 1986; Murphy *et al.*, 1990), and will be discussed in Section 1.8.6.

1.6.3 SDS Gels.

The anionic detergent, sodium dodecyl sulphate (SDS), is a very effective solubilising agent for a wide range of proteins. It masks the intrinsic charge of the polypeptide chain, making net charge per unit approximately constant (Reynolds and Tanford, 1970; Weber *et al.*, 1972). Therefore electrophoretic separation now depends on molecular radius, which is roughly equal to molecular size and occurs by molecular sieving through the gel. This is commonly known as SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulphate) (Chrambach and Rodbard, 1971; Osterman, 1984; Scopes, 1987; See and Jackowski, 1989) and is the most commonly used method for all types of proteins.

For optimal reaction with SDS, a 3-5 minutes incubation in 2-5% w/v SDS in the presence of 2-mercaptoethenol or dithiothreitol to cleave disulphide bonds is a common practice (Weber *et al.*, 1972). The most used buffer system is that of Laemmli (1970) based on the discontinuous buffer system of Ornstein (1964). The buffers are made of differing concentration of Tris/HCL for the resolving and stacking gels. The advantage of this system is that it concentrates sample proteins into sharp bands (or zones) before being resolved (Dunn, 1989).

1.6.4 Substrate Copolymerised Gels.

The substrate copolymerised gel technique is performed using a modification of Laemmli's (1970) procedure with a non-substrate stacking gel. Substrate copolymerised gels are used to localize enzyme activity by molecular weight. These gels differ from the standard SDS-Laemmli gels in two ways, viz., (a) the gels are made by incorporating the protein substrate of interest within the polymerised acrylamide matrix and (b) the

sample is mixed with a higher concentration of SDS to which is added 1 or 2% sucrose and bromophenol blue as a tracking dye (Heussen and Dowdle, 1980; Asahi *et al.*, 1985). In the sample buffer, no reducing agents such as 2-mercaptoethanol and dithiothreitol are included. Also the protein sample is not boiled as this would render the enzymes inactive. However, standard polypeptides of known molecular weight are boiled for approximately five minutes and run on the same gel as the protein sample. This method therefore allows detection of proteinases and the determination of their molecular weight.

The most commonly used substrate to detect proteinases is gelatin, first introduced by Heussen and Dowdle, 1980. The gelatin is dissolved in hot water and added to the resolving gel mixture before pouring into the gel apparatus to allow it to polymerise.

After electrophoresis, the SDS can be removed to allow the enzymes to renature by washing the gel in the nonionic detergent triton X-100 for 2 hours (Heussen and Dowdle, 1980; Lacks and Springhorn, 1980; Asahi *et al.*, 1985). The gel can then be incubated in a suitable buffer to allow breakdown of the gelatin by the enzyme(s). After 12 to 24 hours incubation, enzymes can be denatured using 50% trichloroacetic acid (TCA).

1.6.5 Staining and Destaining Proteinase Gels.

At the end of electrophoresis, the protein band must be visualised. This involves staining with any organic dye that binds to the protein (Scopes, 1987). Fixing of proteins on the gel by denaturation also removes non-protein which may interfere with staining procedures (Harris, 1989). The common fixatives are either methanol/acetic acid/water (3:1:6) (Heussen and Dowdle, 1980), or a protein precipitant, TCA or perchloric acid at 5-15% concentration (Scopes, 1987).

Dyes commonly used for staining are coomassie brilliant blue R-250 (CBB-R), coomassie brilliant blue G-250 (CBB-G) (Tal *et al.*, 1985), amido black (Heussen and Dowdle, 1980) and nigrosine (Scopes, 1987). These stains are used principally due to their sensitivity of detecting small amounts of proteins and their uniform staining with all protein

types. The staining time depends on the thickness of the gels, hence the popular use of thin (≤1mm) gels.

For substrate copolymerised gels, the staining procedure is performed as for denatured proteins except that sample proteins containing proteolytic "species" appear as clear bands on a coomassie stained background after destaining. The limitation with polyacrylamide-gelatin gels for analysing crude samples is that, some proteinases may be irreversibly denatured by the SDS present in the polyacrylamide gels or have different pH optima. The other limitation of the gelatin/SDS-PAGE is that only proteinases of relatively high activity can be investigated, and there is the potential that proteins of identical size cannot be separated in this system. Notwithstanding the above, this technique gives the best and sharpest resolution and clearest zones relative to other methods (Scopes, 1987).

1.6.6 Isozyme (Multilocus Enzyme) Electrophoresis.

The term "isoenzyme" (Markert and Moller, 1959) refers to different molecular forms of enzymes with a common ability to digest a particular substrate but which differ in electrophoretic mobility. Allozymes (Prakash *et al.*, 1969) on the other hand, are a subset of isozymes which are variants of polypeptides produced by different allelic alternatives of the same genetic locus, which differ in electrophoretic mobility. Isozyme (multilocus) electrophoresis relies on indexing allelic variation in sets of randomly selected structural genes of the chromosomal genome, which provides a basis for overall levels of single- and multi-locus genotypic variation in populations (Selander *et al.*, 1986).

The use of a starch gel as a support medium for separation of serum proteins was first introduced by Smithies in 1955. The small pore size allows the retardation of large molecules which had not been achieved with other support materials. This was latter followed by the demonstration that enzymes could be visualised directly on starch gels when stained with specific histochemical stains (Hunter and Markert, 1957).

The isozyme technique involves boiling hydrolysed starch in a buffer, removing air bubbles and allowing the starch to cool thereby forming a

gel. The ratio of starch to buffer can be varied to alter the size of the pores. The pore size produces the sieving effect in the gel, therefore separation can be achieved both on size and charge. There are two types of buffer systems, continuous and discontinuous. For both systems, various mixtures of buffer ions are used to achieve better resolution.

There are two forms of starch gel electrophoresis (SGE): (1) horizontal and (2) vertical. For horizontal SGE, a poured gel is allowed to cool in a gel mould without further preparation. The advantage of a horizontal gel is that it can be cut to obtain slices which can be stained for different enzymes. Vertical gels, on the other hand, are poured into a double sided mould having a "gel comb" as in SDS-PAGE, to make the gel wells for holding tissue extracts (Brewer, 1970; Murphy *et al.*, 1990). In general, vertical gels require more starch and larger tissue extracts and allow fewer samples to be run per gel and therefore are uneconomical. A possible disadvantage with horizontal gels is electrodecantation, whereby high molecular weight (M_r) enzymes drop to the bottom of the gel which may make slices from upper regions of horizontal gels inferior or inadequate for resolving these proteins. However, horizontal gels are the most widely used (Murphy *et al.*, 1990). Horizontal gels were chosen for this study.

1.6.7 Histochemical Enzyme Staining.

A way of determining the distance enzyme components travel through a starch gel is by use of specific enzyme-staining techniques (Vallejos, 1983; Scopes, 1987; Murphy *et al.*, 1990). The use of such techniques is restricted to non-denaturing conditions. This is done without fixing the protein zones, but the reagents should be able to diffuse in the gel. The reagents used for staining include substrates on which the enzyme reacts and which can be detected by a dye or substances able to fluoresce at 340nm (Murphy *et al.*, 1990).

The available detection mechanisms are (1) production of a purple colour (formazan) by reduction of nitro blue tetrazolium (NBT) or a methyl thiazolyl tetrazolium (MTT), using as intermediate an electron acceptor phenazine methosulphate (PMS) or a redox indicator 2,6-dichlorophenol

indophenol (DCPIP) which undergoes a colour change upon oxidation, (2) nonfluorescence of β-nicotinamide adenine dinucleotide (NAD) which is formed from fluorescent NADH, (3) fluorescence of methylumbelliferone, (4) fast diazo dyes and (5) an oxidised form of o-dianisidine dihydrochloride producing an insoluble brown precipitate (Murphy *et al.*, 1990). Many stains also contain requisite molecules, cofactors and coupling enzymes.

1.7 APPLICATIONS OF ENZYME ANALYSIS TECHNIQUES.

The difficulty in using *in vitro* biochemical assays to investigate pathogenicity and virulence is that pathogenicity occurs and is measured only *in vivo*, where the microbe grows under different nutritional and environmental conditions from those provided in laboratory cultures. Like any other biological property, pathogenicity is determined by both the microbe genome and environmental conditions (Smith, 1984). Therefore either because of a phenotypic change or selection of types, a pathogenic microbe *in vitro* may not produce all determinants of pathogenicity that are found *in vivo* during infection. In short, organisms grown *in vitro* culture may be deficient in virulence determinants (Smith, 1984, 1989). However, Mahan *et al.*, (1993) have developed a genetic system, termed *in vivo* expression technology (IVET), which depends on the induction of genes in the host, the idea being that some of the genes may encode products highly expressed in the host which are required for the infection process, including previously unidentified virulence factors. This system is based on the bacterium *Salmonella typhimurium*. No doubt the application of IVET will be investigated for fungi in the future.

1.7.1 Biochemical Assays.

An underlying aim of biochemical assays is the determination of quantitative variations in e.g. proteolytic and/or elastolytic enzyme production which may be responsible for differences in virulence between strains. Such assays have been widely used. Some of the common substrates for screening include hide powder-azure (azocoll) (Depiazzi and Richards, 1979; Stewart, 1979; Rippon, 1982; Gadasi and Kobiler, 1983), casein (McConnell, 1950; Nakamura, 1959; Stewart, 1979), fibrinogen (Stewart, 1979), elastin (Rippon, 1967; Rippon and Varadi, 1968; Rippon and Garber, 1969; Rippon and Soo Hoo, 1971; Cheung and Maniotis, 1973; Miller, 1974; Stewart, 1979; Schultz *et al.*, 1982), egg albumin (Minocha *et al.*, 1972) and gelatin (McConnell, 1950; Baxter, 1968).

Studies by Minocha *et al.* (1972) using egg albumin as a substrate to correlate proteolytic enzyme production to virulence demonstrated that strains of zoophilic *T. mentagrophytes* obtained from lesions with

markedly severe inflammation produced a higher amount of proteolytic enzymes than strains from less inflammatory lesions. Similar results for *T. mentagrophytes* were obtained by Rippon and Garber (1969). Additional studies with eighteen isolates of *T. mentagrophytes* indicated a similar association with elastase activity and inflammation (Rippon, 1967, 1982a).

In an earlier survey of pathogenic fungi for elastase activity as a possible indicator of pathogenicity conducted by Rippon and Varadi (1968), the authors found differences which correlated with mating types among isolates of *N. fulva*. All their seven "+" isolates were positive for elastase activity and all eleven "-" strains were negative. Thus it was suggested that there may be a correlation not only between elastase activity and inflammation but also between mating type and virulence.

Vanbreuseghem (Gordon, 1981) questioned the use in the above studies of a member of the *M. gypseum* complex, a geophile with a frequent contact with man but rarely producing lesions. *M. canis* can be considered a species which may better provide some explanation(s) on whether differences in virulence of mating types might be related to differences in their ability to produce extracelluar enzymes. However, the disadvantage with *M. canis* is the predominance of the "-" mating type with almost a complete absence of the "+" mating type. The predominance of the "-" mating type from both carriers and clinical material has been suggested to be due to a selective advantage of the "-" over the "+" mating types in its actual pathogenicity (Weitzman *et al.*, 1978).

Other experimental studies of this aspect have given conflicting results (Weitzman *et al.*, 1967; Gordon *et al.*, 1967; Weitzman *et al.* 1971; Kwon-Chung, 1971). The experimental animal studies by Weitzman *et al.* (1967) and Gordon *et al.* (1967) with the three species of the *M. gypseum* complex had shown that *N. fulva* was less virulent than either *N. gypsea* or *N. incurvata*. These results are essentially in agreement with those of Rippon and Garber (1969) except that Weitzman *et al.* and Gordon *et al.* (1967) found no significant differences in virulence between mating types of any of the three species when tested in both rabbits and guinea pigs, but the association of elastase production with more inflammatory infections irrespective of species involved was confirmed.

The production of elastase by bacteria is also known to be of significant clinical importance (Werb *et al.*, 1982). A correlation between elastinolytic activity and virulence of bacteria in the skin was demonstrated by Stewart (1979), using the bacterium *Bacteriodes nodosus* causing foot rot, an ulcerative and erosive inflammation in sheep and cattle. The presence of elastinolytic activity correlated with the severity of infection in the sheep from which the organisms were cultured (Stewart, 1979; Werb *et al.*, 1982). Also Depiazzi and Richards (1979) using hide powder-azure were able to differentiate between foot-rot and foot-scald strains on the basis of their relative rate of decline in proteolytic activity over a period of time. The proteolytic activity of the foot-scald strains declined at a much faster rate than foot-rot strains, a finding which was confirmed by Stewart (1979) with the elastin-agar test. *Pseudomonas aeruginosa*'s ability to produce an elastase enzyme is reported to be an important factor in the pathogenesis of infections by this organism. It is responsible for severe skin infections in burned and immuno-compromised hosts (Werb *et al.*, 1982).

1.7.2 Substrate Copolymerised Gels.

Copolymerised gels using gelatin as a substrate to detect proteinases has been widely used (Unkeless *et al.*, 1974; Granelli-Pipemo and Reich, 1978; Heussen and Dowdle, 1980). This method is capable of revealing the number and identity of catalytically active "species" appearing in proteolytic reactions and can be used for impure systems (Granelli-Pipemo and Reich, 1978).

The technique is applicable to a broad spectrum of proteolytic events. For instance, Heussen and Dowdle (1980) employed SDS-copolymerised plasminogen and gelatin substrates to detect plasminogen activators. They found the technique was useful in discriminating between plasminogen activators of different types. Similarly, Hanaoka *et al.* (1979), employed SDS-PAGE without a substrate in the gel, and eluted different proteins of the HeLa cell nuclei. Gadasi and Kobiler (1983) investigated the effect of amoebic proteolytic enzymes on baby hamster kidney (BHK) cell proteins using SDS-PAGE. They found the enzymes were able to digest the proteins participating in the attachment of the BHK cells to the substrate consequently causing detachment of the cells.

Other studies by Tzeng *et al.* (1983) and McKerrow *et al.* (1985) of elastinolytic proteinases secreted by *Schistosoma mansoni* which may contribute to its virulence, were able to purify extracts and determine the proteolytic enzymes by using gelatin substrate gels. In another study employing gelatin copolymerised gels, Keene *et al.* (1986) determined a number of proteinases secreted by *Entamoeba histolytica* which probably contribute to its pathogenicity. They found that lysate showed significant proteolytic activity between M_r 50 000 - 70 000.

A more recent study by Reed *et al.* (1989) using a combination of isozyme and substrate gel techniques found that isozyme patterns of clinical isolates of *E.histolytica* correlated with a specific putative virulence factor, that is, a neutral thiol proteinase enzyme. They found that only pathogenic strains of *E. histolytica* degraded gelatin in substrate gels. A proteinase enzyme with a possible role in the pathogenicity of *E. histolytica* in symptomatic patients was further supported by the presence of circulating antibodies to *E. histolytica* in infected hosts.

Other studies by Apodaca and McKerrow (1989a, 1989b and 1990) using copolymerised substrate gels (gelatin and elastin) investigated the expression and regulation of proteinases of *T. rubrum* using different N and C sources for culturing. They found that a high molecular weight enzyme of >200 KDa was expressed in all their culture conditions, while a 71 KDa M_r was expressed in a N-depleted medium. Other proteolytic "species" of 124 KDa and 27 KDa were secreted in sulfur-depleted cultures.

1.7.3 Isozyme (Multilocus Enzyme) Electrophoresis.

The taxonomy of dermatophytes has posed major difficulties due in part to polymorphism, where successive cultures from the parent stock show variation (Ajello, 1962b) and to pleomorphism and inconsistent or lack of sporulation. A number of methods, including morphological features, physiological tests (Georg and Camp, 1957a; Philpot, 1977b; Shadomy and Philpot, 1980), serological typing (Philpot, 1978b; Mackenzie, 1988) and serotyping using monoclonal antibodies (Polonelli *et al.*, 1986) have

been used to characterise dermatophyte strains in systematics and epidemiology. However, these detect phenotypic variation difficult to relate to allelic variation at specific gene loci.

DNA hybridization which provides estimates of total nucleotide sequence divergence has been used in dermatophyte taxonomy to a limited extent (Davidson *et al.*, 1980, Davidson and Mackenzie, 1984). However, according to Selander *et al.* (1986), this method has large experimental error associated with DNA hybridization and the technique is not very sensitive in detecting genetic relationships of closely related strains. The results obtained between different laboratories or replicates within the same laboratory tend to vary, and in practice DNA hybridization provides information on the degree of similarity of a set of strains to one or a few reference strains rather than a complete matrix of coefficients of genetic relatedness between all pairs of strains.

The value of isozyme electrophoresis in population genetics is that electromorphs (mobility variants) provide indirect information about DNA (Chang *et al.*, 1962; Avise, 1975) and the segregating proteins which usually differ by at least one amino acid can be directly equated with alleles (alternate forms of a gene) (Avise, 1975; Crawford, 1983; Selander *et al.*, 1986). Despite a certain number of limitations, the equation between phenotype and genotype remains reasonably well-defined for isozymes, especially when contrasted with the situation for morphology and secondary chemistry (Crawford, 1983).

Studies based on *Entamoeba histolytica* trophozoite isozyme patterns from asymptomatic and symptomatic human cases all over the world have demonstrated that nonpathogenic species of amoebae found in the intestine of man have a distinctive isozyme pattern distinguishable from pathogenic species (Mirelman, 1987). The isozyme patterns of hexokinase and phosphoglucomutase, whose bands migrate differently in the two cases, can be used as markers to distinguish between pathogenic and nonpathogenic strains (Sargeaunt, 1987). Gharbia and Shah (1990) found the electrophoretic migration of 2-oxoglutarate reductase (OGR) alone a reliable marker to differentiate species of *Fusobacterium*, and glutamate dehydrogenase and OGR migration differentiated all the *Fusobacterium* species except *F. varium* and *F. mortiferum*.

An important observation made using isozyme analysis of *Escherichia coli* and *Shigella* species derived from different sources was that very closely related bacteria may show very different disease types and/or host specificities (Selander and Musser, 1990). These studies of subspecific structure have identified several species in which clones vary markedly in host association, disease specificity and virulence. The implication of this finding is that comparative molecular studies of strains differing in pathogenicity would contribute information of significance to an understanding of fungal pathogenesis.

Cruickshank (1990), using pectic isozymes as a taxonomical criterion for *Rhizoctonia*, found correlations between isozyme patterns and anastomosis groups and their subgroups recognised by morphology and pathology. Oudemans and Coffey (1991) compared 3 morphologically distinct species of *Phytophthora*, *P. cambivora*, *P. cinnamoni*, and *P. cactorum* using isozyme analysis and found that the 3 species were clearly separated, and each further subdivided into electrophoretic types. An analysis of mitochondrial DNA restriction fragment length polymorphism of selected strains from each isozyme group was found to support the isozyme data (Millis *et al.*, 1991).

Studies of dermatophytes by Jones and Noble (1981) and Tucker and Noble (1990) using PAGE and isozymes revealed species specific protein patterns which allowed some species to be recognised despite morphological variation. Additional studies of *M. canis* and *M. distortum* showed the two species were indistinguishable and that *M. canis* apparently had little genetic diversity. They suggested a clonal origin of *M. canis* spread via pet animals or perhaps the involvement of some aspect of pathogenicity as revealed by the protein patterns shared by clinical strains.

Despite the great potential of isozyme technology, this method like others before it has certain limitations and problems. The major problem according to Achtman and Plaschke (1986) is that results of another laboratory can be repeated only by using identical methods on the same selected reference strains. Additionally, the resolution obtained within any group of microorganisms seems to be solely a function of the

number of enzymes examined. This method reveals more and more subdivisions as more enzymes are examined. Although this provides extra information and the additional subdivisions do not affect the branch of the genetic tree, the numerous "twigs and leaves" may obscure the primary structure from non-specialists.

1.7.4 Methods of Genetic Analysis using Isozyme Data.

Among the advantages of the electrophoretic technique is that it can provide not only taxonomical information of a species but also indicate genetic similarities and variation within the species and between species, including information on variance in pathogenicity among isolates (May *et al.*, 1979; Cochrane *et al.*, 1989; Selander and Musser, 1990). Other advantages over conventional systematic criteria, such as morphology, are objectivity (Crawford, 1983), since the enumeration of alleles and their frequencies are objective determinations based on the mobility of bands on gels. Further, there is the constancy of genic characters, i.e. the amount of genetic information, which depends on the number of loci examined and electrophoretic techniques are known to yield precise data on genetic contents of organisms (Avise, 1975).

For isozyme data, there are two frequently used methods of analysis depending on whether the genetics of the banding patterns are known. In the case where it is known, for each enzyme, distinctive mobility variants are designated as electromorphs and numbered in order of decreasing rate of anodal migration. Electromorphs of an enzyme are equated with alleles at corresponding structural gene loci, and absence of enzyme is attributed to a null allele. Distinctive profiles of electromorphs at the enzyme loci corresponding to unique multilocus genotypes are designated as electrophoretic types (ETs).

Genetic diversity can then be calculated as $h = 1 - \Sigma x_i^2[n/(n-1)]$ where x_i is the frequency of the ith allele at the locus, n is the number of isolates or ETs in the sample, and n/(n-1) is a correction for bias in small samples (Nei, 1978).

Genetic distance (D) between pairs of isolates or ETs can be expressed by one of several coefficients. Most authors calculate genetic distance

between pairs of isolates as the proportion of loci at which dissimilar alleles occur i.e., the proportion of mismatches (Selander *et al.*, 1986).

From a matrix of coefficients of genetic distance, various multivariate statistical methods may be used to represent overall relatedness among isolates or ETs.

However, when the genetics of the banding patterns are not known, phenotypic variation can be measured by number of bands per isolate and frequency of occurence (isolates with a given "band" per total number of isolates). The frequency of phenotypes can then be calculated for each population. From such data, the Shannon-Wiener diversity measure can be calculated for each enzyme system with the formula $H = - \sum p_i \log_2 p_i$ where H is phenotypic diversity and p_i is the frequency of the ith phenotype in the population (Lewontin, 1972; Gaur *et al.*, 1981; Magurran, 1988).

Phenotypic relatedness between pairs of isolates has been expressed by most authors using the normalised percent disagreement (PTC). From the matrix of PTC a number of multivariate statistical methods may be used to represent overall phenotypic similarity among isolates. The commonly used method to transform PTC data into a similarity phenogram is the unweighted pair group method of averaging (UPGMA) (Sneath and Sokal, 1973).

CHAPTER 2

MATERIALS AND METHODS.

2.1 MYCOLOGICAL TECHNIQUES.

2.1.1 Principal Media.

(a) Sabouraud Dextrose Agar (SDA) (Oxoid)

Mycological peptone	10g
Dextrose	40g
Agar	15g
Distilled water (DW)	1000ml
pH adjusted to 5.6	

(b) Sabouraud with Antibiotics.

Sabouraud's medium as above, with chloramphenicol 50mg/10ml 95% ethanol and cycloheximide 500mg/10ml acetone. Both were added after the medium had been heated to boiling and cooled down to about 45°C just before distributing into bottles or tubes prior to autoclaving.

(c) Sabouraud Broth.

Neopeptone (Difco)	10g
Dextrose	20g
DW	1000ml

(d) "Takashio" Medium (Diluted Sabouraud).

This has the same components as SDA but is based on 2% dextrose (Georg, 1959; Vanbreuseghem, 1978), less peptone and is enriched with 0.1% salts. The composition of "Takashio" medium used in this study was:

	1/8 Dilution	1/10 Dilution
Neopeptone (Difco)	1.25g	1.0g
Dextrose	2.50g	2.0g
$MgSO_4.7H_2O$	1.0g	1.0g
KH_2PO_4	1.0g	1.0g
Agar	20.0g	20.0g
DW	1000ml	1000ml

(e) Oat-meal Salt Agar.

$MgSO_4.7H_2O$	0.1g
KH_2PO_4	0.5g
$NaNO_3$	1.0g
Oat-meal	10.0g
Agar	10.0g
Water	1000ml

pH was adjusted to 5.6 before autoclaving.

(f) ***Guizotia abyssinica*** **(Niger seed) Agar with Salts.**

Ground *G. abyssinica*	7.5g
$MgSO_4.7H_2O$	1.0g
KH_2PO_4	1.0g
Agar	20.0g
DW	1000ml

G. abyssinica seeds were ground in a coffee grinder, transferred to a 1 L Erlenmeyer flask and 500ml water was added. The suspension was heated in a boiling water bath for 30 min., the ground seed filtered through a cheese cloth and the extract adjusted to 500ml with distilled water. Agar and the salts were added and the medium boiled to dissolve the agar.

All media were autoclaved at 120°C for 20min.

2.1.2 Isolation of Keratinophilic Fungi from Soil.

2.1.2.1 Collection of Soil Samples.

A total of 236 surface soils were collected from different sites in Palmerston North between 7.90 and 4.91. The soil samples were from areas which can be placed into five groups, namely; (a) recreational parks (b) school grounds (c) animal paddocks (d) wasteland (within residential areas) and (e) roadsides.

The surface debris was cleared and the soil up to about 10cm deep scooped into a plastic bag to an amount between 150-200g. The location of the site was noted at the time of collection. The bags were taken to the laboratory for processing. If the samples could not be processed the same day, they were stored at 4°C until the next day.

The pH of 200 soils was measured by placing 10g of soil in 50ml of double distilled water (DDW) in a 125ml Erlenmeyer flask. The soil was swirled and allowed to settle for 5 to 10 min. before measuring the pH using an Orion Research Digital Ionalyser pH meter (Watson Victor Ltd., New Zealand).

2.1.2.2 The Keratin-baiting Technique.

Each sample of soil was mixed well in the bag and smaller duplicate samples, approximately 50g each, were placed into empty sterile Petri dishes and baited with sterilised natural wool, cut into small pieces.

One Petri dish was moistened with 5-10ml of sterile distilled water (SDW) only and the second with SDW containing antibiotics actidione (cycloheximide) 500mg/ml and chloramphenicol 50mg/ml.

All baited samples were incubated at 25°C in the dark and moistened at intervals of 3-4 days to prevent the soil from drying. The plates were inspected daily for up to 5 weeks before being discarded.

The presence of keratinophilic fungi was confirmed by low power microscopic examination and fragments of colonised wool were

inoculated onto slopes of SDA containing antibiotics. These were incubated for 2 weeks at 25°C.

The term "keratinophilic" in this part of the study is defined as applying to those fungi able to grow on and be isolated from the keratin bait by culturing on SDA with antibiotics.

2.1.3 Isolation of Keratinophilic Fungi from Cats and Dogs.

2.1.3.1 The Hairbrush Technique.

A total of 165 cats and 53 dogs from the Society for the Prevention of Cruelty to Animals (SPCA) premises, Palmerston North were sampled between 11.90 and 9.91. Further hairbrushes were obtained from two veterinary clinics located in Palmerston North.

The animals were sampled with a plastic brush (Fig.3-6) (Nu-brush Nº2, Consolidated Plastics Ltd., Auckland) over the back, shoulders, hindquarters, legs and tail. After brushing the animal, each brush was kept in a separate plastic bag and returned to the laboratory.

The brushes were inoculated the same day on SDA containing antibiotics by pressing the bristles into the medium. When necessary, hairs were picked off with a sterile loop (bent at an angle of 45°) and inoculated into the agar surface. The plates were incubated at 25°C in the dark for up to 3 weeks. The term "keratinophilic" in this part of the study is defined as applying to those fungi able to be isolated from the brush samples by culturing on SDA with antibiotics.

The used brushes were sterilised by immersing in 0.1% chlorhexidine ("Hibitane" - I.C.I. (New Zealand) Ltd., Wellington) solution for 24 hours before cleaning with hot water. Any residual hairs were picked off by forceps. After drying the brushes, they were further sterilised under ultraviolet (U.V.) light for 30 minutes and placed in new plastic bags until required for reuse.

Occasionally brushes were checked for the effectiveness of the cleaning process in destroying fungal fragments and other microorganisms on the

brushes by inoculating sterilised brushes onto SDA. All gave negative results.

2.1.3.2 Other Sources.

In addition to isolates obtained by the hairbrush technique, 22 cultures of *M. canis* obtained from clinical cases were submitted from the Faculty of Veterinary Sciences, Massey University.

2.1.4 Identification.

In general, keratinophilic fungi were identified on the basis of their colonial and microscopic morphology on SDA. Identification of fungi by microscopic examination was based on the morphological characteristics of macro- and microconidia and the appearance of the mycelium.

After the primary cultures of *M. canis* and *M. cookei* were isolated, they were subcultured onto "Takashio" medium (1/10), to stimulate increased production of macro- and microconidia for confirmation of identification.

The texts consulted for the identification of the fungi reported in this study included Alexopoulos and Mims (1979), Barnett and Hunter (1972), Barron (1968), Carmicheal (1962), Evans and Richardson (1989), Rebell and Taplin (1978) and Rook *et al.*, (1968).

2.1.5 Determination of Mating Type.

2.1.5.1 Strains.

A. simii "+" (MY036.84) and "-" (MY037.84) tester strains were obtained from the New Zealand Communicable Disease Centre (NZCDC) and *N. otae* "+" (RV42487) and "-" (RV42488) from the Mycology Laboratory, Prince Leopold Institute for Tropical Medicine, Belgium. The mating type of the two *N. otae* tester strains was assigned on the basis of their reaction with "+" or "-" *A. simii* strains.

2.1.5.2 Culture Media.

Three different media were tested for mating studies, namely; oatmeal salt agar (Weitzman and Silva-Hunter, 1967), *G. abyssinica* with salts agar (Takashio and De Vroey, 1976) and "Takashio" medium (1/8 and 1/10) (Takashio, 1970). *M. canis* reacted better on 1/8 while *M. cookei* isolates reacted better on 1/10 "Takashio" medium. These two media were therefore used in all the mating studies with the two *Microsporum* species.

2.1.5.3 Determination of Mating Type using the Stockdale Technique.

Isolates of *M. canis* and *M. cookei* were paired about 1cm apart with the tester strains of *A. simii* "+" and "-". The mating type was assigned based on the hyphal stimulation obtained between the *Microsporum* isolates and either "+" or "-" *A. simii.* The incubation period was between 3 to 5 weeks.

Mating type of the *M. canis* isolates was confirmed by pairing with the tester strains of *N. otae. M. cookei* isolates designated '+' and "-" were paired with each other using all possible combinations.

2.1.6 Maintenance of Cultures.

Three methods were used for maintaining cultures:

2.1.6.1 Subcultures.

The isolates were maintained on slopes of "Takashio" medium (1\10) in universals and stored in the dark at room temperature. They were subcultured approximately every 3 months. Almost all the cultures were able to be maintained morphologically stable. Before the mating experiments and other studies, isolates were rejuvenated by subculturing.

2.1.6.2 Water Cultures (Castellani, 1963).

The isolates were subcultured on slopes of "Takashio" medium (1/10) to increase spore production. After 2 weeks the culture was flooded with 4ml SDW. The water was pipetted rapidly to dislodge the spores, but care taken not to disrupt the agar surface. The suspension was transferred to a bijou bottle, capped and sealed with parafilm (American National Can.). Storage was at 4°C.

2.1.6.3 Lyophilisation.

Microsporum isolates to be lyophilised were subcultured on "Takashio" medium (1/10) in Petri dishes. Five vials for lyophilisation were prepared. 20% sterile skim-milk (0.2ml) was introduced into each sterile freeze drying vial plus a labelled piece of paper identifying the isolate by a code and the fungal suspension introduced. The vials were closed with a cotton plug and placed in a vacuum machine (Edwards High Vacuum, Geo. W. Wilton & Co Ltd., Auckland) under 0.1 torr for 6 hours (to remove the water from the milk). After 6 hours, secondary freezing was for another 2 hours and the vials sealed under vacuum.

The lyophilised samples were randomly checked for viability soon after freeze-drying and after 6 to 10 months. The cultures were stored at 4°C.

2.1.7 Proteinase Production in Broth Cultures.

2.1.7.1 Shake and Stationary Cultures.

Cultures for proteinase enzyme production were grown in sterile Sabouraud broth. The medium was dispensed in 100ml amounts into 250ml Erlenmeyer flasks for shake cultures and in 50ml amounts in 300ml bottles for stationary cultures before sterilising.

Spore suspensions were prepared as for water cultures (Section 2.1.6.2).

100µl of well agitated suspension was the standard inoculum for 100ml of liquid broth and 50µl for 50ml broth. The inoculated broth media were incubated at 25°C (±0.5) in the dark for 2 weeks. The shake cultures were incubated on a continuous orbital shaker at 140 rpm.

2.1.7.2 Estimation of Oxygen Concentration In Cultures.

The oxygen concentration in 2 weeks shake and stationary cultures was measured using a calibration method following the protocol described by Chick (personal communication, 1993).

The method for calibrating the oxygen electrode involved a procedure using the reaction between sodium dithionate and dissolved oxygen which goes virtually to completion.

$$Na_2S_2O_4 + O_2 + H_2O \text{ ---> } NaHSO_3 + NaHSO_4$$

5ml of liquid broth equilibrated with air at 25°C was added to the reaction vessel. The oxygen probe was inserted into the reaction vessel and the stirrer turned on. The chart recorder pen (Sekonic; SS-250F) connected to an oxygen monitor (YSI model 53) was set to 98% full scale deflection. When a steady trace was obtained, about a "pinch" of sodium dithionate was added and the electrode probe replaced. After a few minutes a new steady trace was obtained which corresponded to a zero oxygen concentration.

The process was repeated for uninoculated culture broth.

The calibrations for both DDW and culture broth were very similar. Since the oxygen in water equilibrated with air at 25°C is 0.258µmoles ml^{-1}, the concentration of oxygen in culture broth equilibrated with air at 25°C was taken as 0.258µmoles ml^{-1}.

The concentration of oxygen in the test broth was therefore taken as:

$$0.258 \times \text{the vol. of sample} \times \frac{\text{chart divisions sample}}{\text{chart divisions control}} \text{ µmoles of oxygen.}$$

2.2 BIOCHEMICAL ASSAYS FOR PROTEOLYTIC ENZYMES.

2.2.1 Gelatin-Agar Plate Method.

The method of Frazier (1926) was used to screen relative proteinase liberation by isolates.

Reagents.

Solution A.

5g sodium chloride, 0.5g potassium dihydrogen phosphate and 1.5 dipotassium hydrogen phosphate were dissolved in 100ml distilled water.

Solution B.

4g gelatin and 0.05g dextrose dissolved in 400ml distilled water plus 5ml 0.8% nutrient broth (Difco).

The two solutions were mixed together and heated. 3% agar was added and the pH adjusted to 7.0 before autoclaving.

Medium at 25ml/plate was dispensed by a Petrimat (Struers, Denmark). Duplicate plates were inoculated for each isolate and incubated at 25°C for 7 days.

After 7 days incubation the plates were flooded with 5 ml of a solution of 15g mercuric chloride dissolved in 20ml HCl (conc.) and 100ml water. The mercuric chloride was left for 15 min. to precipitate the undigested gelatin. A clear zone around the colony appeared surrounded by a cloudy precipitate of undigested gelatin. The remaining mercuric chloride was washed from the plate with water.

2.2.2 Azocollytic Assay.

Estimation of proteolytic activity (proteinase) using azocoll was a modification of the method of Walter (1984) and Sarath *et al.* (1989).

Release of dye from 250µl of 2% azocoll (cowhide with a red dye attached) suspended in 50mM Tris/HCl, pH 8.0, was tested with duplicate aliquots of each *Microsporum* isolates. Preliminary studies with some earlier obtained strains had indicated that the assay was reproducible between the different experimental trials. Fungal mycelium and spores were separated by centrifugation from Sabouraud broth cultures at 13000 rpm for 7.5 min.. 150µl of the supernatant was added to the azocoll suspension to start the reaction. A reagent blank with 250µl azocoll and 150µl 50mM Tris/HCl buffer, pH 8.0 instead of broth was included to monitor if the substrate was "bleeding". A blank consisted of 50mM Tris/HCl buffer, pH 8.0 and 150µl of boiled broth.

Incubation was at 37°C in a shaking water bath for 60 min. for both *M. canis* and *M. cookei*. After incubation the reaction was stopped with 1.2ml of 10% trichloroacetic acid (TCA) which precipitated the unhydrolysed protein. The reaction mixture was left for 5 min. to ensure complete precipitation and the tubes centrifuged at 13000 rpm for 7.5 min. to pellet the undigested protein. The absorbance (A) of the supernatant was read at 520nm using a Shimadzu UV-160A spectrophotometer. For this method, proteolytic activity was defined according to Walter (1984), where one unit is the amount of enzyme which catalyses the release of azo dye equal to 0.001 min^{-1} with the catalytic concentration (proteinase units) in the sample being $(10^3/0.2 \times A/t)PU\ ml^{-1}$.

2.2.3 Elastinolytic Assay.

The method of Lowry *et al.* (1951) was used for protein estimation. The final colour is produced as a result of (a) biuret reduction of protein with copper ions in alkali and (b) reduction of the phosphomolybdic reagent by the tyrosine and tryptophan present in the biuret protein.

Reagents.

1. Reagent A, 2% Na_2CO_3 in 0.1M NaOH.
2. Reagent B, 0.5% $CuSO_4.5H_2O$ in potassium (or sodium) tartrate.
3. Reagent C, alkaline copper solution - mix 50ml of reagent A with 1ml of reagent B (prepared fresh).
4. Reagent D, Folin-Ciocalteau reagent (Merck, 6100 Darmstadt). 30ml Folin solution diluted with 60ml DDW, prepared just before use.

Assay.

The method of Gazzinelli and Pellegrino (1964) was used to assay protein degradation by elastase enzymes. Elastinolytic activity was determined in duplicate by incubating 150µl of the supernatant from a centrifuged Sabouraud broth culture with 200mg/ml of elastin (Sigma) suspended in 50mM Tris/HCL, pH 8.0. The assay was at 37°C in a water bath for 60 min.. A blank reagent consisted of 2ml elastin with 150µl of 50mM HCL.

After incubation, the reaction was stopped by adding 3ml ice-cold distilled water and the mixture centrifuged at 30000 rpm for 20 minutes at room temperature.

Colour Reaction.

For the colour reaction, 1.0ml of supernatant was mixed with 1.0ml of reagent C and left for 10 min. at room temperature. A negative colour blank reagent was 1.0ml of water. A positive colour reagent consisted of 1.0ml of 0.5M tyrosine dissolved in 0.2M HCL.

The colour was developed by adding 100µl of the Folin-Ciocalteau reagent D. The mixture was left for a minimum of 30 min. at room temperature for the colour to develop. Absorbancy representing solubilised elastin was measured at 660nm and expressed as $\mu g\ ml^{-1}$.

Calibration was carried out using 1.0µg/ml to 20.0µg/ml samples of tyrosine.

Preliminary studies had indicated reproducibility of the assay.

2.2.4 Keratinase Assay.

Keratin degradation was assayed in duplicate following the method of Apodaca and McKerrow (1989a, 1989b, 1990) using keratin azure (Sigma), a dyed wool product. 100µl of the supernatant from a centrifuged Sabouraud broth culture was incubated at 37°C with 5mg of keratin suspended in 50mM Tris/HCL buffer, pH 8.0 for 72h.. The final reaction volume was 1 ml. After incubation, the reaction was stopped by immersing the tubes in ice-cold water for 10 min.. The tubes were spun in a microfuge for 10 minutes at 13000 rpm. The degradation was measured by determining the A_{595} of the supernatant using a Shimadzu UV-160A spectrophotometer. A change of 0.01 A_{595} units per hour was equal to 1 unit of keratinase activity (KU ml^{-1}).

2.2.5 Characterisation of *Microsporum* Enzymes.

Fungal culture filtrates of *M. canis* (WG09) and *M. cookei* (H219) were used for inhibition studies. First a range of inhibitor concentrations were tested to arrive at an optimal inhibitory concentration for each. Stock solutions of the inhibitors were dissolved in the solvents listed in Table 2-1.

1,10-phenanthroline (PT), pepstatin (PEP) and L-trans-epoxysuccinyl leucylamido (4-guanidino)-butane (E-64) were stored at -20°C; phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetate (EDTA), *p*-chloromercuric acid (*p*CMB) and α-1-proteinase (α_1-P) at 4°C. Iodoacetic acid (IAA) was prepared fresh just before the experiment.

Table 2-1: Inhibitor concentrations.

Inhibitor	Solvent /Buffer	Concentration	
		M.canis	*M. cookei*
α_1-P	0.1M PO_4 buffer 0.5M NaCl, pH 7.5	0.2mM	0.4mM
PMSF	ethanol	100µgml^{-1}	100µgml^{-1}
PT	methanol	20mM	20mM
EDTA	DW	20µM	10µM
*p*CMB	0.2M NaOH/HCL	1µM	1µM
E-64	DW	0.01mM	0.02mM
IAA	DW	20mM	20mM
PEP	methanol	1.0mM	1.0mM

For inhibition assays, the appropriate inhibitor was pre-incubated with 150µl of culture filtrate for 20 minutes at 25°C. To start the reaction, the substrate was added to the assay mixture. The reaction was carried out at 37°C for 1 hour. An equal volume of the solvent for each inhibitor alone was also tested with the culture broth without substrate. Inhibition was calculated relative to the solvent controls after estimating absorbancy at 520nm for azocoll and 660nm for elastin.

2.3 PROTEINASE ASSAYS USING SDS-SUBSTRATE COPOLYMERISED GELS.

The general proteinase and elastase activity of shake and stationary culture filtrates (from Sabouraud broth cultures) were assayed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gels were copolymerised with either gelatin for proteinases or elastin for elastases. This allowed the determination of the number of proteolytic and elastinolytic "species" present in the filtrate. This procedure is sensitive enough to detect proteinases of less than 10µl of unconcentrated medium or lysate (Banda *et al.*, 1987).

Several preliminary determinations were made using resolving gels containing 10%, 11% and 12% acrylamide (see Table 2-2) for both shake and stationary filtrates. 10% and 12% gave reasonably good resolutions of proteolytic and elastolytic enzymes for both.

2.3.1 Reagents and Solutions.

(a) Acrylamide Solutions.

(i) Resolving gel

Acrylamide	30g
Methylene bis acrylamide	1g
Distilled water (DW)	100ml

(ii) Stacking gel

Acrylamide	30g
Methylene bis acrylamide	1.6g
DW	100ml

The acrylamide solutions were prepared by adding the acrylamide to 70ml of DW and stirring to dissolve. Methylene bis acrylamide was dissolved in this solution and the whole made up to 100ml with DW.

(b) Buffer Solutions.

(i) Lower gel buffer (1.5M Tris-HCL, pH 8.8, 0.4% SDS)

Tris	18.15g
DW	70ml
Adjust with HCL to	pH 8.8
10% SDS solution	4.0ml
DW to	100ml

(ii) Upper Gel Buffer (0.5M Tris-HCl, pH 6.8, 0.4% SDS)

Tris	6.0g
DW	70ml
Adjust with HCL to	pH 6.8
10% SDS solution	4.0ml
DW to	100ml

For both gel buffers, Tris was dissolved in 70ml DW and then adjusted to the required pH with HCL. 4ml of 10% SDS solution was then added and the solution made up to 100ml. These buffers are 4x the normal working concentration.

(iii) Reservoir (Running) Buffer (pH 8.3)

Tris	6.07g
Glycine	28.8g
10% SDS solution	20ml
DW	2000ml

No adjustment of pH is required for the buffer (to maintain optimal ion concentration for electrophoresis).

(iv) Initiator (10% ammonium persulphate)

Ammonium persulphate	0.1g
DW	1ml

A fresh solution was prepared daily.

(v) Sample buffer

Sucrose	1g
SDS	2.5g
Bromophenol blue (BPB)	0.005g
0.5M Tris-HCL, pH 6.8	100ml

The chemicals were dissolved in 50ml, 0.5M Tris-HCL, pH 6.8 and then made up to 100ml.

(vi) Buffer for Standards (Molecular weight markers)

DW	4.0ml
0.5M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
β-mercaptoethanol	0.4ml
0.1% (w/v) Bromophenol blue	0.2ml

(c) Stain stock (1% Coomassie Blue R-250)

Coomassie blue R-250	2.0g
DW	to 200ml

Stain (0.125% Coomassie Blue R-250, 50% methanol, 10% acetic acid):

Coomassie Blue R-250	62.5ml stain stock
Methanol	250ml
Acetic acid	50ml
DW	500ml

After dissolving the dye in DW, the solution was filtered before use.

2.3.2 Preparation of Gels.

(a) Resolving gel.

Soluble elastin (Elastin Products) and gelatin (Sigma, Bovine skin: type B) were each dissolved in water at a concentration of 0.1% which in preliminary runs was found to give optimum enzyme zones on the gel when stained. This is approximately 5x the normal working concentration. In the case of gelatin, dissolving was assisted by heating for 45 seconds in a microwave.

For gels of 10% and 12% acrylamide, the stock solutions stored at 4°C were mixed in a 250ml Erlenmeyer flask as in Table 2-2. After adding all the chemical solutions, the flask was swirled to evenly mix the chemicals but at the same time avoiding formation of air bubbles. The mixing ensured even polymerisation. The yet unpolymerised acrylamide solution was poured into the mould (approximately 25ml/0.75mm thick gel, Protean^{R}II XI Cell Apparatus, Bio-Rad) using a 5ml pipette. Bubble formation inside the gel was avoided during pouring. The acrylamide column was overlayed carefully with DW, to prevent direct contact between unpolymerised gel and the air, to create anaerobic conditions to allow complete polymerisation. Polymerisation took about 45 min..

(b) Stacking gel.

Before use, the Laemmli (1970) stacking gel around a 15 well comb was prepared. The solutions were mixed as in Table 2-2. The DW on the resolving gel was decanted and 1ml of unpolymerised stacking gel poured on top and rocked several times to remove any water still on top of the resolving gel. This allowed the stacking gel to adhere to the resolving gel. Fresh stacking gel solution was then added to fill the remaining upper surface. A comb was pushed (to cast the wells) between the plates into the stacking gel and left to polymerise for about 20 minutes.

After polymerisation, the comb was removed, the wells rinsed with water and the apparatus assembled ready for running.

Table 2-2: Recipes for gel preparation using SDS-Discontinuous buffer system.

	Resolving gel (pH 8.8)			Stacking gel (pH 6.8)
	30% Acrylamide 1% bis Acrylamide[1]			30% Acrylamide 1.6% bis Acrylamide[1]
Final Acrylamide conc[n]	7%	10%	12%	4%
	ml	ml	ml	ml
Lower Tris buffer, pH 8.8	10.00	10.00	10.00	-
Upper Tris buffer, pH 6.8	-	-	-	2.50
Acrylamide	9.40	13.40	16.05	1.30
Distilled water	20.60	12.60	9.90	6.15
0.1% Gelatin or Elastin	4.00	4.00	4.00	-
Ammonium persulphate[2]	0.20	0.20	0.20	0.05
TEMED[3]	0.02	0.02	0.02	0.01
Total Volume (Approx.)	40.00	40.00	40.00	10.00

1. Methylene bis Acrylamide
2. Fresh 10% solution in double distilled water
3. N,N,N^1,N^1 - Tetramethylethylenediamine

2.3.3 Sample Preparation and Electrophoresis.

Culture filtrate was separated from mycelium by centrifugation at 13000 rpm for 5 min. at 4°C. A 75µl aliquot of filtrate was mixed with 25µl sample buffer (3:1 ratio). Aliquots of 20µl were loaded for the gelatinase assay, and 60µl for the elastase assay, using a Hamilton micro-syringe (Hamilton Company, Reno, Nevada 89510). Initially, several well loadings of the diluted culture filtrate were tried ranging from 5µl to 80µl. Of these, 20µl gave the optimum band intensity and resolution for gelatinase and 60µl for elastases. The micro-syringe was rinsed in tank (reservoir) buffer in between each loading. The gels were run at 4°C to reduce enzyme interaction with the substrate, minimise enzyme denaturation and allow more accurate separation by molecular weight. The gels were run at 20mA/gel. The bromophenol blue (BPB) in the sample and standard buffers allowed the progress of electrophoresis to be monitored. Electrophoresis was performed until the dye reached about 1 to 1.5cm from the bottom (between 4 to 4.5 hours).

Trypsin positive controls (10µl of 0.001mg/ml diluted 1 to 250µl with sample buffer) were included on the gelatin/SDS-PAGE runs. Elastase (type I from porcine pacrease; Sigma) positive controls were included on the elastin/SDS-PAGE runs.

2.3.4 Developing the Gel.

After electrophoresis was complete, the proteins were allowed to renature by removing the SDS. This was accomplished by soaking in 2.5% (w/v) triton X-100 with gentle shaking for 60 min. at room temperature.

The gel was then rinsed with 50mM Tris-HCL, pH 8.0 buffer and incubated in fresh buffer with gentle shaking overnight. At the end of incubation the gel was fixed in 100ml of 50% TCA for 60 min.. After fixing, TCA was removed using a vacuum aspirator. The aspirator facilitated the change of solutions with minimal contact with the gel. The gel was then stained using 100ml of coomassie blue R-250 solution for 60 min.. Excess stain was removed with a mixture of 45% (v/v) methanol, 10% (v/v) acetic acid and water for 20 min.. At the end of destaining the

gel was transferred into distilled water. The areas containing enzymes showed as clear zones against a blue background.

2.3.5 Gel Scanning.

A gel scanner model 1312 (ISCO, Inc., Box 5347, Lincoln, Nebraska 68505, USA) was used on automatic mode (a dual beam mode using a reference wavelength outside the major absorbance region for colour stained gels). Automatic mode provided improved sensitivity, with the ability to ignore variations (e.g. changes in thickness and cracks)

Operating conditions were:

Slit aperture 0.15mm x 3.3mm linear scanning mode; scan speed 150cm/hr; wavelength filter 580nm; sensitivity 0.2; calibration 4.5; coarse adjustment 6 or 7 and rise time 2.5 seconds.

Gel scanning was performed on:

(i) A coomassie blue stained column only.
(ii) Trypsin columns for the first gels for both shake and stationary culture filtrates.
(iii) The columns representing different *Microsporum* isolates (normally 10 isolates per SDS-PAGE).

2.3.6 Gel Photography.

For a permanent record, the gels were photographed on an illuminated opal-white screen. The gels were positioned in place with care to avoid breakage and to avoid trapping air bubbles. All coomassie blue R-250 stained gels were photographed using a standard procedure after initial investigation for optimum contrast. The film used was Tech/Pan, black and white.

2.4 MULTILOCUS ENZYME ELECTROPHORESIS USING STARCH GELS.

2.4.1 Preparation of Mycelial Homogenates.

The mycelial growth was separated from the liquid broth using Whatman Nº1 filter paper. The operation was conducted at 4°C in a cold room. For enzyme extraction the mycelium was transferred to a mortar and immediately frozen with liquid nitrogen. The frozen mycelium was crushed to a fine powder with a pestle and the homogenate subdivided into approximately 1.5g quantities and placed in eppendorf tubes. These were stored at -70°C until required for electrophoresis. At this temperature, the homogenates could be stored for up to 1 month without significant loss of enzyme activity.

2.4.2 Enzyme Extraction.

The enzymes were extracted from the homogenate by adding 500µl buffer (50mM Tris-HCL, pH 7.1) (Micales *et al.*, 1986) to the 1.5g quantities of freeze-dried material before thawing (500µl sample buffer was found to not dilute the enzyme extract below detection level). The samples were allowed to thaw at room temperature, and the mycelial debris separated by centrifugation at 13000 rpm for 5 min. at 4°C.

2.4.3 Enzyme Selection.

Initially using a few extracts of both *M. canis* and *M. cookei*, 28 different enzymes (see appendix C) were tested for their ease of detection and the suitability of buffers. Eight enzyme systems were finally chosen. These screening experiments helped in determining the active enzyme systems in the two species and the buffers which provided optimal resolution for each enzyme system. The remaining enzyme systems were difficult to demonstrate with satisfaction, either because of poor staining or because of difficulty in finding suitable buffers.

The enzymes selected, their names, symbols and Enzyme Commission (EC) numbers as recommended by the International Union of Biochemistry (IUBNC, 1984) are listed in Table 2.3.

Table 2-3: List of enzymes, abbreviations and enzyme commission (E.C.) code.

Enzyme	Symbol	EC Number
1. Catalase	CAT	1.11.1.6
2. Esterase	EST	3.1.1.-
3. Glucose-6-phosphate dehydrogenase	G6P	1.1.1.49
4. Glucose-6-phosphate isomerase	GPI	5.3.1.9
5. Leucine aminopeptidase	LAP	3.4.11.1
6. Malate Dehydrogenase	MDH	1.1.1.37
7. Peptidase	PEP	3.4.- -
8. Peroxidase	PER	1.11.1.7

2.4.4 Starch Gel Apparatus.

The starch gel apparatus was locally constructed equipment. It consisted of a cooling platform made of aluminium raised to the level of the buffer trays (height ≈6.4cm) using a block put at the bottom of the aluminium cooler. The platform contained convoluted pipes through which cooled water at 4°C was pumped using a multiTemp thermostatic circulator (LKB Bromma). This provided continuous cooling of the gel during electrophoresis of the enzymes. The equipment was operated at room temperature.

2.4.4.1 Preparation of Gels.

The starch gels used in this study were all at 12% and were prepared with 36g of refined starch (Starch Art Corp, Box 268, Smithville, Texas 78957) per 300ml buffer. The starch was weighed and placed in a 1 L Büchner flask to which 90ml of the gel buffer was added along the side of

the flask. The suspension was vigorously swirled until the mixture was free of lumps and swirled continuously to prevent the starch from settling down. The remaining gel buffer was meanwhile heated to boiling in a 500ml Erlenmeyer flask using a microwave. The gel buffer was heated for 3 or 3.5 min.. The boiling gel buffer was first swirled to release steam and then rapidly poured into the Buchner flask containing the starch suspension. The hot starch suspension was vigorously swirled until it was evenly mixed. The suspension was heated in a microwave for 30 or 40 seconds to dissolve the starch. The starch solution was then degassed for about 20 to 30 seconds to remove air bubbles, using a vacuum water aspirator. A rubber stopper with one glass tube was tightly placed into the mouth of the flask and the vacuum hose attached to the tube. A finger tip covered the side opening of the Buchner flask, so controlling the operation of the vacuum. Vacuum was applied until all small bubbles were removed from the starch solution and large bubbles distributed uniformly in the mixture. The vacuum was then released slowly to stop the formation of further air bubbles.

The solution was poured onto a mould in the centre and into the corners and then gently rotated on the bench to spread the starch evenly over the mould. The moulds were made from glass with perspex for the ridges (dimension 13cm x 26cm x 0.6cm). Air bubbles were removed before the starch solidified. The mould containing the starch was left for 60 min. on the aluminium platform cooler. After 60 min. cooling, the starch gel was ready for running. All the moulds were "washed" inside with a cloth moistened with 2% (v/v) dichlorodimethyl silane in carbon tetrachloride to prevent the gel sticking to the bottom of the mould. Although some authors (Murphy *et al.*, 1990) recommend storing the gels at 4°C for next day use, it was found that fresh gels made and used the same day gave the best results.

2.4.4.2 Loading of Samples onto Gels.

An origin was cut using a scalpel blade and ruler across the width of the gel approximately 6cm from the cathode edge. The origin was cut perpendicular to the gel surface and reached the bottom of the gel. The cathodal part of the gel was partially separated from the origin to facilitate placement of wicks soaked in the sample fluid.

Small filter paper wicks cut from Whatman Nº3 filter paper were used in this study (Fig.2-1). The size of the rectangular wicks were approximately 5-7 x 10-15 mm.

The wicks were soaked with 70µl (except for runs using glucose phosphate isomerase, which was 50µl) of soluble fungal extract and placed vertically at the origin with the wick reaching the gel bottom. The samples were spaced with 2-3mm between each wick. A maximum of 12 wicks representing 10 different isolate samples, a positive control and a tracking dye were able to be loaded on one gel. The isolates chosen as positive controls acted as "standards" allowing comparison between different gels while the tracking dye was for monitoring the progress of the electrophoresis. The composition of the tracking dye was amaranth 0.01g, coomassie blue G 0.01g dissolved in 2.5ml of ethanol and brought up to 7.5ml with DW.

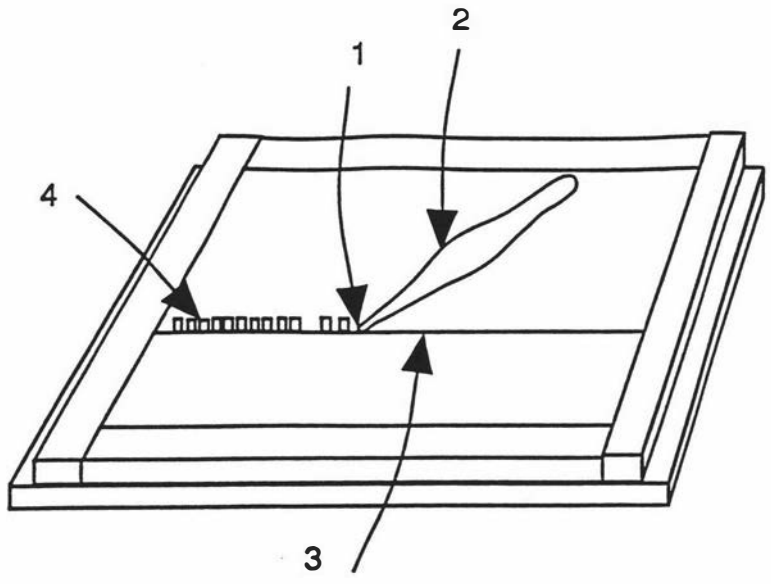
2.4.5 Electrophoresis.

After loading the samples, the 2 pieces of the gel (cathodal and anodal parts) were pushed together for a firm contact to allow free movement of the electrode buffer. Electrophoresis was accomplished by passing current through the gel using 2 electrode buffers wicked onto the gel surface with an absorbent, disposable cloths (Handiwipe, Wettex, Sweden) placed in the buffer trays (Fig.2-3). The cloths were thoroughly washed after use by soaking overnight and dried before reuse. The cloths were folded in 3 layers and the leading edge placed on the surface of the gel ensuring good contact between the cloth and the gel. The gel was then covered with thin plastic (Glad Wrap^{TM}, Auckland) to prevent the gel from drying (Fig.2-2). The wires from the DC power supply were inserted into each electrode buffer tray, with the cathode closest to the gel origin. When power was switched on gel voltage and current adjustments were made to the required levels (Table 2-4 and 2-5). Periodic checks were made during electrophoresis and readjustments made when necessary. After 30 min. of electrophoresis, the current was turned off and the wicks removed. The gels were cooled by circulating cold water at 4°C throughout the time of the electrophoretic run.

Fig.2-1: Loading of samples on starch gel.
1, Whatman Nº3 filter paper soaked with fungal extract and placed vertically at the origin. 2, forceps. 3, gel is cut (origin) 6cm from the cathode. 4, 2-3mm spacing between each wick.

Fig.2-2: a layer of thin polyethene (Glad Wrap™) is placed over the gel leaving at least 1-2cm at each end.

Fig.2-3: Preparation of gel for electrophoresis.
1, Plastic buffer dish is filled 2/3 full with electrode buffer solution. 2, Platinum electrodes are placed in the buffer. 3, Absorbent wick (Handiwipe) is placed in the buffer solution and firmly placed to the surface of the gel. 4, The gel mould is raised about 6.4cm using a wooden block placed beneath the glass plate.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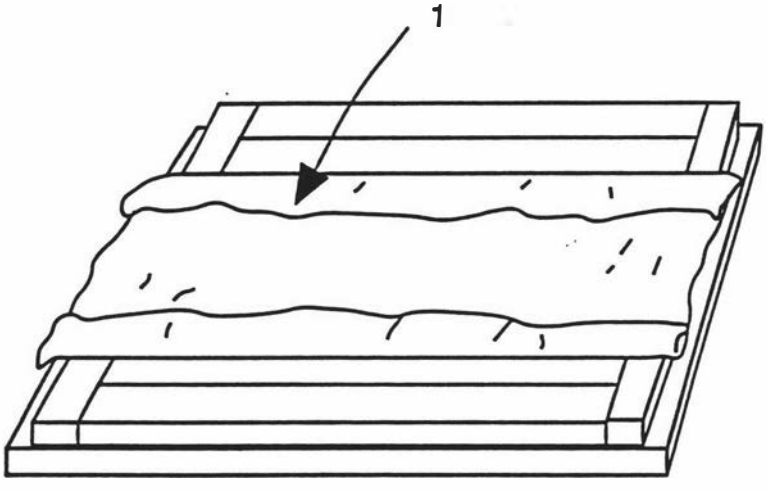
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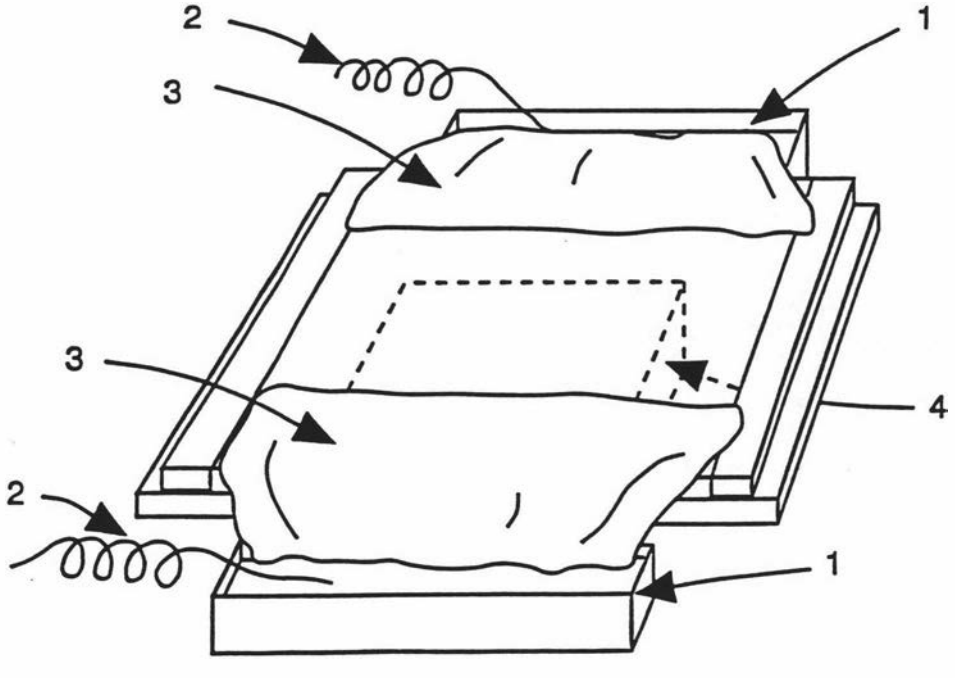
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2.4.5.1 Electrophoretic Buffers.

Two procedures were employed during electrophoresis:
(a) Continuous buffer electrophoresis or
(b) Discontinuous buffer or multiphasic electrophoresis.

For all enzyme electrophoresis runs, fresh electrode and gel buffers were used.

(a) Continuous buffer system.

This uses the same buffer ions in the support matrix and the electrode bridge compartments, but at different concentrations. The continuous buffer systems were of two types, namely Tris[hydroxymethyl]aminomethane (BDH) (Tris)-citrate and Tris-phosphate, at different concentrations.

Table 2-4: Buffers used for the continuous buffer system.

Enzyme	Electrode buffer	Gel buffer	Run (V)
CAT	Tris-citrate (pH 6.3) 8.24g Tris, 7.78g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	Tris-citrate (pH 6.7) 0.73g Tris, 0.63g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	200
EST	Tris-citrate (pH 7.0) 16.35g Tris, 7.78g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	Tris-citrate (pH 7.0) electrode buffer diluted 1:15	150

Table 2-4 contd.

GPI	Tris-citrate (pH 8.0) 8.24g Tris, 7.78g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	Tris-citrate (pH 8.0) electrode buffer diluted 1:29	130
LAP	Tris-citrate (pH 6.3) 27g Tris, 18.07g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	Tris-citrate (pH 6.7) 0.97g Tris, 0.63g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	200
MDH	Tris-citrate (pH 6.3) 8.24g Tris, 7.78g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	Tris-citrate (pH 6.7) 0.73g Tris, 0.63g citric acid monohydrate, 1 litre DDW, pH adjusted with NaOH	200
PEP	Tris-NaH_2PO_4 (pH 7.4) 12.11g Tris, 15.60g NaH_2PO_4, 1 litre DDW pH adjusted with NaOH	Tris-citrate (pH 7.4) 1:20 dilution of electrode buffer	200

(b) Discontinuous buffer system.

This uses different buffer ions in the support matrix and electrode bridge compartments (Table 2-5).

Table 2-5: Buffers used for the discontinuous buffer system.

Enzyme	Electrode buffer	Gel buffer	Run(mA)
G6P	Borate (pH 8.5) 18.55g boric acid 1 litre DDW, pH adjusted with NaOH	Tris-EDTA (pH 8.5) 4.6g Tris, 0.09g EDTA 1 litre DDW, pH adjusted with 0.4M citric acid monohydrate	40
PER	Borate (pH 8.5) 18.55g boric acid 1 litre DDW, pH adjusted with NaOH	Tris-EDTA (pH 8.5) 4.6g Tris, 0.09g EDTA 1 litre DDW, pH adjusted with 0.4M citric acid monohydrate	40

2.4.6 Slicing and Staining.

At the end of the electrophoresis, a piece of gel on the dye marker front was cut away as an identifying mark and to indicate the dye front. The gel was then transferred to a plexiglass mould with 3mm ridges to slice the gel into 2 pieces. The slicing wire, held tight between 2 ends of a metal holder, was rested on the ridges and the slicer glided through the gel gently at a constant speed. A downward pressure was applied to prevent raising the wire and to keep it taught.

At the end of the slicing, the two gels were peeled off and placed in staining trays.

Enzyme activity staining allowed the location of specific enzymes to be revealed.

The staining chemicals were weighed into labelled 50ml or 100ml Erlenmeyer flasks as the gel was still running. When electrophoresis was complete, the staining components were mixed together in a 150ml flask and poured over the gel in the staining tray. The tray was shaken gently to allow maximum contact of the gel slice with the staining solution. The tray was then placed in the dark in an incubation room at 37°C on a shaker until the bands appeared. The incubation periods were different for the various enzymes. Incubation was in the dark (except for peroxidase and catalase) as the tetrazolium dyes and the diazonium salts are sensitive to light (Vallejos, 1983). The staining was stopped by discarding the staining solution followed by two to three washes with water.

Since no one staining method can be used for all the enzymes, 3 different staining methods were used as listed in Tables 2-6, 2-7 and 2-8.

(a) Substrates, buffers and dyes used in the "chemical detection" method for EST and LAP (Tables 2-6).
(b) Substrates, buffers and dyes used in the Electron transfer dye method for G6P and MDH (Table 2-7).
(c) Substrates, buffers and dyes used in the Enzyme-linked method for PEP and GPI (Table 2-8).

Table 2-6: Substrates, buffers and dyes used in the "chemical detection" method for EST and LAP.

Enzyme	Substrate compound	Buffer & salt	Dye
EST	α-naphthyl acetate 40mg/4ml in 2ml, 50% acetone	0.05M sodium phosphate pH 6.5 (100ml)	Fast blue B 50mg
LAP	L-leucine-β-naphthyl amide hydrochloride, 100µl of 10mg/ml in 50% acetone	0.1M potassium phosphate, pH7.0 (50ml) 0.1M $MgCL_2$ (1ml)	Black K 30mg/ml
CAT[i]	hydrogen peroxide (33µl/100ml)		50:50 mix of 2% $K_3[Fe(CN)_6]$ 2% $FeCL_3$
PER[ii]	hydrogen peroxide (0.3%)		1% pyrogallol

(i) The gel slice was incubated for 15 min. in 100ml solution containing hydrogen peroxide. At the end of the incubation the solution was poured off and the gel rinsed with water and immersed in potassium ferricyanide/ferric chloride mixture until clear zones appeared against a blue background.

(ii) The gel slice was incubated in 1% pyrogallol for 60 sec., the solution was drained and the gel sprayed with 0.3% (v/v) of hydrogen peroxide and left for approximately 5-10 min. for the enzyme zones to appear.

Table 2-7: Substrates, buffers and dyes used in the electron transfer dye method for G6P and MDH.

Enzyme	Substrate compound	Buffer (0.2M Tris/HCL)	Salt	Coenzyme	Dye
	Dehydrogenases:				
G6P	glucose-6-phosphate disodium hydrate (100mg)	50ml, pH 8.0	0.1M $MgCL_2$ (1ml)	NADP 10 mg/ml	NBT 15mg/ml PMS 5mg/ml
MDH	1M DL-malic acid adjusted to pH 7.5 with 10M NaOH	100ml, pH 7.5	0.1M $MgCL_2$ (5ml)	NAD 30mg	MTT 20mg PMS 4mg

MTT = Methyl thiazolyl tetrazolium; PMS = Phenazine methosulphate
NADP = Nicotinamide adenine dinucleotide phosphate; NBT = Nitro blue tetrazolium; NAD = β-Nicotinamide adenine dinucleotide

Table 2-8: Substrates, buffers and dyes used in the enzyme-linked method for PEP and GPI.

Enzyme	Substrate compound & coupling enzyme	Buffer & salt	Dye
PEP	Peptide (8mg) Peroxidase (20mg) Snake venom (10mg)	0.05M sodium phosphate, pH 6.5 (100ml) 0.1M $MgCL_2$ (0.5ml)	3A9EC 10mg/3ml in DMF
GPI	D-fructose-6-phosphate 40mg; G6PDH (3.8µl) NAD (20mg/2ml)	0.2M Tris-HCL pH 7.0 0.1M $MgCL_2$ (5ml)	NBT 5mg/ml PMS 5mg/ml

DFM = dimethyl formamide; 3A9EC = 3-amino-9-ethylcarbazole

2.4.7 Gel Fixation.

After staining and stopping the enzyme reaction, the gels were fixed in 100ml of "wash".

The composition of the 2 fixatives were:

(a) "Alcohol Gel Wash" (Siciliano and Shaw, 1976)

Ethanol	1000ml
Acetic acid	400ml
Glycerine	200ml
Water	800ml
Total	2400ml

(b) Glycerine and Water (Vallejos, 1983)

Glycerine	500ml
Water	500ml
Total	1000ml

The "alcohol gel wash" fixative fixed the enzyme zone stains, toughened the gels and bleached out the background better than glacial/methyl alcohol/water (5:5:1) mixture and 50% ethanol. The glycerine/water fixative was used for catalase and peroxidase isozymes. This fixative is only good at preserving the colour of the enzyme zones but does not toughen the gel. Following fixation, permanent records of the gels were made on 35mm film (Kodak, T-MAX 100).

2.4.8 Safety Precautions.

Since the majority of the chemicals used for both SDS-PAGE and starch gel electrophoresis are very dangerous and some are potential carcinogens, care was taken when handling the chemicals. A weighing balance was placed in a fume cupboard for weighing and in all cases a face mask (Dust and Mist Respirators, Gerson, Middleboro, Mass-02346) and disposable gloves were worn.

2.5 Statistical Analyses.

The data concerning the fungal isolates from the soil and small animals was analysed for significant differences between the means using a *t*-test by SAS (SAS Institute Inc., 1988).

The data for enzyme expression by *M. canis* and *M. cookei* isolates for the two treatments (continuous shake and stationary culture) were compared by the weighted mean using the inverse of the standard error (SE). This was performed using the statistical package, SAS (SAS Institute Inc., 1988). The trends in expression of the three enzymes (proteinase, elastase and keratinase) were analysed by the product moment correlation coefficient (Pearson correlation matrix) using the statistical package, SYSTAT (Wilkinson, 1989).

Phenotypic variation using isozyme data between isolates of the two species *M. canis* and *M. cookei* and isolates from the three regions (Auckland, Palmerston North and Wellington) was calculated as the number of bands observed and frequency of occurence (number of isolates with a given "band" per total number of isolates.

When calculating phenotype frequency, single-banded isozymes will give same percentages for electromorphs using R.f. values and phenotype frequency. However, for multi-banded isozymes the R.f. values only allow to indicate the relative mobility but are not useful for calculating phenotype frequencies of the isozymes (Gaur *et al.*, 1981; Hamrick *et al.*, 1986)

2.5.1 Phenotypic Diversity.

Calculations of phenotypic diversity was by the Shannon-Wiener information measure ($H = - \sum p_i \ln p_i$) (Lewontin, 1972; Gaur, Lightwardt and Hamrick, 1981; Villeneuve *et al.*, 1989; Magurran, 1988; Grandtner and Fortin, 1989; Anagnostakis, 1992), using frequencies of isozymes, and normalised by $H = H/H_{max}$ (Sheldon, 1969), in which H is the usual Shannon-Wiener diversity measure over phenotypes, and Hmax is ln(N), the maximum diversity for the sample of size N and was performed by the statistical package SAS (SAS Institute Inc., 1988).

Shannon and Wiener independently derived the function which has become known as the Shannon measure of diversity (Magurran, 1988). The log to the base 2 (=$\log_2$) is often used in calculating Shannon-Wiener diversity measures, although any log base may be adopted. The $\log_2$ was used in this study in part to allow comparison with other reported studies in the literature, since it is more widely used.

2.5.2 Apportionment of Diversity.

Total diversity (H_T) of the *M. canis* isolates from Auckland, Palmerston North and Wellington was apportioned by calculating the mean phenotypic diversity for each region (sub-population) for each of the four enzyme systems from which the overall mean was calculated for the within population diversity.

This allows to subdivide total phenotypic diversity (H_T) into diversity within populations (H_S) and diversity among populations (D_{ST}) using the the method of Nei (1975):

$$H_T = H_S + D_{ST}$$

where:

H_T = calculated by obtaining the weighted average phenotypic frequencies over all populations ($H = -\sum Pi \log_2 Pi$) (refer 2.5.1)

H_S = the weighted average over all populations of the value H ($-\sum Pi \log_2 Pi$) for each population

D_{ST} = obtained by subtraction

Differentiation among populations can be calculated as:

$$G_{ST} = D_{ST}/H_T$$

where G_{ST} can vary between 0 ($H_S = H_T$) and 1 (H_S=0, that is the population is fixed for different phenotypes).

2.5.3 Phenotypic Identity.

Phenotypic similarities between pairs of *M. canis*, *M. cookei* and *Diheterospora* populations were calculated using Hedrick's identity measure (Hedrick, 1971). Phenotypic identity was calculated for each enzyme system, and a mean identity calculated from these values.

$$I_{x.y} = \frac{\sum P_{j.x} P_{j.y}}{\frac{1}{2}\left(\sum P^2_{j.x} + \sum P^2_{j.y} \right)}$$

$P_{j.x} + P_{j.y}$ = is the frequency of the jth phenotype in populations x and y.

I = Identity.

P = Phenotypic frequency.

2.5.4 Estimation of Phenotypic similarity using the Cluster Method.

To generate a dendrogram showing phenotypic similarity, an electromorphic matrix (Table 4-45) containing the isozyme phenotypes of all isolates was analysed by cluster analysis. Normalised percent disagreement (PTC) was used as a distance measure and the dendrogram computed by the average-linkage method of clustering (UPGMA) (Sneath and Sokal, 1973). The calculations were performed using the statistical package, SYSTAT (Wilkinson, 1989).

CHAPTER 3

RESULTS

3.1 KERATINOPHILIC FUNGI FROM SOIL.

3.1.1 Frequency and Distribution of Genera.

Of the 236 soils sampled, the total number of samples yielding keratinophilic fungi in culture on SDA with antibiotics following use of the keratin-baiting technique is shown in Table 3-1. The application of the term "keratinophilic" is defined in methods Section 2.1.2.2.

Table 3-1: Soil samples yielding keratinophilic fungi in culture. (n=236)

Culture				
Growth		No growth		Total
Antibiotic* solution	SDW	Antibiotic* solution	SDW	positive samples
137	174	99	62	168
58.1%	73.7%	41.9%	26.3%	71.2%

* = actidione & chloramphenicol.

Of the soils collected from different sites, 210 (89%) showed fungal colonisation by direct microscopy of the baits but only 168 (71.2%) produced fungal growth after portions of the bait were transferred to SDA with antibiotics. Culture of baits from soils moistened with antibiotic solution produced 137 fungi and those moistened with water only, 174. Table 3-2 shows the fungal genera isolated from both treatments.

Table 3-2: Fungal genera isolated from 236 soil samples by the keratin-baiting technique.

	Nº isolations			
	Treatment Group*			
Genus	Antib. soltn	Isol. rate (%)	Water only	Isol. rate (%)
Cephalosporium	0	0.0	5	2.1
Chrysosporium +	2	0.8	5	2.1
Conidiobolus	0	0.0	1	0.4
Cladosporium	1	0.4	0	0.0
Cunninghamella	34	14.4	11	4.7
Diheterospora	11	4.7	39	16.5
Fusarium	19	8.0	31	13.1
Geotrichum	3	1.3	0	0.0
Gliocladium	60	25.4	32	13.6
Microsporum +	11	4.7	12	5.1
Mucor	1	0.4	0	0.0
Paecilomyces	35	14.8	65	27.5
Penicillium	5	2.1	8	3.4
Rhizopus	1	0.4	0	0.0
Scopulariopsis	1	0.4	1	0.4
Trichoderma	12	5.1	12	5.1
Trichophyton +	28	11.9	77	32.6
Trichothecium	1	0.4	1	0.4
Verticillium	11	4.7	9	3.8

Isol = isolation; antib soltn = antibiotic solution.

* the two treatments were replicates for each sample.

+ = regarded as true keratinolytic spp.

Comparison of the two treatments showed no significant differences ($P \geq 0.05$) in isolation rates of fungi. A total of 19 genera were isolated, 17 from antibiotic treated samples and 15 from the water only treatment. Isolation rates in the two groups are shown in Table 3-2. Of the 19 genera, the most abundantly distributed at the five sites (Table 3-3) were *Cunninghamella*, *Diheterospora*, *Fusarium* (except at river/roadsides), *Gliocladium*, *Paecilomyces*, *Trichoderma* and *Trichophyton* spp.. The other genera including *Microsporum* and *Chrysosporium* spp. were in very low numbers or absent. *Fusarium* spp. were more common in areas of high human activity, such as recreational parks, cleared areas and schools.

3.1.2 Keratinolytic Species.

The species of the truely keratinolytic genera *Chrysosporium*, *Microsporum* and *Trichophyton* isolated from the soils are shown in Table 3-4. *T. ajelloi* was by far the most frequent (Fig. 3-4).

Table 3-3: Keratinolytic fungal species isolated from 236 soil samples by the keratin-baiting technique.

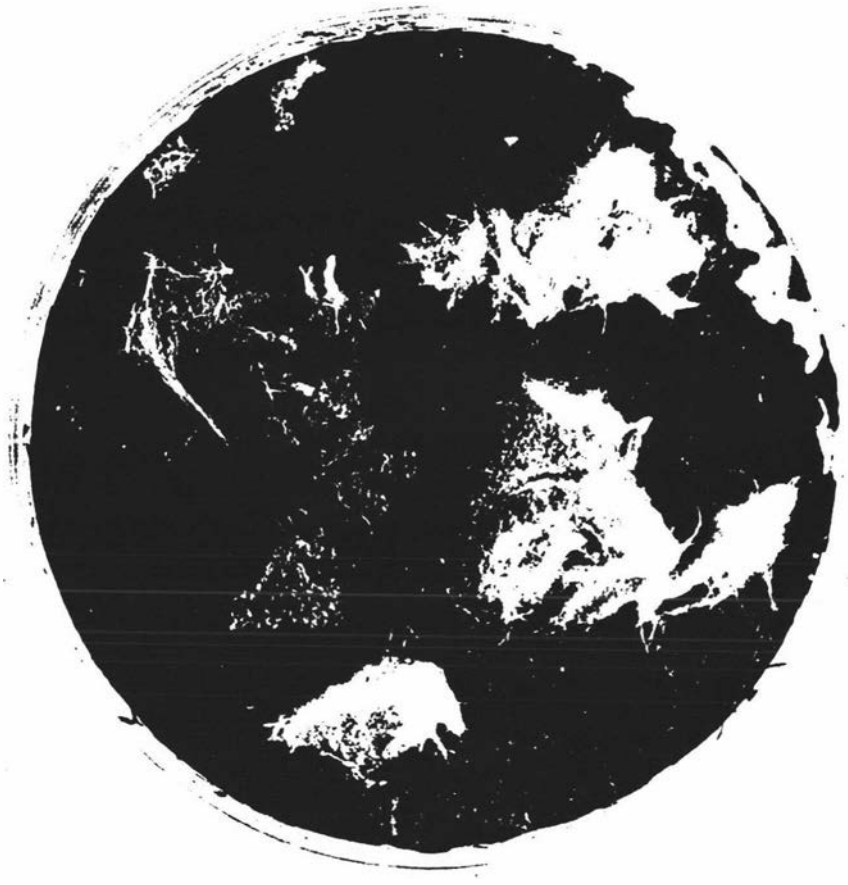
Species	Treatment group: Antibiotic Solution	Isol. rate (%)	Water only	Isol. rate (%)	Nº Pos. samples in 236
Microsporum cookei	8	3.4	8	3.4	16
M.gypseum complex	3	1.3	4	1.7	5
Trichophyton ajelloi	27	11.4	74	31.4	90
T.terrestre	1	0.4	3	1.3	4
Chrysosporium spp.	2	0.8	5	2.1	7

Isol = isolation; Pos = Positive

Keratinolytic fungal species were analysed separately by a *t*-test. There was no significant difference ($P \geq 0.05$) in isolation rates between the two treatment groups.

Fig.3.1: Keratin-baiting technique using sheep wool for the isolation of keratinophilic fungi.

Fig.3.2: Spontaneous cleistothecium development on soil by *T. ajelloi*.



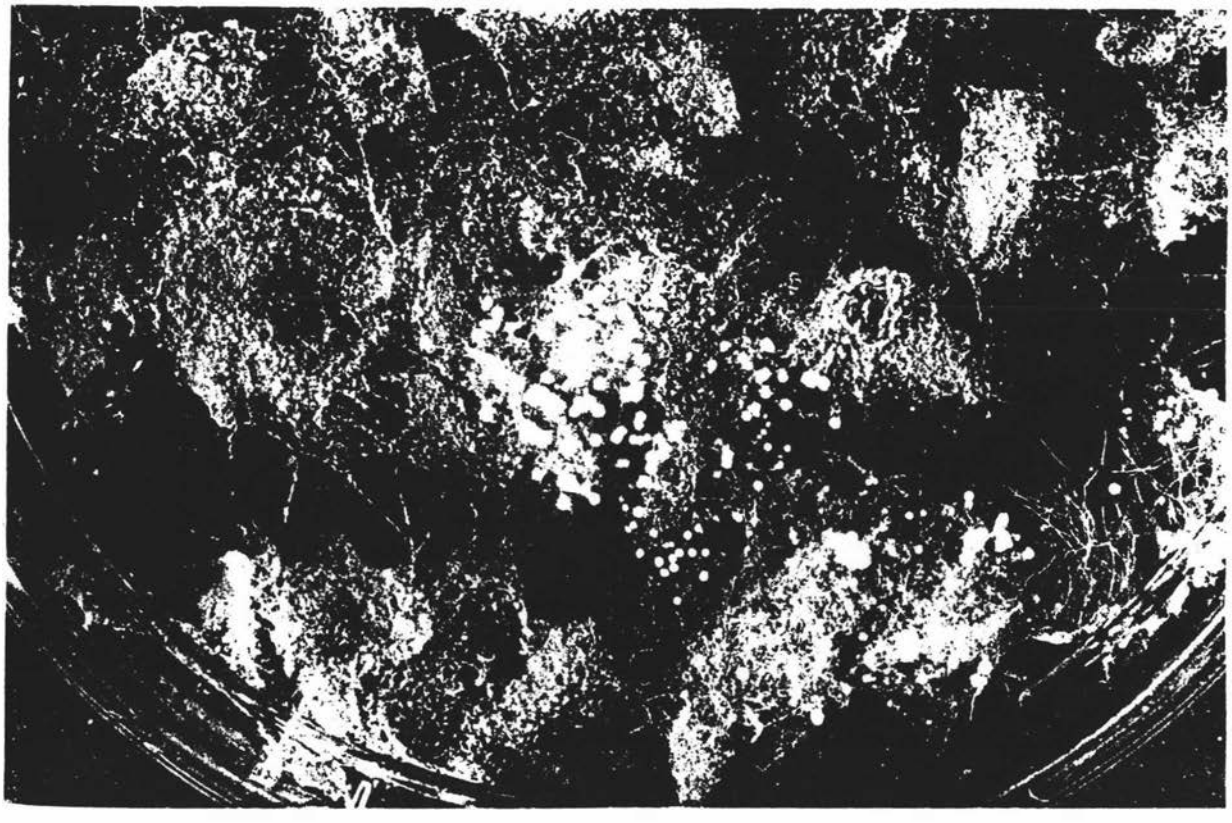


Table 3-4: Distribution of fungi isolated from soil samples from different sites.

	Total number of soil samples from each site (in brackets)												
Genus	Parks (75)	%	Cleared areas (44)	%	School grounds (28)	%	Paddocks (24)	%	River/Road side (29)	%	Sites with no detalls (36)	%	Nº Pos. samples in 236
Cephalosporium	3	4.0	1	2.3	0	0.0	1	4.2	0	0.0	0	0.0	5
Chrysosporium +	0	0.0	4	9.1	0	0.0	1	4.2	0	0.0	2	5.6	5
Conidiobolus	0	0.0	1	2.3	0	0.0	0	0.0	0	0.0	0	0.0	1
Cladosporium	0	0.0	1	2.3	0	0.0	0	0.0	0	0.0	0	0.0	1
Cunninghamella	14	18.7	10	22.7	8	28.6	3	12.5	4	13.8	0	0.0	39
Diheterospora	15	20.0	14	31.8	3	10.7	9	37.5	6	20.7	0	0.0	47
Fusarium	24	32.0	11	25.0	6	21.4	4	16.7	2	6.9	0	0.0	47
Geotrichum	0	0.0	3	6.8	0	0.0	0	0.0	0	0.0	0	0.0	3
Gliocladium	22	29.3	28	63.6	8	28.6	9	37.5	10	34.5	0	0.0	77
Microsporum +	7	9.3	5	11.4	3	10.7	0	0.0	1	3.4	4	11.1	21
Mucor	1	1.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1
Paecilomyces	36	48.0	17	38.6	14	50.0	8	33.3	13	44.8	0	0.0	88
Penicillium	7	9.3	2	4.5	1	3.6	2	8.3	1	3.4	0	0.0	13
Rhizopus	0	0.0	0	0.0	0	0.0	0	0.0	1	3.4	0	0.0	1
Scopulariopsis	2	2.7	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2
Trichoderma	9	12.0	4	9.1	3	10.7	4	16.7	4	13.8	0	0.0	24
Trichophyton +	20	26.7	18	40.9	9	32.1	15	62.5	8	27.6	24	66.7	94
Trichothecium	0	0.0	1	2.3	0	0.0	0	0.0	1	3.4	0	0.0	2
Verticillium	6	8.0	5	11.4	3	10.7	0	0.0	2	6.9	2	5.6	18

Pos = postive

An analysis of the isolation rates of *M. cookei* and *T. ajelloi* in relation to the pH of the soil indicated that most isolations were from soils of pH 5.1-7.0.

Table 3-5: Distribution of *M. cookei* and *T. ajelloi* in relation to soil pH (200 samples).

Soil pH	*M. cookei* Nº	% Isolates	*T. ajelloi* Nº	% Isolates
4.1 - 5.0	0	0.0	7	10.3
5.1 - 6.0	6	46.2	32	47.1
6.1 - 7.0	7	53.8	25	36.8
7.1 - 8.0	0	0.0	2	2.9
8.1 - 8.5	0	0.0	2	2.9
	13		68	

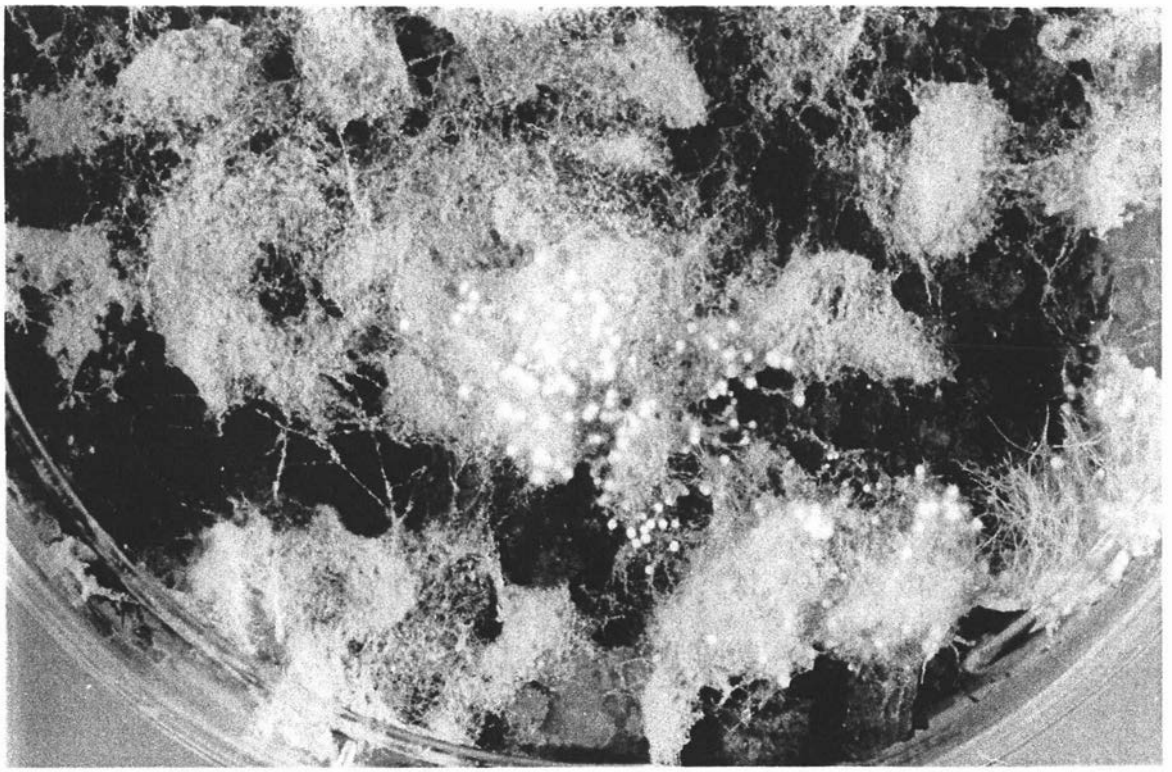


Fig.3-3: Distibution of *M. cookei* in relation to soil pH (200 samples).

(A= pH4.1-5.0, B= pH5.1-6.0, C= pH6.1-7.0,
D= pH7.1-8.0, E= pH8.1-8.5)

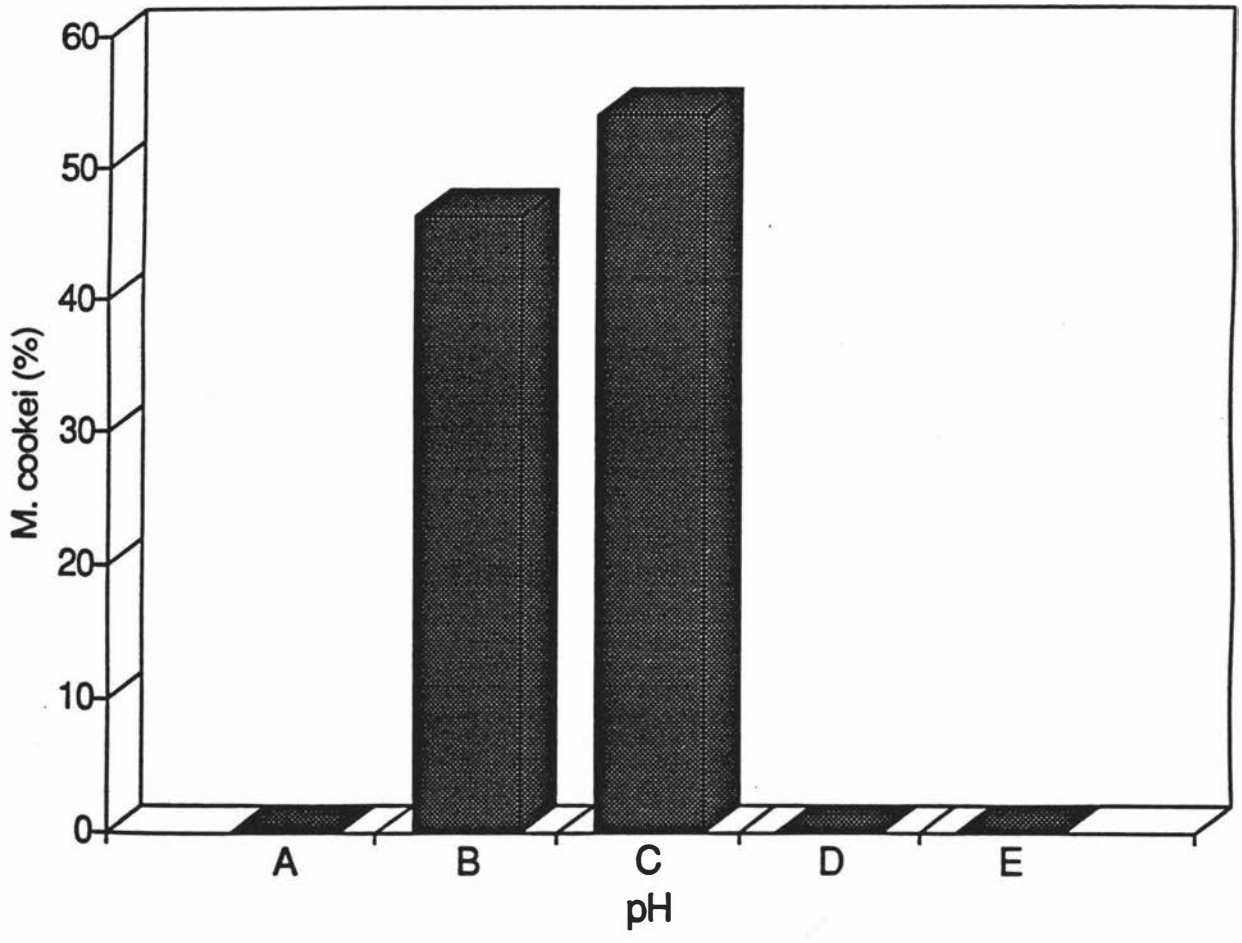
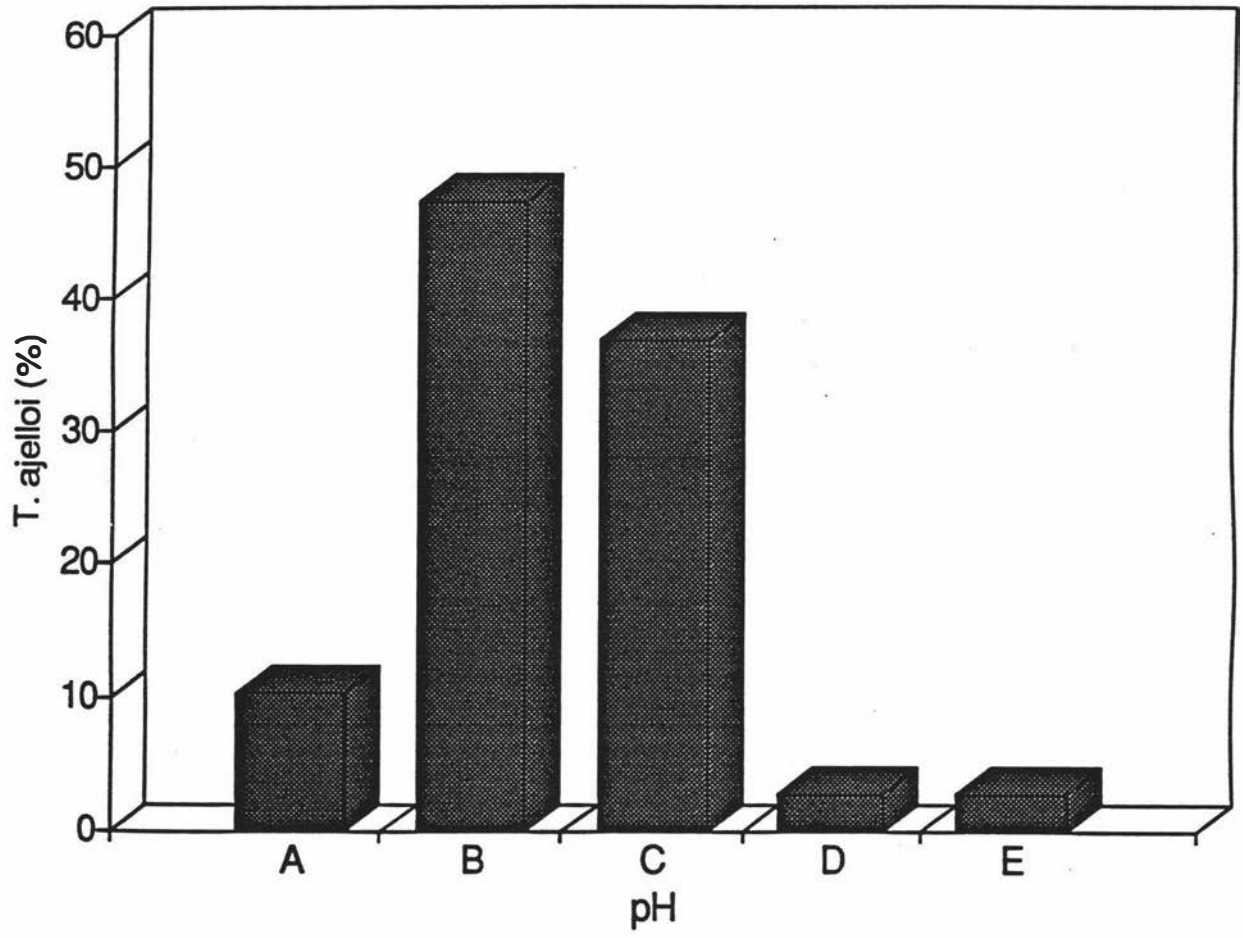


Fig.3-4: Distibution of *T. ajelloi* in relation to soil pH (200 samples).

(A= pH4.1-5.0, B= pH5.1-6.0, C= pH6.1-7.0,
D= pH7.1-8.0, E= pH8.1-8.5)



3.2 KERATINOPHILIC FUNGI FROM CATS AND DOGS.

3.2.1 Fungi Isolated using the Hairbrush Technique.

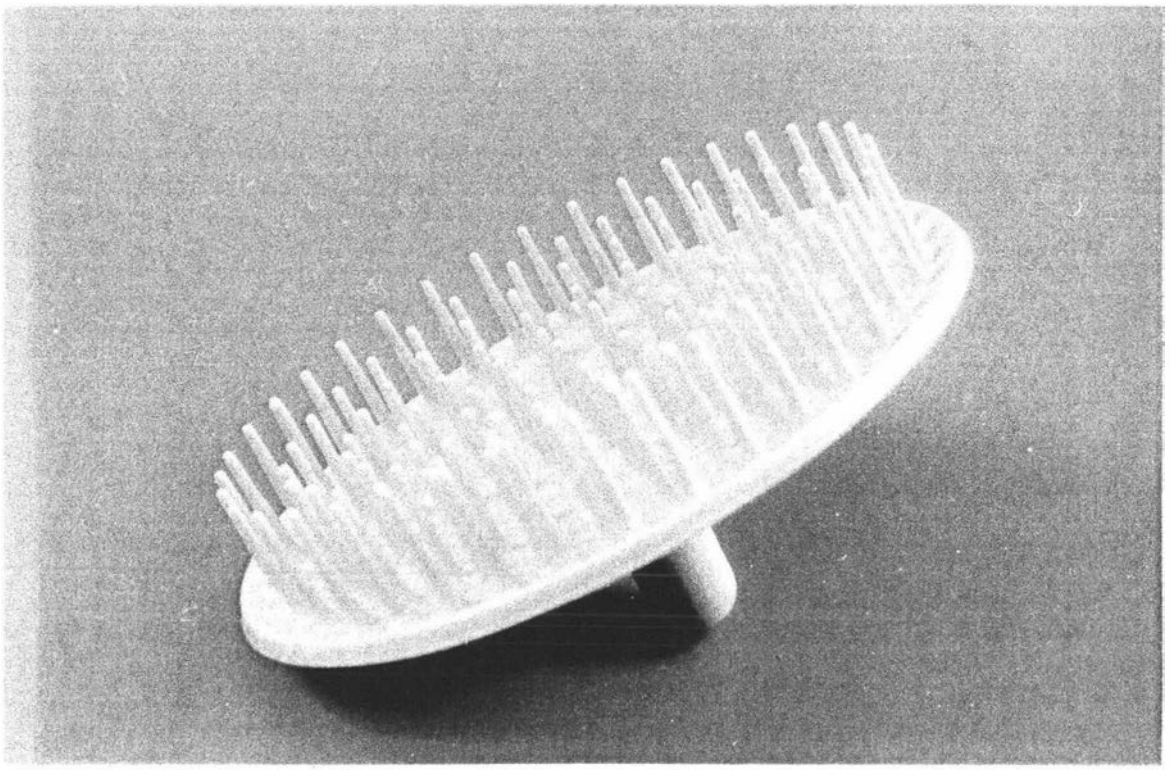
During the investigation of keratinophilic fungi from cats and dogs, the animals were sampled by the hairbrush technique and the samples cultured on SDA incorporating antibiotics to suppress non-keratinophilic fungi (Section 2.1.3.1). Fungi isolated from the hair of the animals and able to grow on SDA with antibiotics were considered keratinophilic. The principal source of samples was the SPCA premises in Palmerston North.

Table 3-6: Isolations from cats and dogs examined at the SPCA premises, using the hairbrush technique.

Source	Nº Examined	Samples positive for fungi	% positive	Nº of clinical cases
Cats	178	105	59.0	2
Dogs	59	32	54.2	0
Total:	237	137	57.8	2

Fig.3-5: The plastic Nu-brush used for screening carrier animals.

Fig.3-6: Growth of *M. canis* from a Nu-brush tested cat.



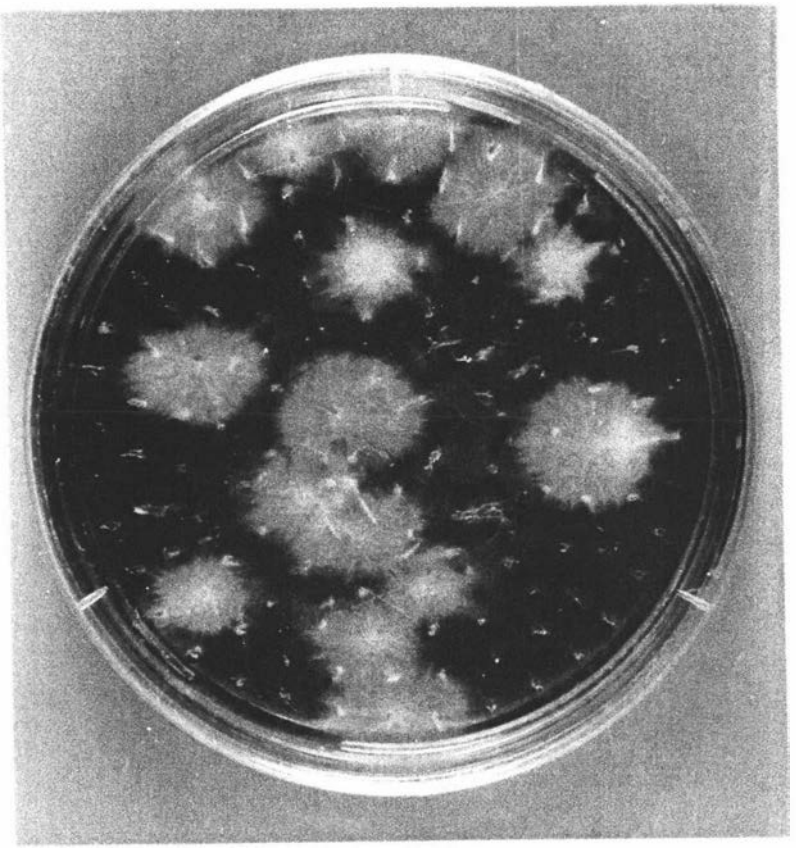


Table 3-7: Fungi isolated from cats and dogs using the hairbrush technique and cultured on SDA with antibiotics.

Genus	Cats (178)	Isol. rate (%)	Dogs (59)	Isol. rate (%)	Total (237)	Isol. rate (%)
Alternaria	11	6.2	6	10.2	17	7.2
Anixiopsis	1	0.6	0	0.0	1	0.4
Arthroderma +	1	0.6	1	1.7	2	0.8
Aspergillus	7	3.9	6	10.2	13	5.5
Beauveria	8	4.5	2	3.4	10	4.2
Cephalosporum	0	0.0	1	1.7	1	0.4
Chrysosporium +	42	23.6	14	23.7	56	23.6
Cladosporium	16	9.0	8	13.6	24	10.1
Diheterospora	2	1.1	0	0.0	2	0.8
Geotrichum	0	0.0	2	3.4	2	0.8
"*Graphium*"	1	0.6	0	0.0	1	0.4
Microsporum +	37	20.8	3	5.1	40	16.9
Mucor	2	1.1	0	0.0	2	0.8
Paecilomyces	3	1.7	1	1.7	4	1.7
Penicillium	21	11.8	4	6.8	25	10.6
Rhizopus	1	0.6	0	0.0	1	0.4
Scopulariopsis	0	0.0	1	1.7	1	0.4
Trichoderma	11	6.2	1	1.7	12	5.1
Trichophyton +	26	14.6	3	5.1	29	12.2
Trichosporon	1	0.6	1	1.7	2	0.8
Trichothecium	1	0.6	0	0.0	1	0.4
Yeasts	3	1.7	4	6.8	7	3.0

+ = regarded as keratinolytic

The number of different genera isolated was 17 (18 +"*Graphium*") and 15 from cats and dogs respectively with a total of 20 (21 +"*Graphium*") genera isolated from both animals (Table 3-7). The fungus termed "*Graphium*" was identified on the basis of its similarity to *Petriella* as described by Barron (1968).

Isolations from cats were predominantly those considered keratinolytic fungi, with *Chrysosporium*, *Microsporum* and *Trichophyton* spp. representing 23.6%, 20.8% and 14.6% respectively. The predominant keratinolytic fungus from dogs was *Chrysosporium* (23.7%) with *Microsporum* and *Trichophyton* each with 5.1%. The other fungi common on cats were *Cladosporium* (9%) and *Penicillium* (11.8%) while *Alternaria* and *Aspergillus* each with 10.2% and *Cladosporium* (13.6%) were common on dogs.

A *t*-test performed by the statistical package SAS (SAS Institute Inc., 1988) showed a significant difference ($P \leq 0.05$) between the two animals as carriers of fungi, cats being the more important reservoir.

3.2.2 Keratinolytic Species.

Overall, keratinolytic fungi were more common than other fungi with *Chrysosporium* at 23.6%, *Microsporum* 16.9% and *Trichophyton* 12.2%. *Penicillium* (10.6%) and *Cladosporium* (10.1%) were the only non-keratinolytic fungi common on both cats and dogs.

Table 3-8: Keratinolytic fungi isolated from small animals using the hairbrush technique.

Species	Cats (178)	Isol. rate (%)	Dogs (59)	Isol. rate (%)	Total (237)	Isol. rate (%)
Microsporum canis	33	18.5	3	5.1	36	15.2
M.cookei	1	0.6	0	0.0	1	0.4
Trichophyton ajelloi	6	3.4	0	0.0	6	2.5
T.mentagrophytes	7	3.9	0	0.0	7	3.0
T.terrestre	18	10.1	3	5.1	21	8.9
Arthroderma curreyi	1	0.6	1	1.7	2	0.8
Chrysosporium spp.	42	23.6	13	23.7	55	23.2

The *Microsporum* spp. comprised mainly the pathogenic *M. canis*, while *Trichophyton* spp. were predominantly the nonpathogenic *T. terrestre*.

Cats were a common source of *M. canis*. Isolations of *T. terrestre* were more frequent from cats than from dogs but *Chrysosporium* spp. were equally prevalent on both animals.

There was still a significant difference ($P \leq 0.05$) between cats and dogs as carriers of keratinolytic fungi, with cats being the favoured host.

The SPCA premises was a good source of *M. canis*, 33 isolates being obtained from 178 cats examined (18.5%) and 3 from 59 dogs (5.1%). *T. mentagrophytes* was isolated from 3.9% of the cats. Although the brush technique is not used as a quantitative test, 6 of the 178 cats examined at the SPCA, produced a large number of colonies of *M. canis* on culture, indicating that their coats were heavily contaminated with fungal spores. On close examination, two of these were found to have clinical disease (Table 3-6). Six small rugs of blanket used in the cages for cats were also sampled. One of the rugs produced a positive *M. canis* isolate using the brush technique.

In addition to the isolates of *M. canis* obtained above, a further 25 hairbrush samples yielding 6 isolates of *M. canis* were obtained from veterinary clinics and 14 *M. canis* pure cultures were received from Wellington and Auckland. All isolates are listed in appendix A.

3.2.3 Bimonthly Distribution of *M. canis*.

The bimonthly distribution of *M. canis* isolations from cats and dogs was compared with the average bimonthly rainfall, average bimonthly temperature and average bimonthly humidity. It can be noted from Table 3-9 and Fig.3-7 that over a ten month period the distribution of *M. canis* in small animals peaked in May-June. The relative average bimonthly humidity for the two months was the highest relative humidity recorded for the year.

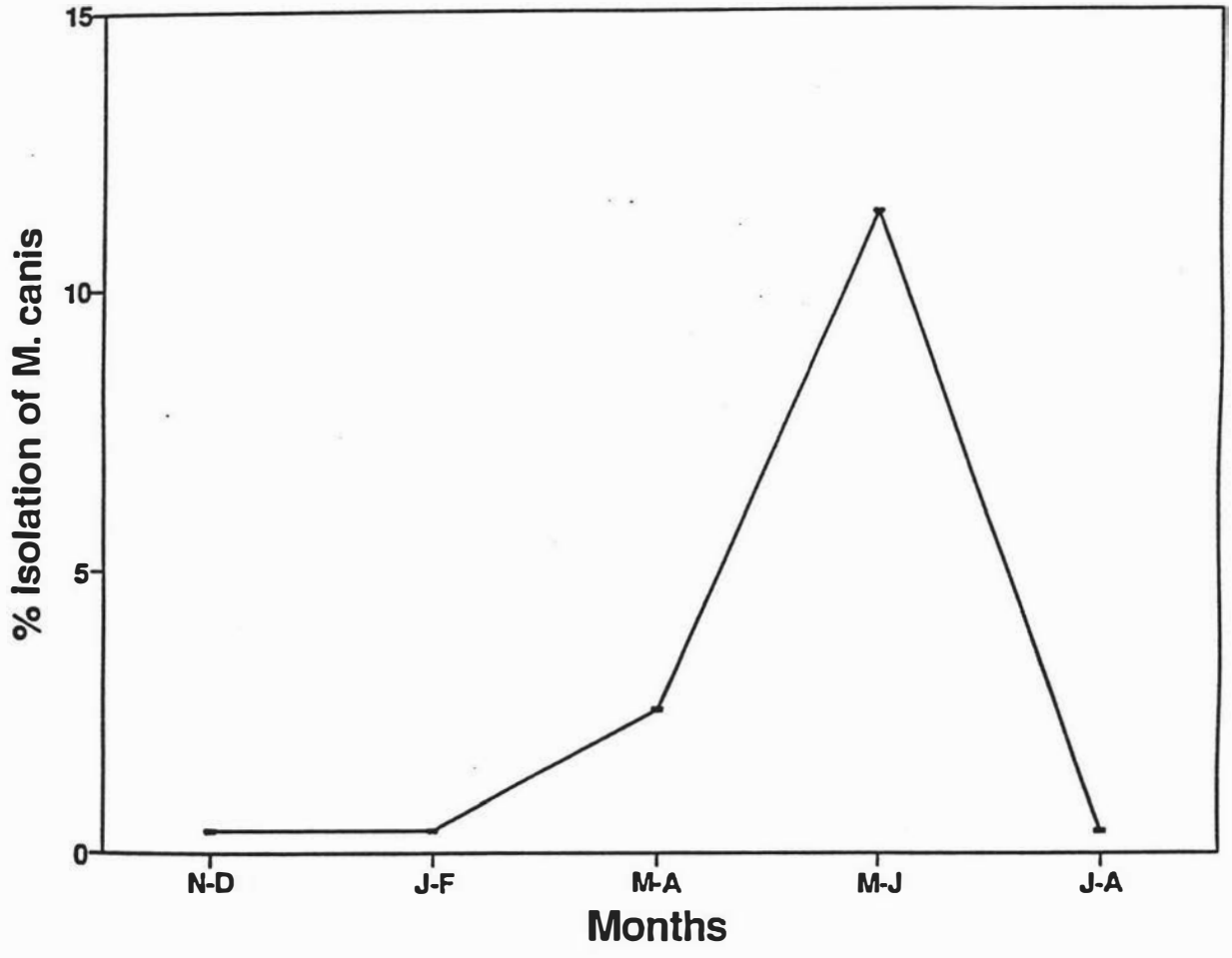
Table 3-9: Bimonthly fluctuations of *M. canis* isolations from 237 small animals, Nov. 1990 - Aug. 1991.

Month[a]	*M.canis*	%	Rainfall (mm)	Temp. (°C)	RH%
November-December	01	0.4	2.46	15.6	76
January-February	01	0.4	4.44	17.5	78
March-April	06	2.5	3.18	14.9	79
May-June	27	11.4	2.68	9.9	87.5
July-August	01	0.4	3.09	9.2	86.5

a: Nov-Dec., 1990; Jan-Aug., 1991

Comparing the data using statistical analysis, the correlation coefficient (*r*) between the bimonthly distribution of *M. canis* in small animals and those of the meteorological factors were calculated using the statistical package, SYSTAT (Wilkinson, 1989). The distribution of *M. canis* was positively correlated with relative humidity ($r = 0.621$) which was significant at the 10% significance level. However, the distribution of *M. canis* was negatively correlated with temperature ($r = -0.508$), but was not significant. Therefore, relative humidity and temperature can be interpreted to have an effect on *M. canis* distribution, which increases with increased relative humidity and decreases with increased temperature.

Fig.3-7: Bimonthly distribution of *M. canis*.



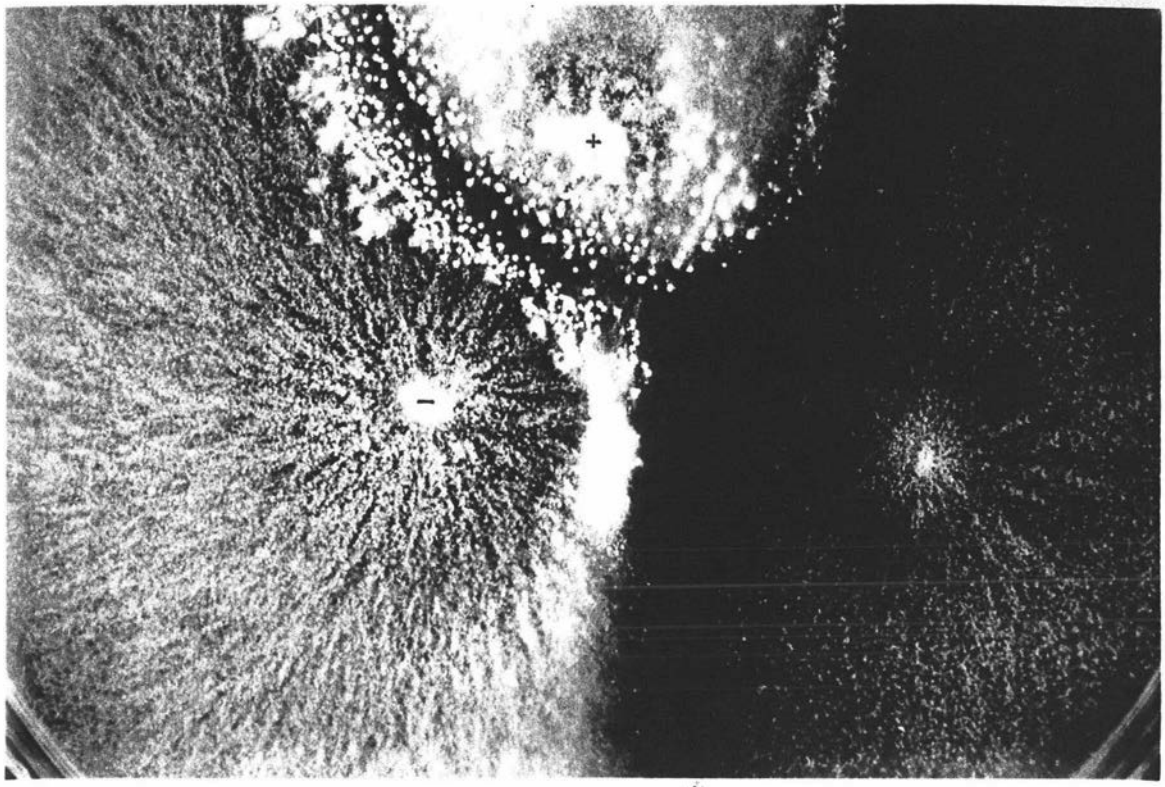
3.3 DETERMINATION OF MATING TYPES OF *MICROSPORUM* ISOLATES USING *A. SIMII* AND *N. OTAE*.

3.3.1 *M. canis*

The mating type of all *M. canis* isolates as determined by A.simii "+" (MY036.84) and "-" (MYO37.84) and the tester strains, *N. otae* "+" (RV42487) and "-" (RV42488) was found to be "-".

Fig.3-8: Zone with cleistothecia formed between *A. simii* "+" (MY03684) and "-" (MY03784) and stimulated growth containing some cleistothecial initials between *A. simii* "+" (MY03684) and *M. canis* (PN11).

Fig.3-9: A cross between the tester strain *N. otae* "+" (RV42487) and *M. canis* (PN05) on SDA (1/8) agar.



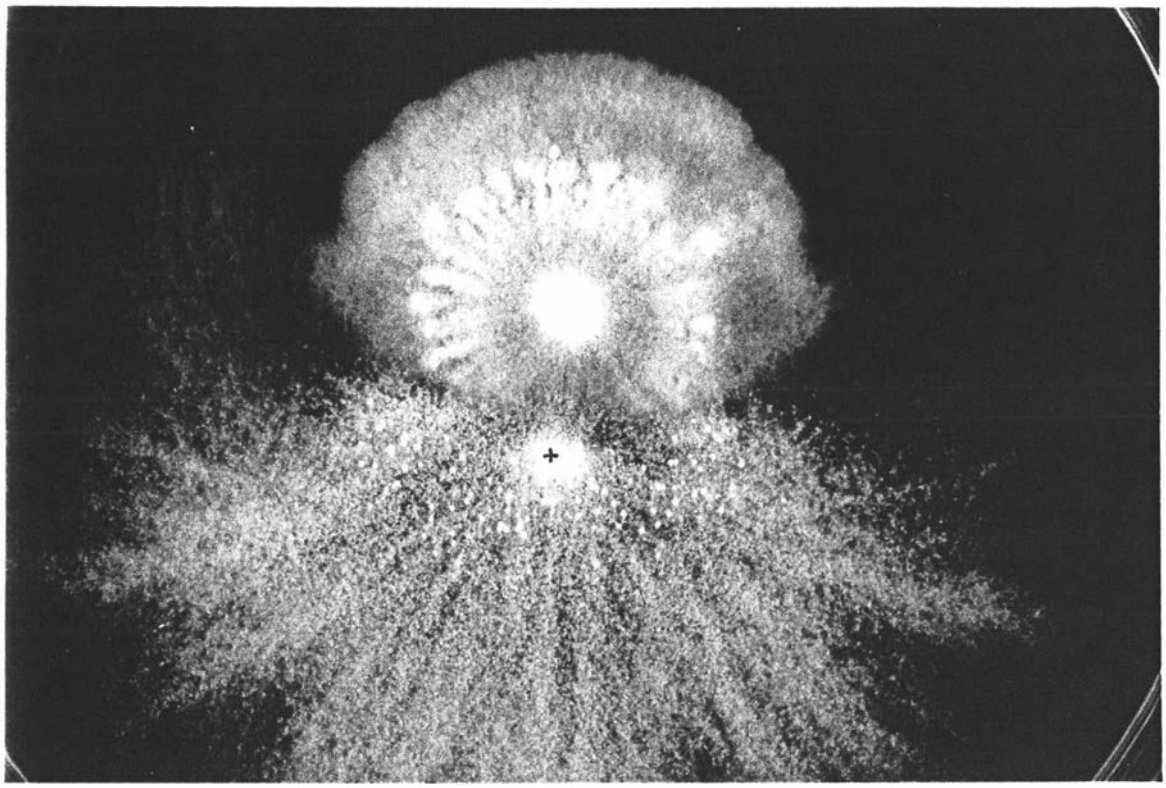
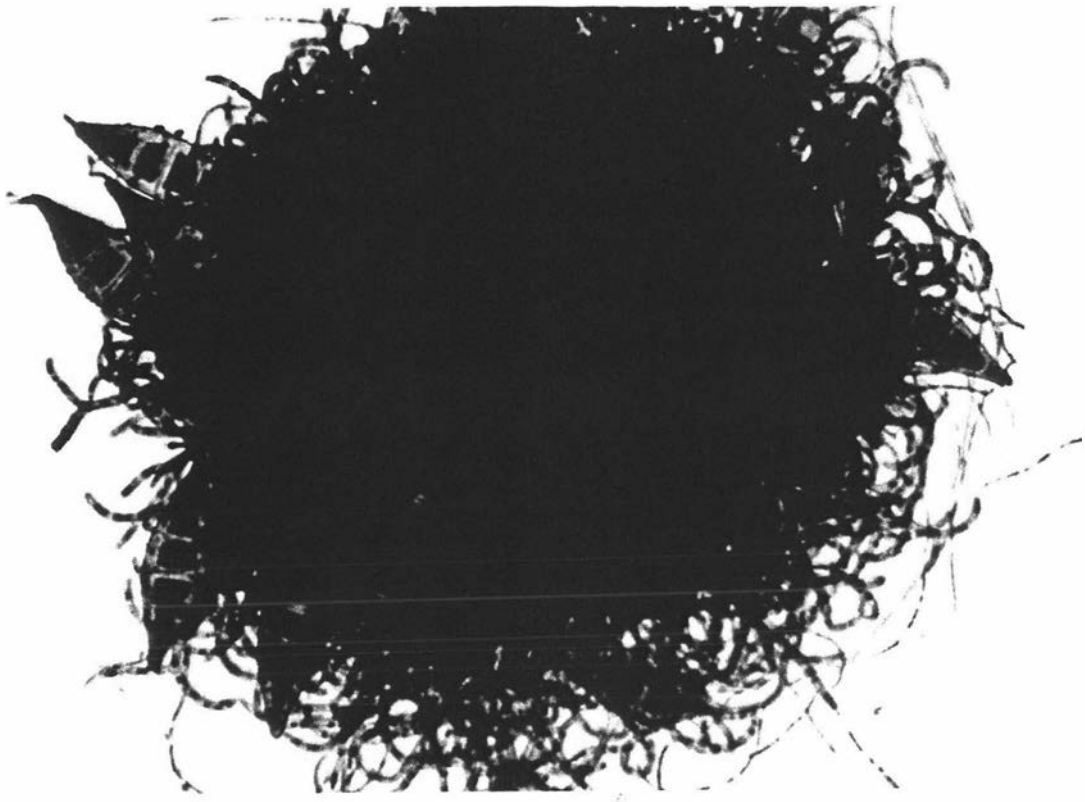
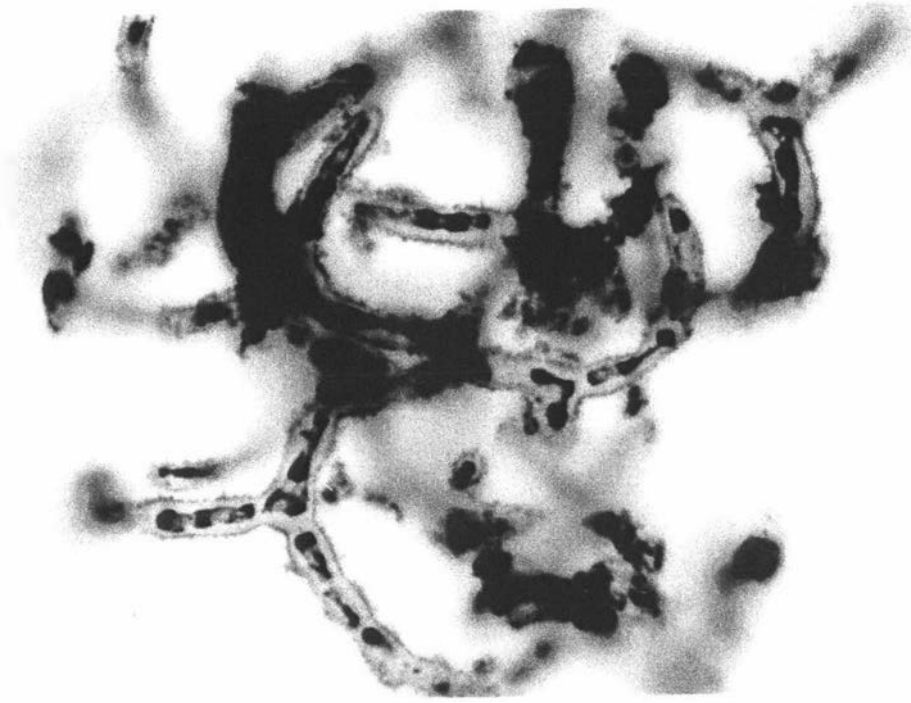


Fig.3-10: Cleistothecia of *N. otae* produced by crossing *M. canis* (PN05) with tester strain *N. otae* "+" (RV42487) (magnification x200).

Fig.3-11: Peridial hyphae of cleistothecia produced by crossing *M. canis* (PN05) with tester strain *N. otae* "+" (RV42487) (magnification x500).





3.3.2 *M. cookei.*

The mating types of the *M. cookei* isolates are shown in table 3-10.

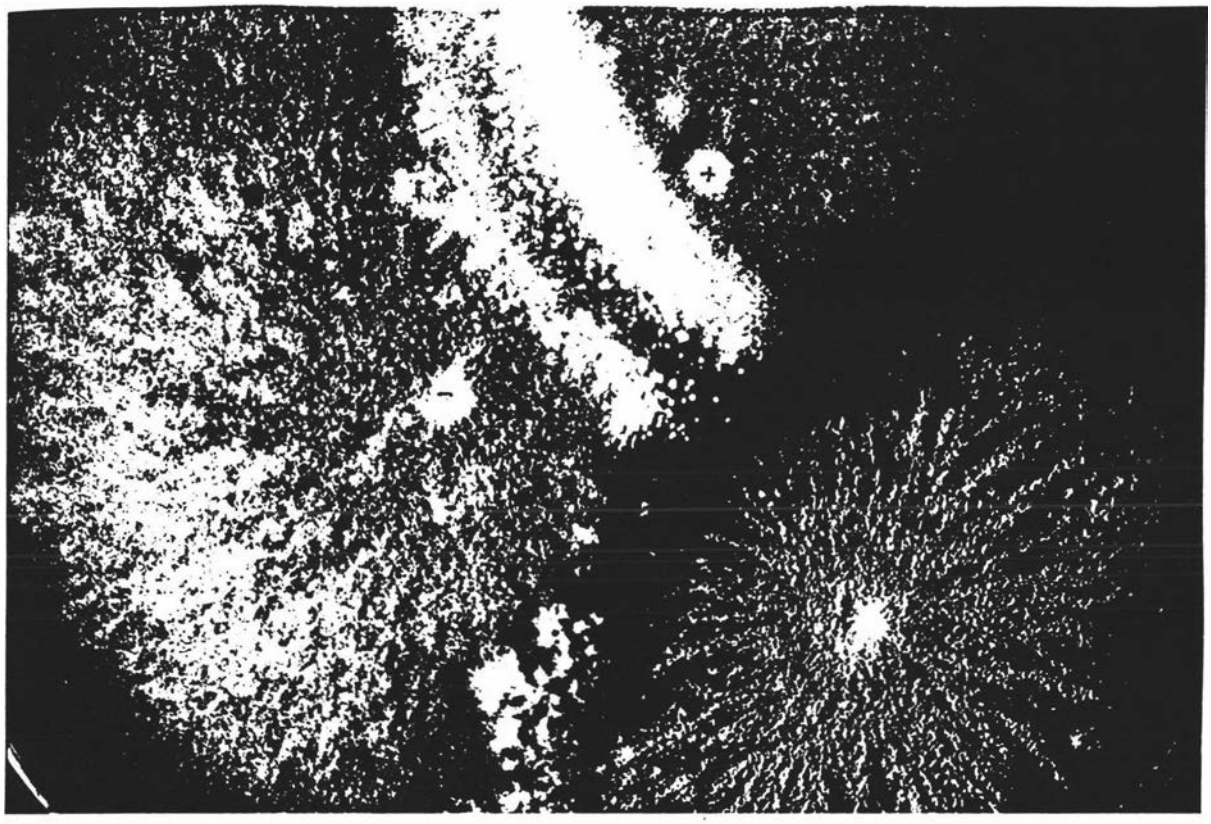
Table 3-10: Determination of mating types of *M. cookei* isolates using *A. simii.*

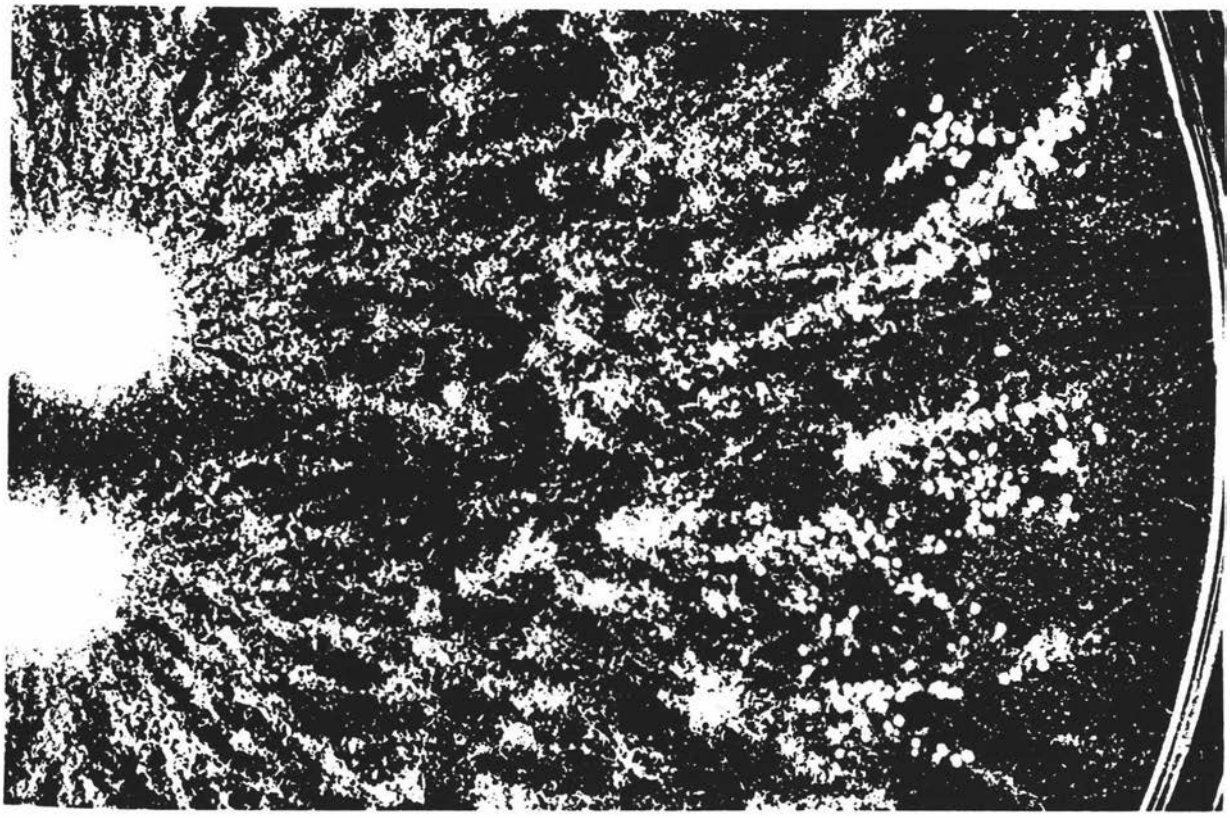
Isolate	Result of pairing		Mating type
	A.simii(+)	A.simii(-)	
H08	+	-	-
H30	+	-	-
A32	+	-	-
S33	-	+	+
A39	+	-	-
A81	+	-	-
A107	+	-	-
A125	-	+	+
A152	+	-	-
H153	-	+	+
H154	+	-	-
A155	-	+	+
H173	+	-	-
H192	-	+	+
H219	-	+	+
H223	-	+	+
H228	+	-	-
C551	+	-	-

+ = stimulated growth; - = no stimulated growth

Fig.3-12: Zone with cleistothecia formed between *A. simii* "+" (MY03684) and "-" (MY03784) and stimulated growth containing some cleistothecial initials between *A. simii* "+" (MY03684) and *M. cookei* (H223).

Fig.3-13: Crossing between (A39) "+" and (H219) "-" strains of *M. cookei* on SDA (1/10) agar.





Of the 72 crosses between "+" and "-" isolates, four were very fertile, 14 were moderately fertile, 31 were weak reactors and the rest were infertile. Table 3-11 summarises the degree of fertility between "+" and "-" mating types.

Table 3-11: Mating studies of "+" and "-" *M. cookei* isolates.

	(+) Isolates					
(-)Isolates	S33	A125	H153	A155	H219	H223
H08	+	+	+	-	+	++
H30	++	-	++	-	+	++
A32	+	+	-	++	+	+
A39	-	-	+	-	+++	+++
A81	+	-	+	-	+	++
A107	-	-	++	+	++	+
A152	+	-	+	-	+	++
H154	-	+	++	-	++	+
H173	-	-	-	++	-	-
H192	+	-	+	-	+	+
H228	++	+	+++	++	+	+
C551	+	+	+	-	+	+++

Number of Cleistothecia

+++ = >50

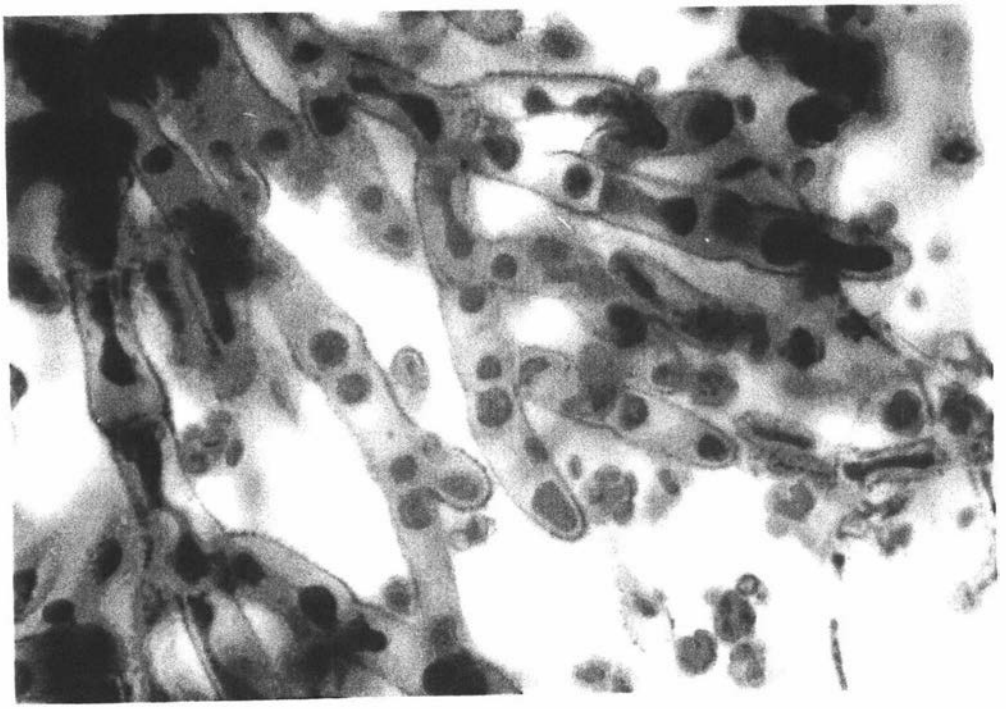
++ = 10 - 50

+ = <10

- = no cleistothecia

Fig.3-14: Cleistothecia of *N. cajetani* produced by crossing "+" and "-" strains of *M. cookei* (magnification x200)

Fig.3-15: Peridial hyphae produced by crossing "+" and "-" strains of *M. cookei* (magnification x500).



3.4 ASSAYS OF PROTEOLYTIC ENZYMES PRODUCED BY *M. CANIS* AND *M. COOKEI*.

3.4.1 Gelatin Hydrolysis.

Gelatin hydrolysis was used as an indictor of relative extracellular enzyme expression by the isolates of *M. canis* and *M. cookei* obtained in this study. Measurement of the colony radius and the extent of gelatin hydrolysis away from the colony margin allowed an expression of proteolytic activity as the ratio extent of hydrolysis : colony radius (Baxter, 1968). Activity among the 52 *M. canis* isolates tested showed little variation, ranging from 1.1 - 2.0 (Table 3-12) but the 18 *M. cookei* isolates (Table 3-13) showed a greater range (1.6 - 4.0). *M. cookei* H223 had the highest activity of all isolates examined by this technique.

Table 3-12: Gelatin hydrolysis by *M. canis* isolates.

Isolate code	Activity (cc/cd)	Isolate code	Activity (cc/cd)	Isolate code	Activity (cc/cd)
PN01	1.4	PN18	1.8	WG34	1.5
PN02	1.6	PN19	1.7	WG35	1.7
PN03	2.0	PN20	1.7	NK36	1.2
PN04	1.7	PN21	1.8	PN37	1.6
PN05	1.7	PN22	1.7	PN38	1.8
PN06	1.7	PN23	1.8	PN39A	1.8
PN07	1.7	PN24	1.7	PN39B	1.7
PN08	1.8	PN25	1.3	PN40	1.8
WG09	1.7	PN26	1.5	PN41	1.7
WG10	1.8	PN27	1.7	PN42	1.6
PN11	1.8	PN28	1.9	PN43	1.7
PN12	1.8	PN29	1.5	PN44	1.4

Table 3-12 contd.

PN13	1.8	WG30A	1.5	AK45	2.0
PN14	1.4	WG30B	1.7	AK46	1.3
PN15	1.8	WG31	1.2	AK47	1.4
PN16	1.8	WG32	1.5	AK48	1.2
PN17	1.8	WG33	1.6	AK49	1.1
				AK50	1.4

cc = radius of hydrolysis (cm); cd = colony radius (cm)
Nk = source of isolate unknown

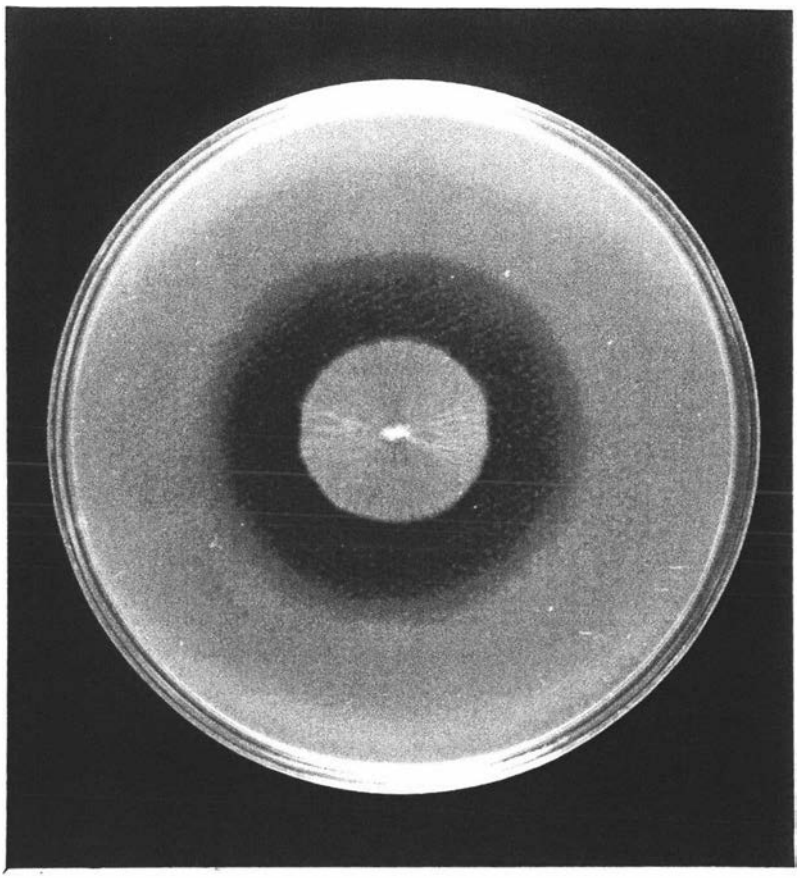
Table 3-13: Gelatin hydrolysis by *M. cookei* isolates.

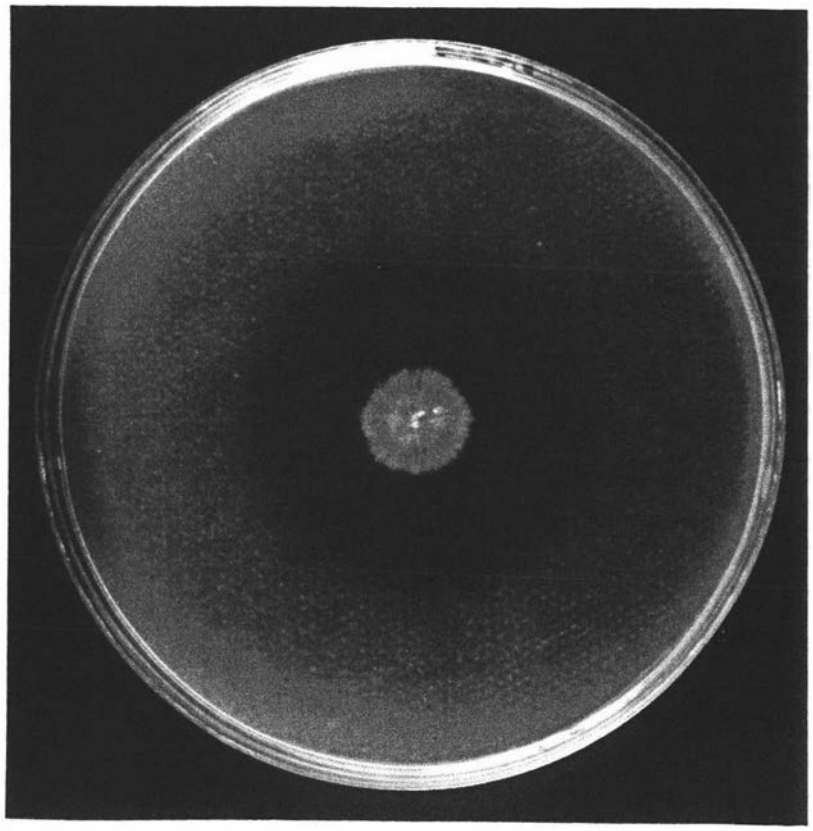
Isolate code	Activity (cc/cd)	Isolate code	Activity (cc/cd)	Isolate code	Activity (cc/cd)
H08	2.2	A107	2.0	C551	2.0
H30	2.5	A125	2.6	H219	1.6
A32	2.2	A153	2.4	H223	4.0
S33	2.5	H154	2.0	H228	2.4
A39	2.4	A155	2.1	HD32	2.2
A81	2.0	H192	1.9	HD28	2.0

cc = radius of hydrolysis (cm); cd = colony radius (cm)

Fig.3-16: Hydrolysis of gelatin by *M. canis* (PN26) using the gelatin plate method.

Fig.3-17: Hydrolysis of gelatin by *M. cookei* (H223) using the gelatin plate method.





3.4.2 Biochemical Assays with *M. canis*.

The results of the assays for proteinase (azocollytic), elastinolytic and keratinolytic activity following growth of *M. canis* in Sabouraud broth in shake and stationary culture at 25°C are displayed in Tables 3-14 and 3-15.

Proteinase activities of the individual isolates varied widely for *M. canis* from 0.13 $PUml^{-1}$ to 6.87 $PUml^{-1}$ for shake (Table 3-14) and 0.04 $PUml^{-1}$ to 3.16 $PUml^{-1}$ for stationary cultures (Table 3-15). *M. canis* PN21 had the highest activity for shake while AK48 had the highest activity for stationary cultures. There was a consistently higher level of proteinase activity shown by individual isolates in shake than in stationary cultures.

Elastase expression in shake cultures ranged from 5.54μgml^{-1} to 9.03μgml^{-1} and in stationary cultures from 6.66μgml^{-1} to 10.52μgml^{-1} with the highest elastase activity obtained in shake culture with AK46 (Table 3-14) and in stationary culture with AK45 (Table 3-15).

Keratinase activity was low in stationary cultures (0.0 to 0.26 $KUml^{-1}$) but ranged from 0.0 to 9.22 $KUml^{-1}$ in shake cultures.

Table 3-14: Enzyme expression of *M. canis* isolates in shake cultures.

Isolate code	clinical cases(+) carriers (-)	Shake cultures		
		Proteinase (±SE)	Elastase (±SE)	Keratinase (±SE)
PN01	-	0.88±0.21	7.79±0.07	0.11±0.03
PN02	-	1.25±0.42	8.05±0.21	0.55±0.54
PN03	-	1.12±0.29	8.15±0.02	0.01±0.01
PN04	-	2.08±0.42	7.92±0.13	0.05±0.01
PN05	+	0.83±0.00	7.80±0.02	0.00±0.00
PN06	+	2.21±0.29	6.25±0.08	0.00±0.00
PN07	+	0.17±0.08	8.10±0.06	0.02±0.00
PN08	+	0.25±0.13	8.91±0.13	0.00±0.00
WG09	+	0.13±0.13	8.39±0.10	0.03±0.03
WG10	+	0.21±0.13	8.06±0.04	0.01±0.01
PN11	-	5.21±0.13	8.03±0.06	0.09±0.04
PN12	-	0.83±0.17	8.27±0.04	0.01±0.01
PN13	-	3.75±0.17	8.34±0.21	0.02±0.02
PN14	-	2.17±0.50	8.58±0.14	0.09±0.03
PN15	-	5.33±0.58	8.20±0.00	0.00±0.00
PN16	-	1.29±0.04	8.61±0.03	0.01±0.01
PN17	-	1.91±0.17	7.33±0.04	0.15±0.15
PN18	-	1.29±0.04	8.44±0.04	0.00±0.00
PN19	-	3.29±0.63	7.06±0.04	0.11±0.02
PN20	-	0.92±0.00	8.91±0.07	0.08±0.05
PN21	N	6.87±0.96	6.25±0.09	0.11±0.11
PN22	-	0.00±0.00	7.92±0.12	4.39±4.30
PN23	-	4.58±0.00	6.05±0.04	8.23±8.07
PN24	-	2.83±1.16	5.79±0.13	0.66±0.65
PN25	-	1.33±0.00	7.00±0.04	1.10±1.08

AK = Auckland; PN = Palmerston North; WG = Wellington

N = unknown; NA = not applicable

Table 3-14 contd.

PN26	-	1.25±0.08	8.37±0.15	5.38±5.27
PN27	-	4.29±0.46	6.05±0.13	9.22±9.04
PN28	-	3.62±0.38	7.09±0.35	1.98±1.94
PN29	+	1.04±0.29	8.70±0.21	5.16±5.06
WG30A	+	1.46±0.29	7.48±0.01	2.42±2.37
WG30B	+	0.75±0.50	6.91±0.03	7.68±7.53
WG31	+	3.33±0.17	5.71±0.15	0.00±0.00
WG32	+	1.25±0.25	6.95±0.05	0.00±0.00
WG33	+	1.58±0.33	6.31±0.12	0.00±0.00
WG34	+	1.96±0.04	7.02±0.12	6.37±6.24
WG35	+	2.87±0.38	6.88±0.06	1.21±1.18
LK36	N	2.12±0.21	6.95±0.06	0.00±0.00
PN37	-	2.41±0.33	5.54±0.03	0.00±0.00
PN38	-	2.62±0.54	6.92±0.04	0.00±0.00
PN39A	-	2.17±0.25	6.39±0.03	3.95±3.87
PN39B	-	1.12±0.21	7.70±0.22	0.00±0.00
PN40	-	4.91±0.33	6.39±0.05	0.00±0.00
PN41	-	2.58±0.42	6.71±0.10	8.23±8.07
PN42	-	3.54±0.13	6.54±0.04	0.00±0.00
PN43	-	6.08±0.25	5.92±0.08	0.00±0.00
PN44	+	1.62±0.13	7.01±0.09	0.00±0.00
AK45	+	1.12±0.29	7.36±0.25	0.00±0.00
AK46	+	1.00±0.08	9.03±0.12	0.15±0.03
AK47	+	1.12±0.21	8.63±0.11	0.07±0.03
AK48	+	1.75±0.00	8.22±0.15	0.08±0.01
AK49	+	0.38±0.38	6.73±0.17	0.00±0.00

AK = Auckland; PN = Palmerston North; WG = Wellington

N = unknown; NA = not applicable

Table 3-15: Enzyme expression of *M. canis* isolates in stationary cultures.

Isolate code	clinical cases(+) carriers (-)	Stationary cultures		
		Proteinase (±SE)	Elastase (±SE)	Keratinase (±SE)
PN01	-	0.58±0.25	8.45±0.19	0.05±0.00
PN02	-	0.63±0.05	8.61±0.05	0.03±0.02
PN03	-	0.13±0.13	8.56±0.07	0.03±0.03
PN04	-	0.33±0.09	8.54±0.04	0.08±0.03
PN05	+	0.79±0.21	7.90±0.10	0.19±0.12
PN06	+	0.50±0.08	8.16±0.13	0.09±0.06
PN07	+	1.04±0.46	8.18±0.09	0.05±0.00
PN08	+	1.66±0.08	8.84±0.07	0.02±0.02
WG09	+	1.17±0.25	9.02±0.01	0.14±0.03
WG10	+	0.88±0.05	8.35±0.12	0.03±0.03
PN11	-	0.79±0.21	9.13±0.22	0.03±0.03
PN12	-	0.17±0.00	8.33±0.18	0.10±0.10
PN13	-	1.71±0.04	9.27±0.00	0.04±0.03
PN14	-	1.08±0.33	8.58±0.02	0.05±0.05
PN15	-	0.38±0.38	9.11±0.48	0.00±0.00
PN16	-	0.38±0.20	9.28±0.34	0.12±0.05
PN17	-	0.00±0.00	9.13±0.07	0.00±0.00
PN18	-	0.25±0.25	9.43±0.20	0.01±0.01
PN19	-	0.13±0.13	9.57±0.34	0.00±0.00
PN20	-	0.04±0.04	8.80±0.29	0.19±0.17
PN21	N	1.16±0.58	8.71±0.14	0.04±0.04
PN22	-	0.50±0.25	8.90±0.09	0.00±0.00
PN23	-	1.20±0.13	7.90±0.15	0.04±0.02
PN24	-	1.12±0.38	6.66±0.08	0.00±0.00
PN25	-	0.71±0.21	8.91±0.06	0.08±0.06

AK = Auckland; PN = Palmerston North; WG = Wellington

N = unknown; NA = not applicable

Table 3-15 contd.

PN26	-	0.58±0.08	9.39±0.11	0.11±0.11
PN27	-	0.80±0.13	7.48±0.13	0.00±0.00
PN28	-	0.41±0.41	8.93±0.09	0.00±0.00
PN29	+	0.71±0.21	9.78±0.26	0.00±0.00
WG30A	+	1.83±0.50	8.41±0.04	0.00±0.08
WG30B	+	0.88±0.13	8.52±0.28	0.08±0.00
WG31	+	1.04±0.13	7.75±0.09	0.00±0.00
WG32	+	0.88±0.13	8.41±0.18	0.07±0.02
WG33	+	1.04±0.46	7.69±0.07	0.11±0.11
WG34	+	1.21±0.29	8.32±0.23	0.04±0.04
WG35	+	0.88±0.54	8.75±0.02	0.05±0.02
LK36	N	0.45±0.13	8.23±0.11	0.00±0.00
PN37	-	0.58±0.42	8.45±0.33	0.09±0.07
PN38	-	0.63±0.05	7.87±0.18	0.05±0.04
PN39A	-	0.09±0.09	8.83±0.09	0.08±0.08
PN39B	-	2.41±0.42	9.34±0.02	0.00±0.00
PN40	-	2.33±0.33	6.94±0.06	0.19±0.01
PN41	-	3.08±0.42	9.23±0.10	0.20±0.02
PN42	-	2.25±0.33	8.31±0.04	0.20±0.03
PN43	-	1.92±0.00	7.29±0.00	0.19±0.04
PN44	+	1.62±0.29	9.06±0.04	0.00±0.00
AK45	+	2.58±0.08	10.52±0.25	0.00±0.00
AK46	+	1.96±0.29	9.62±0.23	0.00±0.00
AK47	+	1.08±0.25	8.98±0.30	0.26±0.04
AK48	+	3.16±0.17	10.04±0.19	0.17±0.02
AK49	+	1.71±0.04	10.00±0.25	0.00±0.00
AK50	+	0.88±0.21	9.83±0.05	0.00±0.00

AK = Auckland; PN = Palmerston North; WG = Wellington

N = unknown; NA = not applicable

3.4.2.1 Comparison of Overall Enzyme Expression of *M. canis* Isolates.

For differences in the overall enzyme expression by *M. canis* isolates for the two treatments (continuous shake and stationary culture), they were compared by the weighted mean using the inverse of the standard error (SE). This was performed using the statistical package, SAS (SAS Institute Inc., 1988).

Table 3-16: Analysis of enzyme expression of isolates of *M. canis* grown in shake and stationary cultures using the weighted mean.

Enzyme	P-value
Proteinase	0.0016
Elastase	0.0001
Keratinase	0.4324

The overall expression of proteinase activity in shake culture was significantly (P=0.0016) more than in stationary culture, while elastase expression was significantly (P=0.0001) more in stationary culture than in shake culture. Keratinase expression, however, was not different (P=0.4324) between the two culture methods.

To investigate any trends in the expression of the three enzymes the results were also analysed by the product moment correlation coefficient (Pearson correlation matrix) using the statistical package, SYSTAT (Wilkinson, 1989).

Table 3-17: Correlations of enzyme activities of *M. canis* using the product moment correlation coefficient (Pearson correlation matrix).

Shake culture	*r*	Sig. level
Proteinase vs elastase	-0.505	*
keratinase vs proteinase	0.031	ns
keratinase vs elastase	0.039	ns
Stationary culture		
Proteinase vs elastase	0.006	ns
keratinase vs proteinase	0.068	ns
keratinase vs elastase	0.256	ns

* = significant

Proteinase and elastase activities in shake cultures was significantly negatively correlated (r = -0.505), i.e. when proteinase expression increased, elastase expression decreased and vice versa. The other correlations for both shake and stationary cultures were not significant.

3.4.2.2 Comparison of Enzyme activities of *M. canis* Isolates using Scattergrams.

Enzyme activities of *M. canis* isolates for the two culture methods and grouped into clinical (infected) cases and carriers, were also compared by scattergrams drawn using Autocard software (version 12). (see Figs.3-18 - 3-26). This was to compare with other authors who have suggested that isolates from inflammatory cases tend to express higher proteinase levels. The dots on the scattergrams represent enzyme activity of individual isolates.

Fig.3-18: Proteinase (azocollytic) ($PUml^{-1}$) expression in shake and stationary cultures of *M. canis* isolates.

Although some isolates had similar proteinase (azocollytic) activity in the two culture methods, there was generally a higher activity in shake culture than stationary culture. (This is a similar observation to that found by gelatin/SDS-PAGE analysis, where a higher number of proteinases were detected in shake culture than stationary culture).

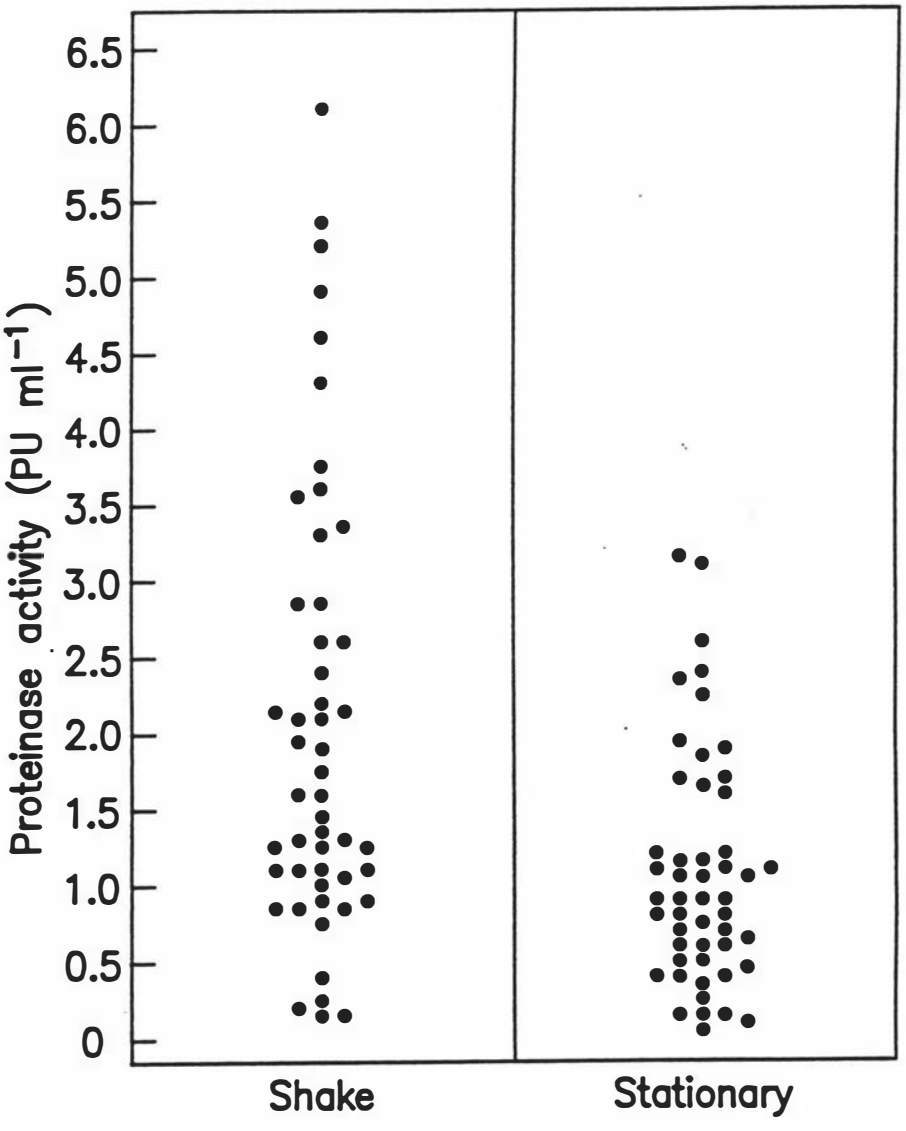

Proteinase activity (PU ml^{-1})
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Shake
Stationary

Fig.3-19: Proteinase (azocollytic) ($PUml^{-1}$) expression in shake cultures of *M. canis* isolates from clinical cases and carriers.

In shake culture, isolates from carriers had a higher baseline level (the minimum activity shown by the isolates) of proteinase activity than isolates from clinical cases. Some isolates from carriers expressed considerably higher proteinase activity than isolates from clinical cases.

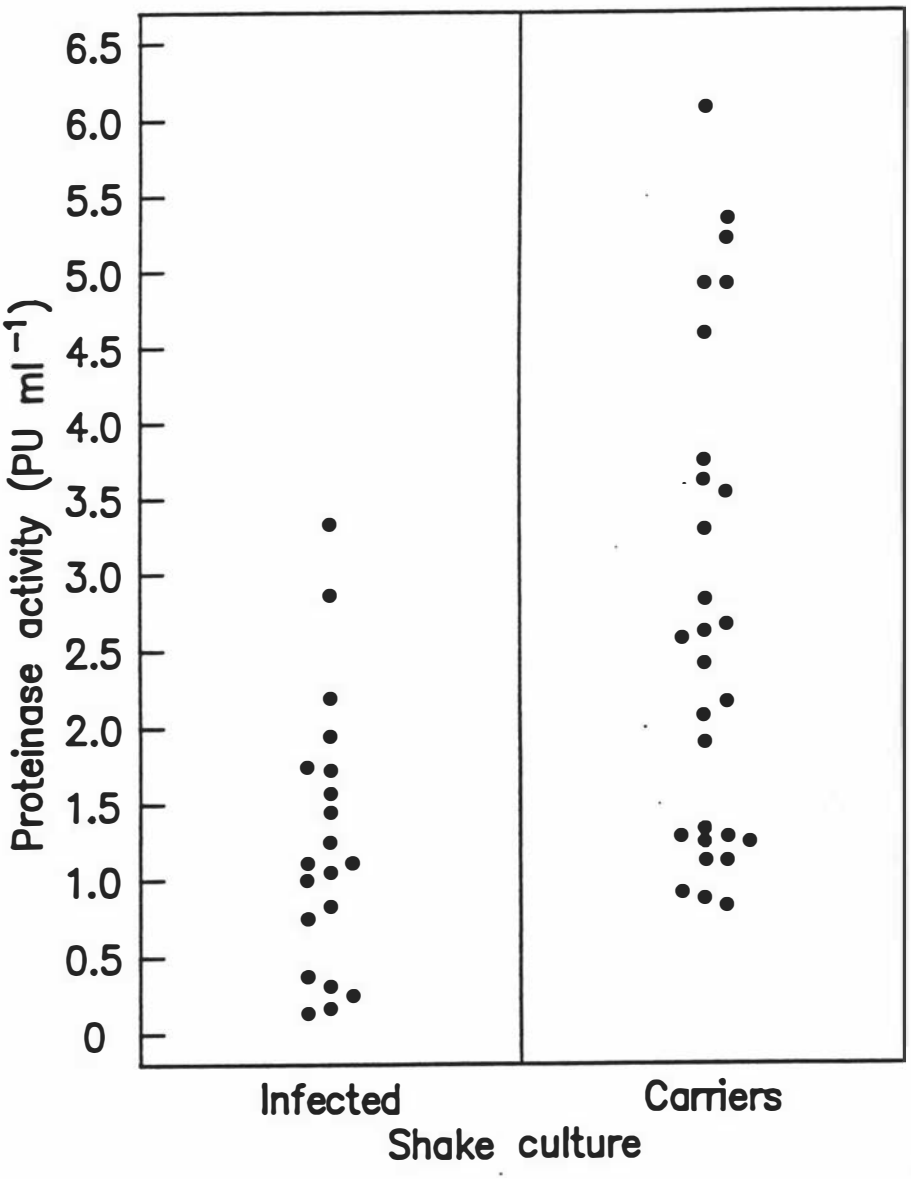

6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Proteinase activity (PU ml^{-1})
Infected
Carriers
Shake culture

Fig.3-20: Proteinase (azocollytic) ($PUml^{-1}$) expression in stationary cultures of *M. canis* isolates from clinical cases and carriers.

The proteinase activity was very similar for stationary cultures of isolates from clinical cases and carriers.

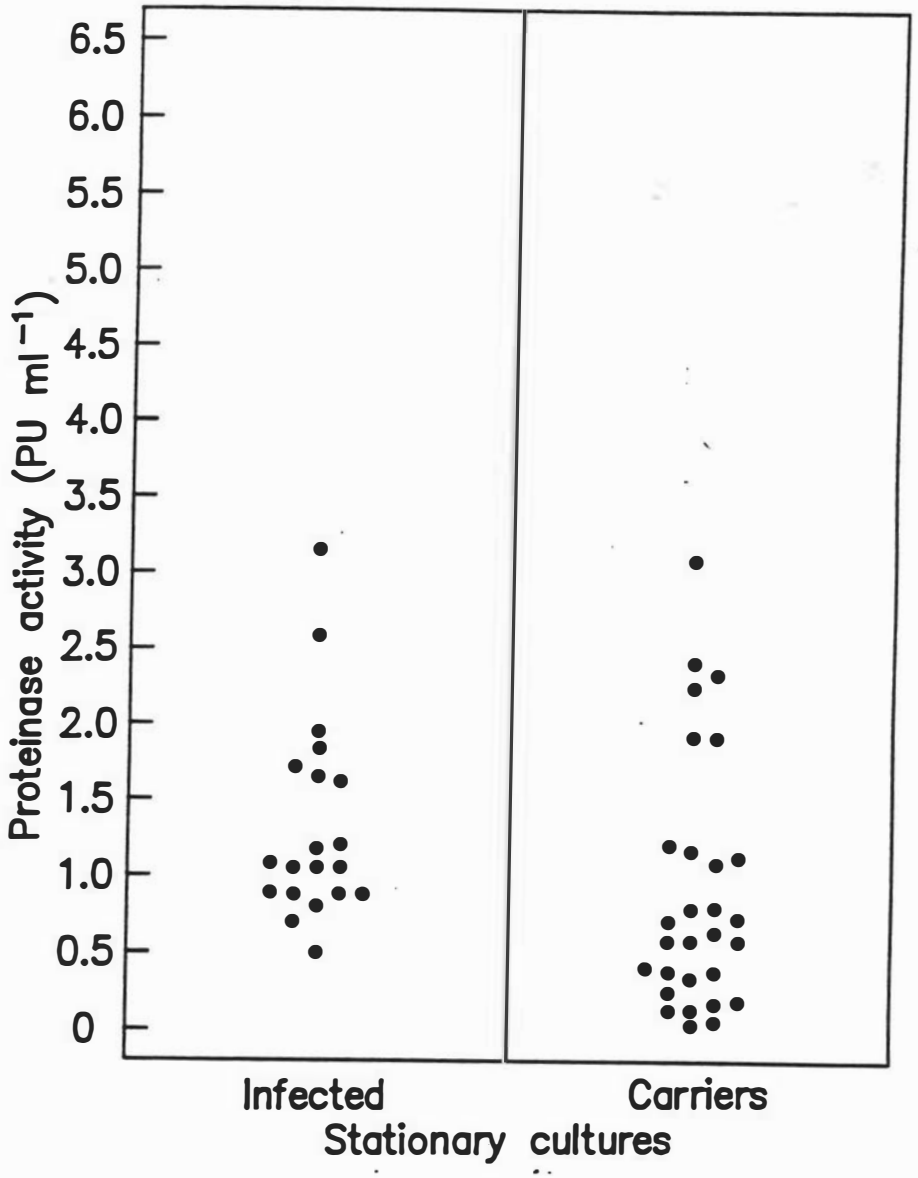

6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Proteinase activity (PU ml⁻¹)
Infected
Carriers
Stationary cultures

Fig.3-21: Elastase ($\mu g ml^{-1}$) expression in shake and stationary cultures of *M. canis* isolates.

Elastinolytic activity in shake culture was less than in stationary culture. Elastinolytic activity in stationary culture had a higher baseline level of enzyme activity than shake culture and most isolates had elastinolytic activity around $8.5 \mu g ml^{-1}$.

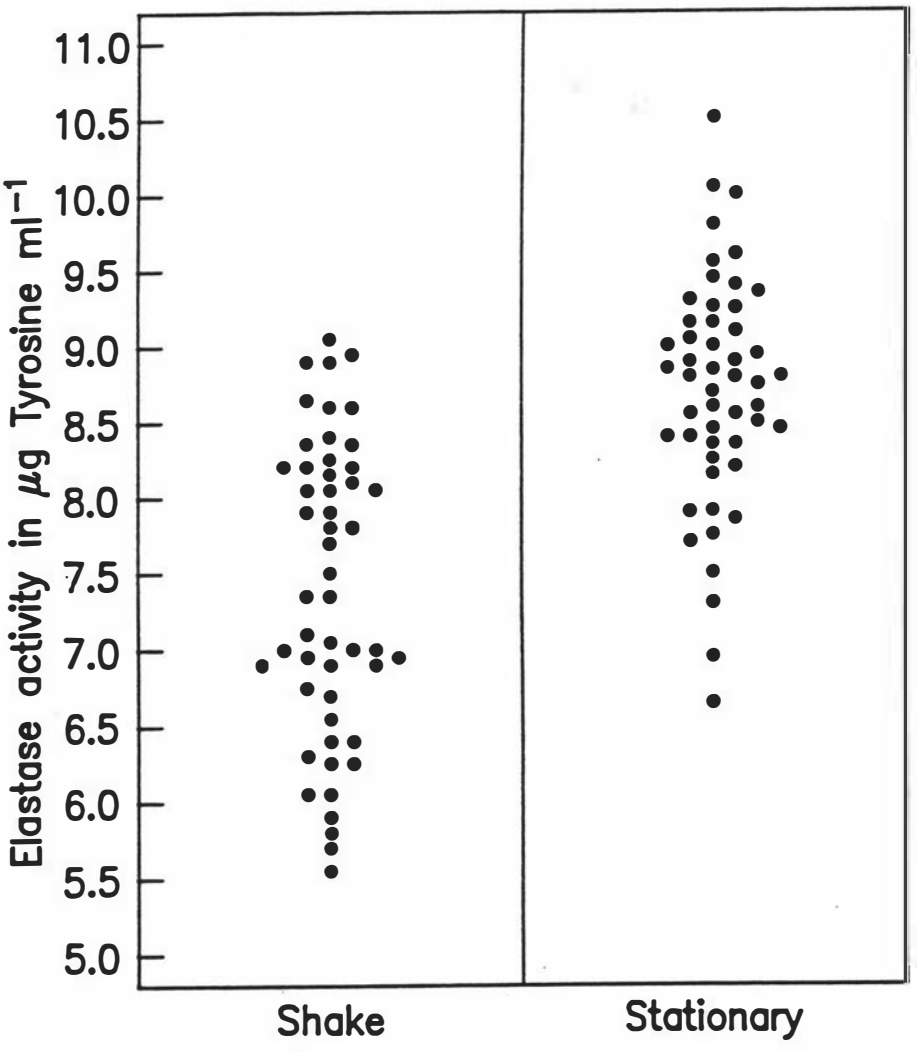

Elastase activity in μg Tyrosine ml⁻¹
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Shake
Stationary

Fig.3-22: Elastase (μgml^{-1}) expression in shake cultures of *M. canis* isolates from clinical cases and carriers.

The spread in elastinolytic activity of 5.5μgml^{-1} to 9.2μgml^{-1} was very similar for the two groups of isolates from clinical cases and carriers.

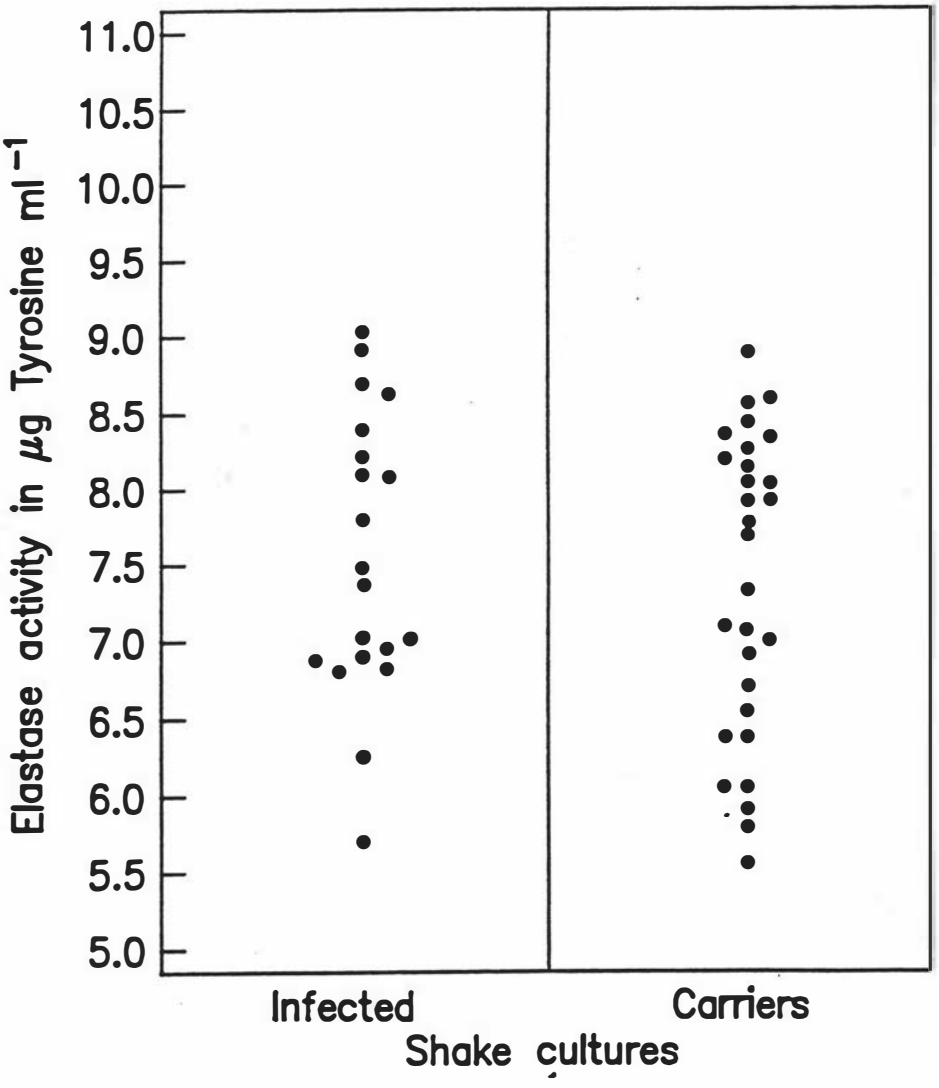

11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Elastase activity in µg Tyrosine ml⁻¹
Infected
Carriers
Shake cultures

Fig.3-23: Elastase (μgml^{-1}) expression in stationary cultures of *M. can* isolates from clinical cases and carriers.

Elastinolytic activity of isolates from clinical cases was slightly highe than isolates from carriers. Most isolates from both clinical cases an carriers had elastinolytic activity between 8.0μgml^{-1} to 9.5μgml^{-1}. A fe isolates from clinical cases had elastinolytic activity of more tha 9.5μgml^{-1}.

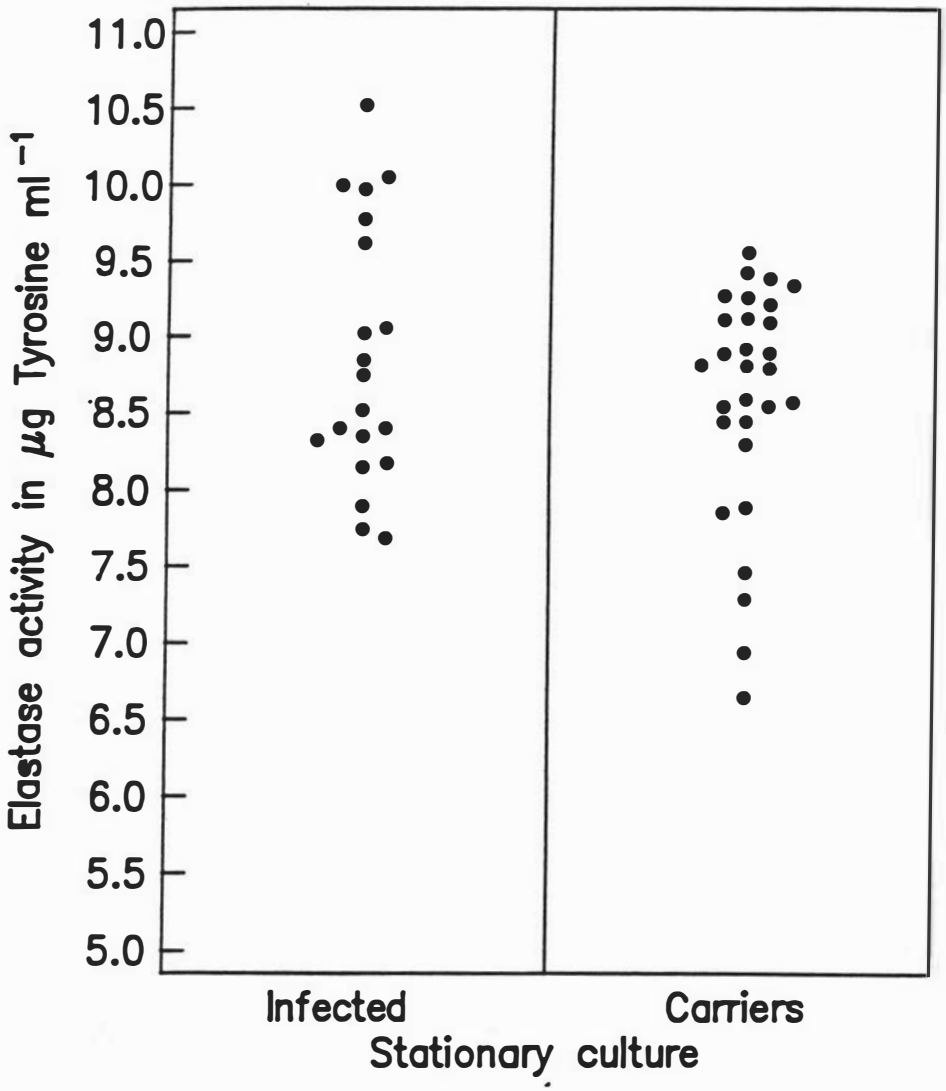

Elastase activity in µg Tyrosine ml⁻¹
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Infected
Carriers
Stationary culture

Fig.3-24: Keratinase ($KUml^{-1}$) expression in shake and stationary cultures of *M. canis* isolates.

There was very little keratinase activity in stationary culture (see also Fig. 3-34). Activity in shake culture ranged from 0.0$KUml^{-1}$ to 9.2$KUml^{-1}$.

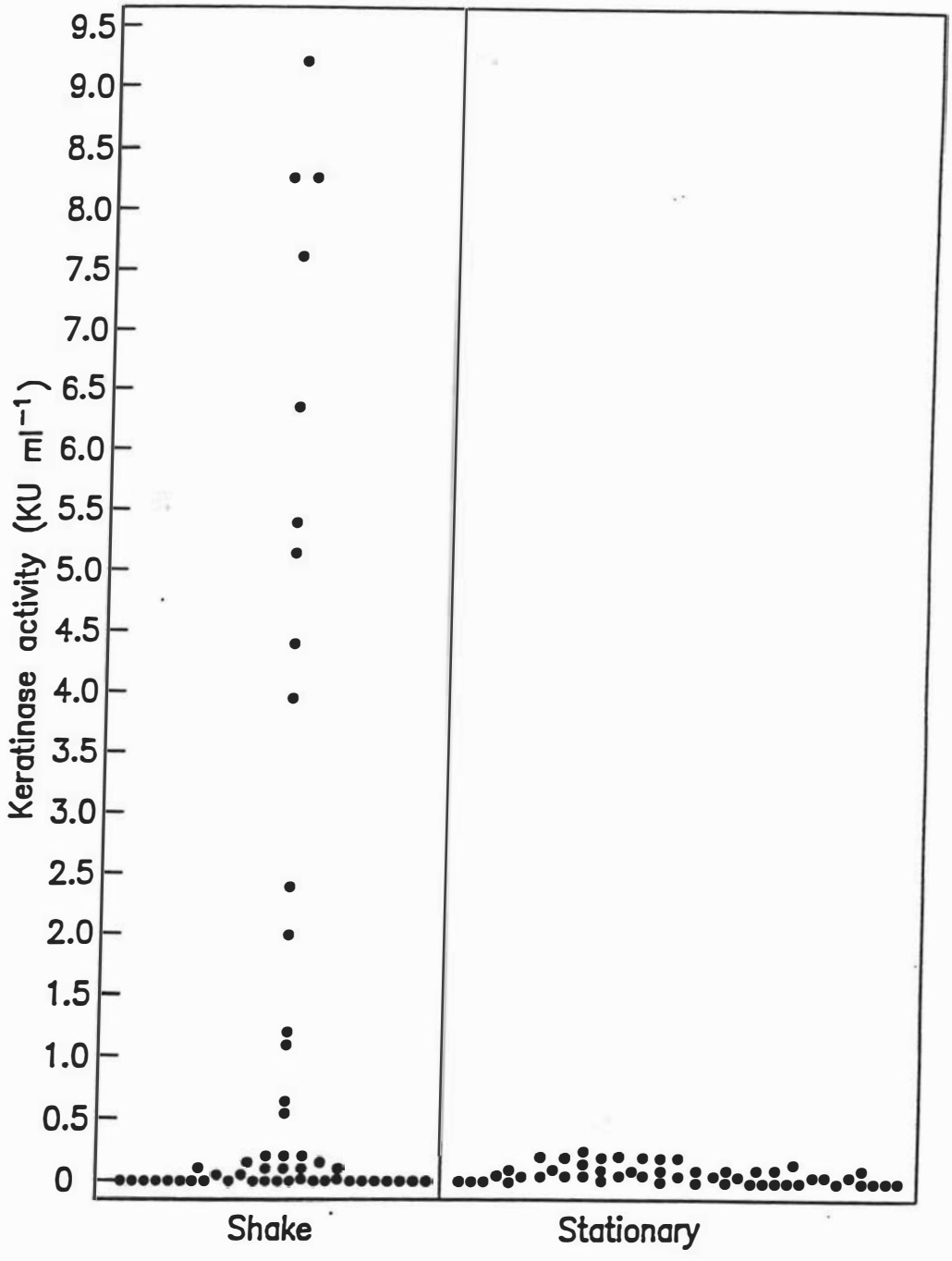

Keratinase activity (KU ml^{-1})
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Shake
Stationary

Fig.3-25: Keratinase ($KUml^{-1}$) expression in shake cultures of *M. canis* isolates from clinical cases and carriers.

Several isolates from both clinical cases and carriers grown in shake culture displayed keratinase activity. Activity was scattered over a wide range.

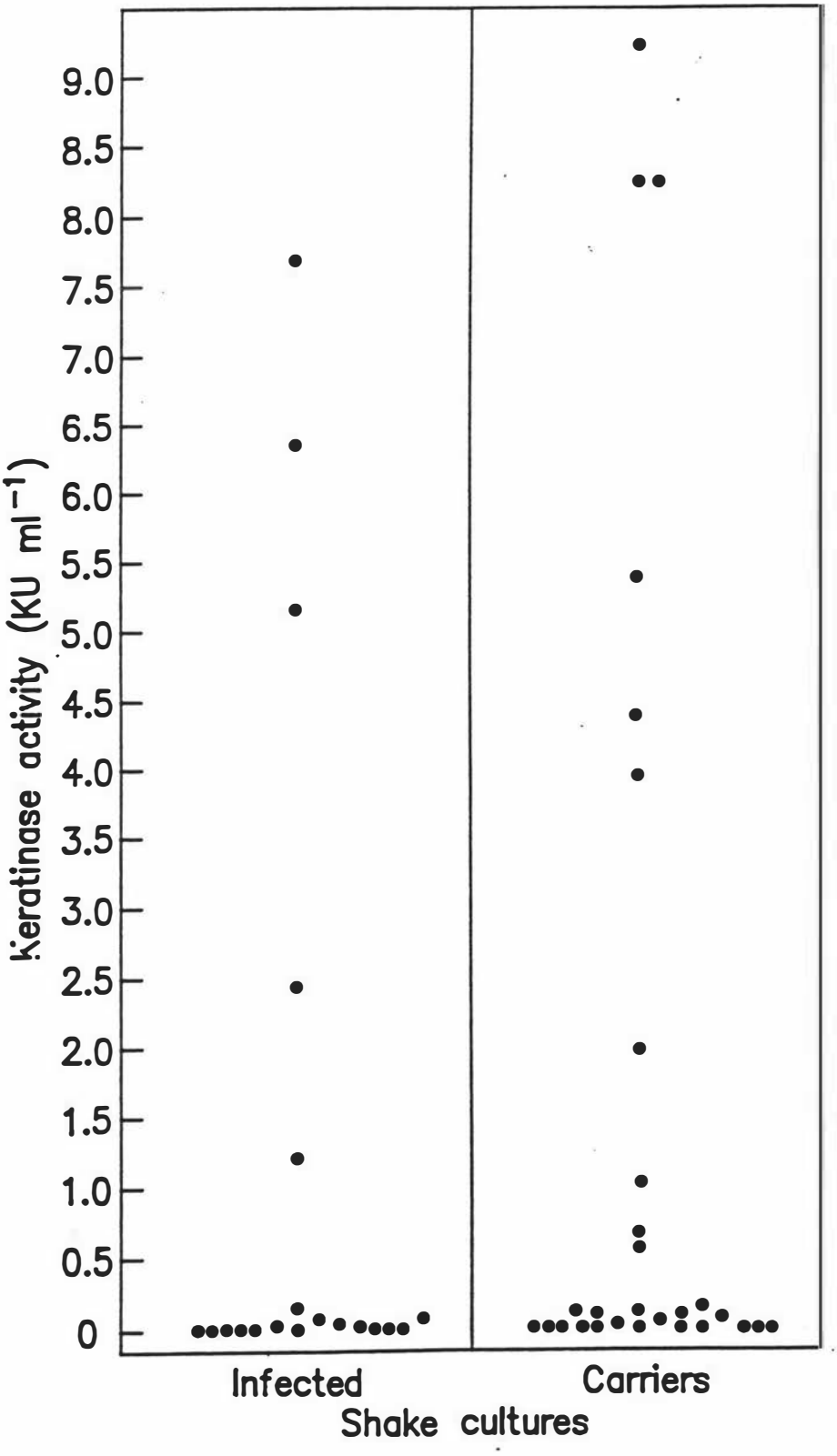
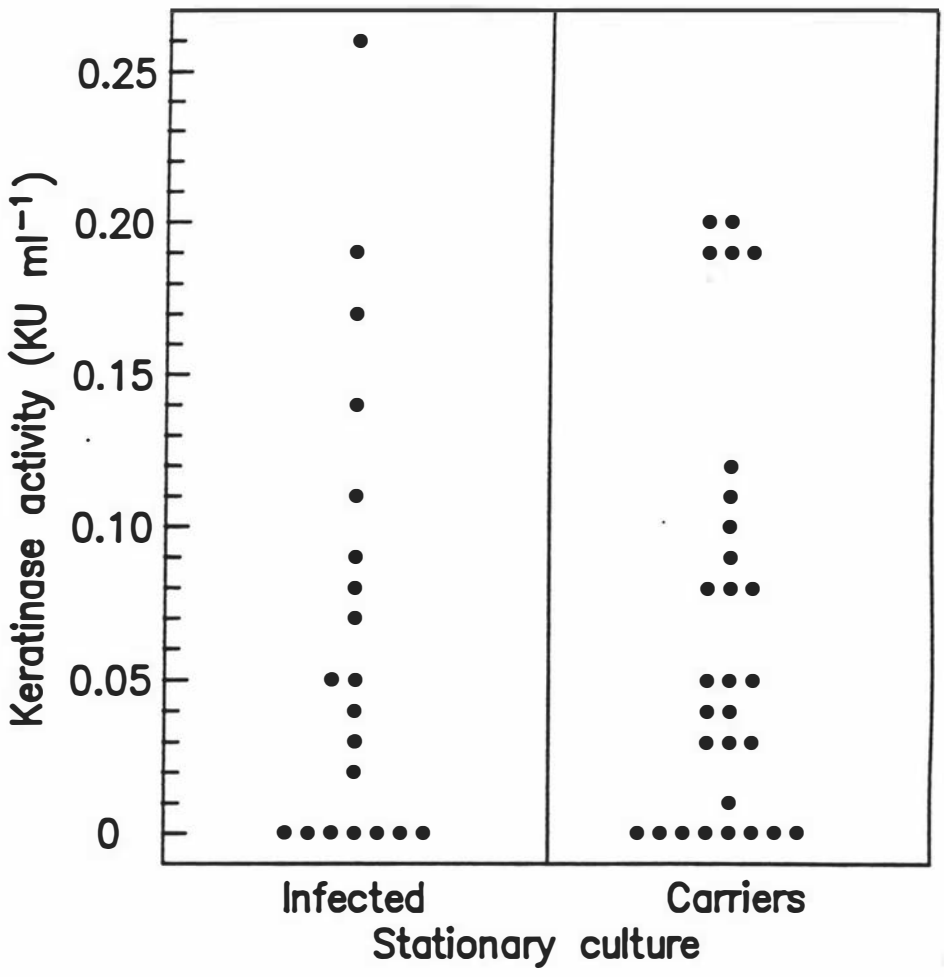

9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Keratinase activity (KU ml⁻¹)
Infected
Carriers
Shake cultures

Fig.3-26: Keratinase ($KUml^{-1}$) expression in stationary cultures of *M. canis* isolates from clinical cases and carriers.

Although there was keratinase activity in isolates from both clinical cases and carriers it was much less than that detected in shake culture.


Keratinase activity (KU ml^{-1})
0.25
0.20
0.15
0.10
0.05
0
Infected
Carriers
Stationary culture

3.4.3 Biochemical Assays with *M. cookei.*

Proteinase expression in shake culture ranged from 0.92PUml^{-1} to 32.09PUml^{-1} (Table 3-18) for S33 and H219 respectively. However, for the remaining *M. cookei* isolates proteinase activity was less than 20PUml^{-1}. Elastase activity ranged from 6.67μgml^{-1} for H08 to 10.02μgml^{-1} for C551 (Table 3-18). Keratinase activity varied widely from zero for A81, A107, A152 and H154 to 10.76μgml^{-1} for S33 (Table 3-18).

Table 3-18: Enzyme expression of *M. cookei* isolates in shake cultures.

Isolate code	Shake cultures		
	Proteinase (±SE)	Elastase (±SE)	Keratinase (±SE)
H08	12.33±0.25	6.67±0.15	2.52±2.47
H30	11.00±0.17	7.04±0.08	0.00±0.00
A32	3.25±0.42	6.75±0.04	6.59±6.46
S33	0.92±0.09	7.26±0.17	10.76±10.55
A39	11.83±0.00	7.71±0.05	9.66±9.47
A81	3.71±0.21	7.70±0.04	0.00±0.00
A107	2.37±0.38	8.72±0.28	0.00±0.00
A125	2.50±0.17	8.99±0.03	3.62±3.55
A152	9.25±0.67	7.70±0.16	0.00±0.00
A153	2.29±0.29	9.67±0.00	5.71±5.60
H154	3.25±0.00	8.97±0.09	0.00±0.00
A155	1.17±0.00	8.36±0.24	8.34±8.18
H173	1.58±0.25	9.70±0.14	7.25±7.10
H192	5.29±0.63	9.18±0.10	2.20±2.15
H219	32.09±1.41	8.05±0.04	1.21±1.18
H223	6.00±0.25	8.07±0.09	2.20±2.15
H228	14.58±0.08	7.14±0.05	6.26±6.13
C551	4.33±0.08	10.02±0.00	0.33±0.32

Proteinase activities in stationary culture for *M. cookei* ranged from 0.50PUml^{-1} to 4.83PUml^{-1} (Table 3-19). This variation was less than that for shake culture (Table 3-18). Elastase activity ranged from 7.76µgml^{-1} to 10.47µgml^{-1} (Table 3-19) which was higher than in shake culture (Table 3-18). There was no keratinase activity observed in stationary cultures of *M. cookei* isolates.

Table 3-19: Enzyme expression of *M. cookei* isolates in stationary cultures.

Isolate code	Stationary cultures		
	Proteinase (±SE)	Elastase (±SE)	Keratinase (±SE)
H08	2.75±0.50	8.30±0.00	0.00
H30	4.83±0.08	9.50±0.00	0.00
A32	3.87±0.21	9.71±0.07	0.00
S33	1.16±0.34	7.76±0.06	0.00
A39	1.37±0.29	8.68±0.13	0.00
A81	2.46±0.21	9.09±0.11	0.00
A107	2.50±0.08	9.74±0.30	0.00
A125	1.75±0.00	9.34±0.25	0.00
A152	2.04±0.13	10.07±0.03	0.00
A153	0.96±0.13	9.38±0.18	0.00
H154	1.37±0.04	9.73±0.04	0.00
A155	3.00±0.25	10.29±0.12	0.00
H173	1.87±0.13	9.55±0.00	0.00
H192	0.50±0.08	9.59±0.03	0.00
H219	4.58±0.08	9.68±0.04	0.00
H223	4.20±0.13	8.90±0.13	0.00
H228	4.33±0.25	8.59±0.11	0.00
C551	1.79±0.21	10.47±0.04	0.00

3.4.3.1 Comparison of Overall Enzyme Expression of *M. cookei* Isolates.

For differences in the overall enzyme expression by *M. cookei* isolates for the two treatments (continuous shake and stationary culture), they were compared in the same way as with *M. canis* (Section 3.4.2.1).

Table 3-20: Analysis of enzyme expression of isolates of *M. cookei* grown in shake and stationary cultures using the weighted mean.

Enzyme	P-value
Proteinase	0.0161
Elastase	0.1377

The overall expression of proteinase activity in shake culture was significantly (P=0.0161) more than in stationary culture, while elastase expression was similar (P=0.1377) in the two culture methods.

Table 3-21: Correlations of enzyme activities of *M. cookei* using the product moment correlation coefficient (Pearson correlation matrix).

Shake culture	*r*	Sig. level
Proteinase vs elastase	-0.344	ns
keratinase vs proteinase	-0.204	ns
keratinase vs elastase	-0.145	ns
Stationary culture		
Proteinase vs elastase	0.017	ns

All the three enzymes (Table 3-21) were tested for any correlations of enzyme expression. All were found not significant, implying that the three enzymes are expressed independently of each other.

3.4.3.2 Comparison of Enzyme activities of *M. cookei* Isolates using Scattergrams.

Enzyme activities of *M. cookei* isolates for the two culture methods were also compared by scattergrams drawn using Autocard software (version 12). (see Figs.3-27 - 3-28).

Fig.3-27: Proteinase (azocollytic) ($PUml^{-1}$) expression in shake and stationary cultures of *M. cookei* isolates.

Proteinase activity was greater in shake culture than in stationary culture. In stationary culture, activity was less than 5 $PUml^{-1}$. In shake culture one isolate showed activity of 32 $PUml^{-1}$.

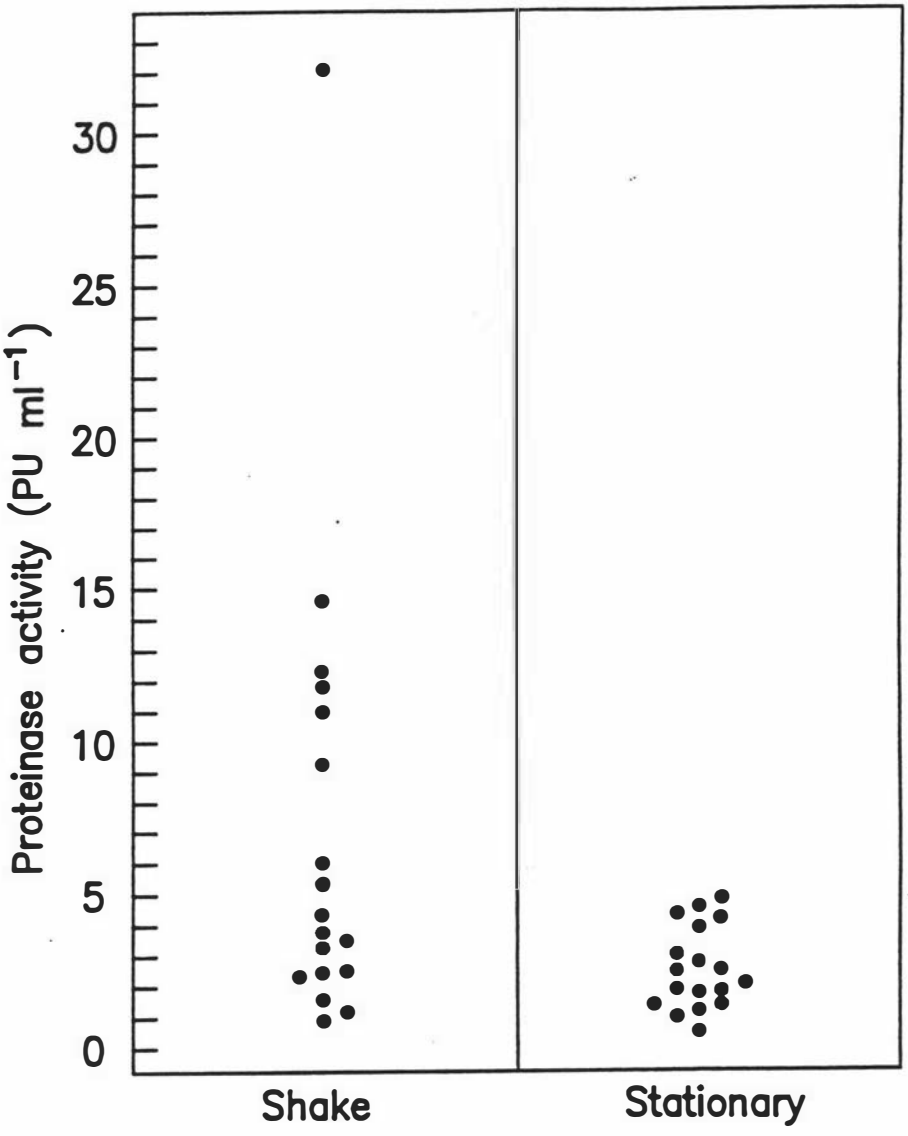
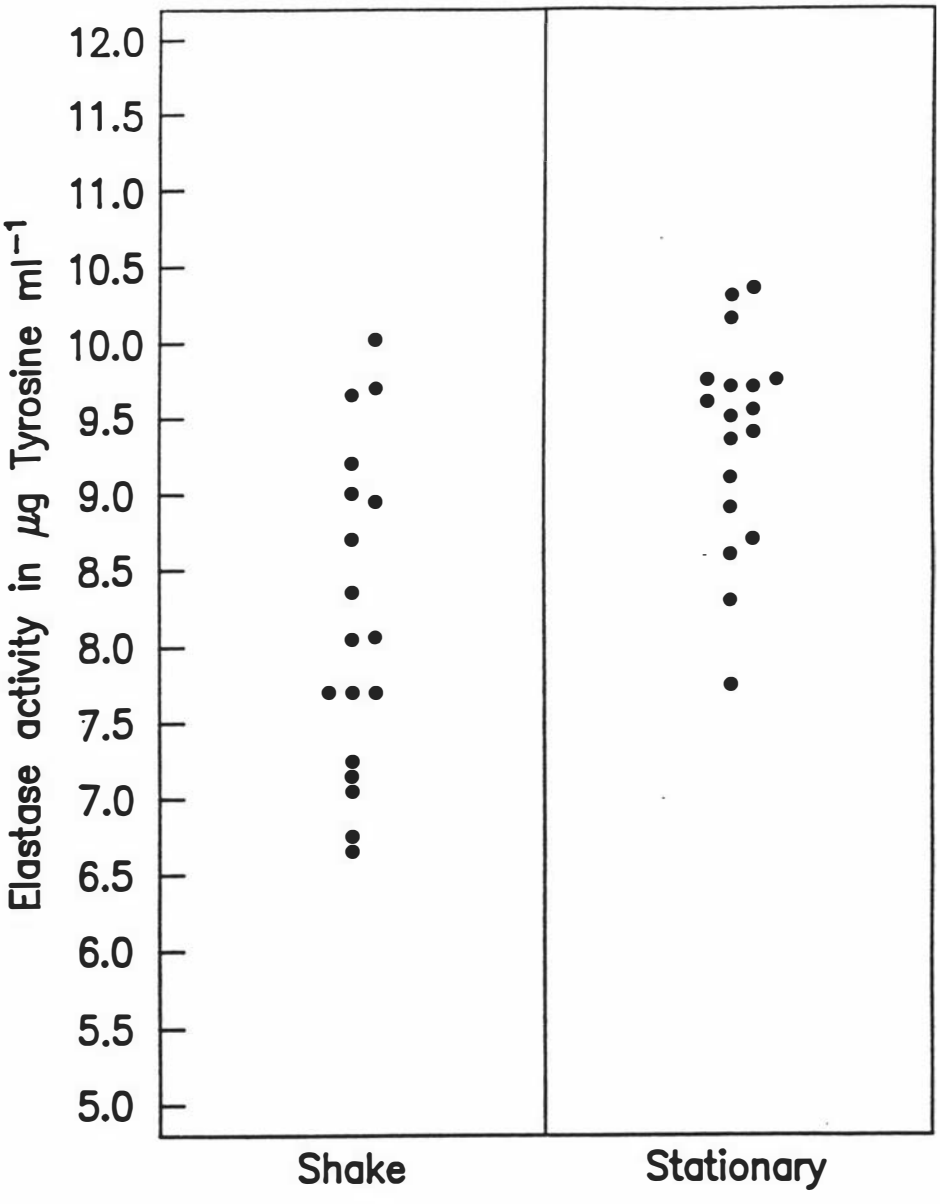

Proteinase activity (PU ml^{-1})
0
5
10
15
20
25
30
Shake
Stationary

Fig.3-28: Elastase ($\mu g ml^{-1}$) expression in shake and stationary cultures of *M. cookei* isolates.

Elastinolytic activity was very similar in the two cultural methods although stationary cultures had a slightly higher baseline level of elastinolytic activity.


Elastase activity in μg Tyrosine ml⁻¹
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
Shake
Stationary

3.4.4 Estimation of Oxygen Concentration in Cultures.

To determine whether oxygen might have an effect on enzyme expression in the two culture methods, continuous shake and stationary, oxygen content was determined after two weeks of incubation in both conditions using five *M. canis* isolates grown in Sabouraud broth and testing aliquot duplicates of each culture, utilising the technique described in Section 2.1.7.2.

Table 3-22: Oxygen concentration in shake and stationary cultures.

Isolate	Oxygen Conc^{n} (μmolesml^{-1}) in broth	
	Shake Cultures	Stationary Cultures
PN01	76	64
PN05	47	65
PN06	78	69
PN08	58	81.5
WG10	84	74

The chi-square test comparing the two culture methods showed no significant difference (X^2 =8.95; P≥0.05) in oxygen concentration.

3.4.5 Characterisation of *Microsporum* Proteolytic Enzymes.

Culture filtrate from *M. canis* (WG09) and *M. cookei* (H219) both had proteinase (azocollytic) and elastinolytic activities. To characterise the enzymes, a number of proteinase inhibitors were used to determine the type of enzymes involved (see Section 2.2.5).

3.4.5.1 *M. canis*.

Table 3-23: Effect of inhibitors on *M. canis* crude filtrate activity.

Inhibitors*	Concentration	Inhibition	
		% relative to solvent controls	
		Azocoll	Elastin
α_1-P	100µg/ml	52.7	ND
PMSF	0.2mM	100.0	0.0
PT	20mM	a	67.2
EDTA	1.0mM	100.0	39.0
$_p$CMB	20mM	73.1	5.0
E-64	20µM	31.2	3.2
IAA	0.01mM	31.6	0.0
PEPS	1µM	45.4	0.0

a: Increased activity ND = not determined

* see Section 2.2.5 or refer to Fig.3-29 for full names.

For *M. canis* using azocoll (Table 3-23), PMSF showed complete inhibition of azocollytic activity while α_1-Proteinase resulted in 52.7% inhibition. EDTA also showed complete inhibition but a stimulation of activity was observed with PT. The remaining inhibitors only showed partial inhibition. For elastin, inhibition was detected with PT and EDTA, with the others showing little or no inhibition.

Fig.3-29: Inhibition of proteinase (azocollytic) activity in *M. canis* filtrate by various inhibitors.

Abbreviations:

PMSF = phenylmethylsulfonyl fluoride; PT = 1,10-phenanthroline; EDTA = ethylenediaminetetraacetate; *p*CMB = *p*-chloromecuric acid; E-64 = L-trans-epoxysuccinyl leucylamido (4-guanidino)-butane; IAA = Iodoacetic acid; PEPS = pepstatin; and α_1-P = α-1-proteinase. (1,10-phenanthroline (PT) gave increased enzyme activity over control - not included on the graph)

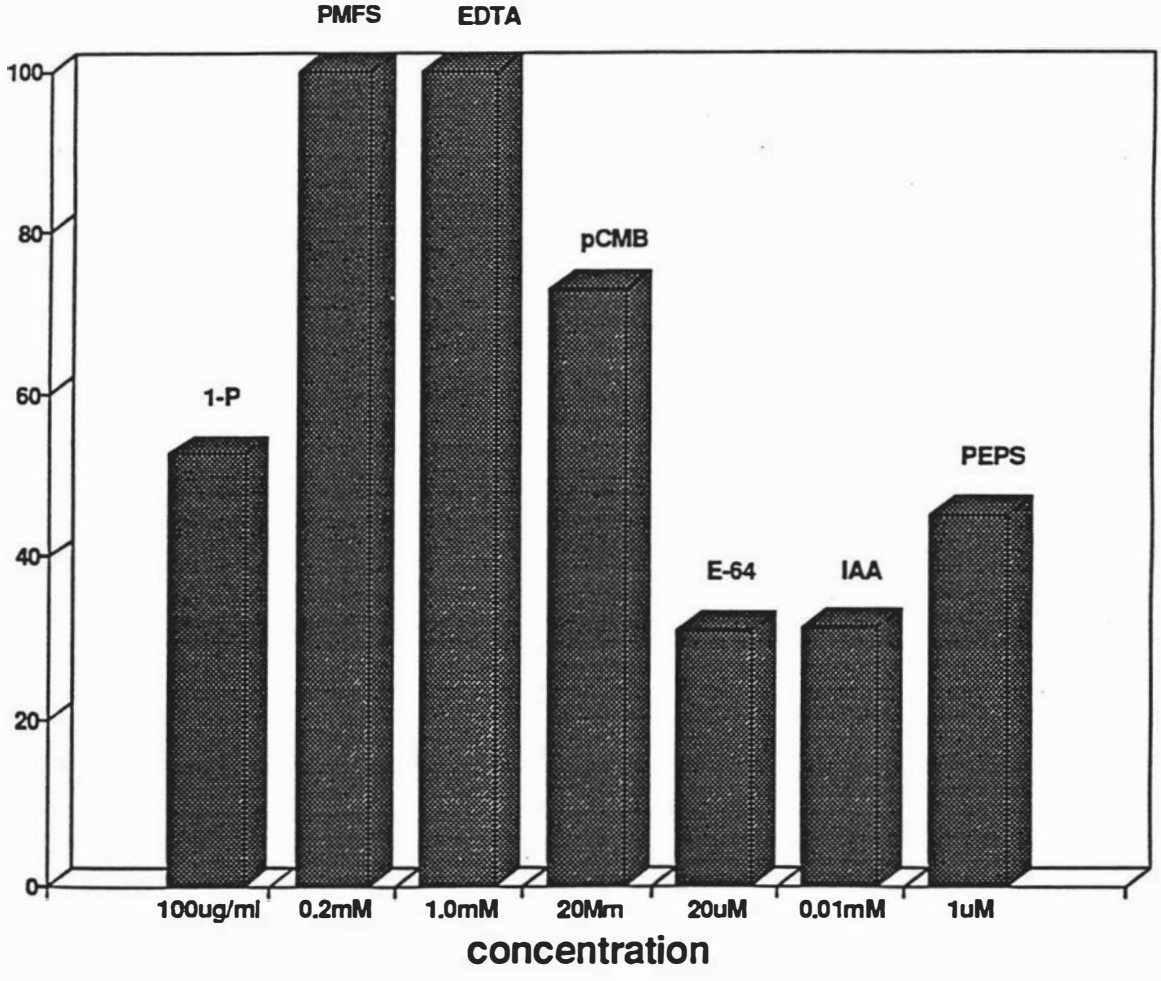
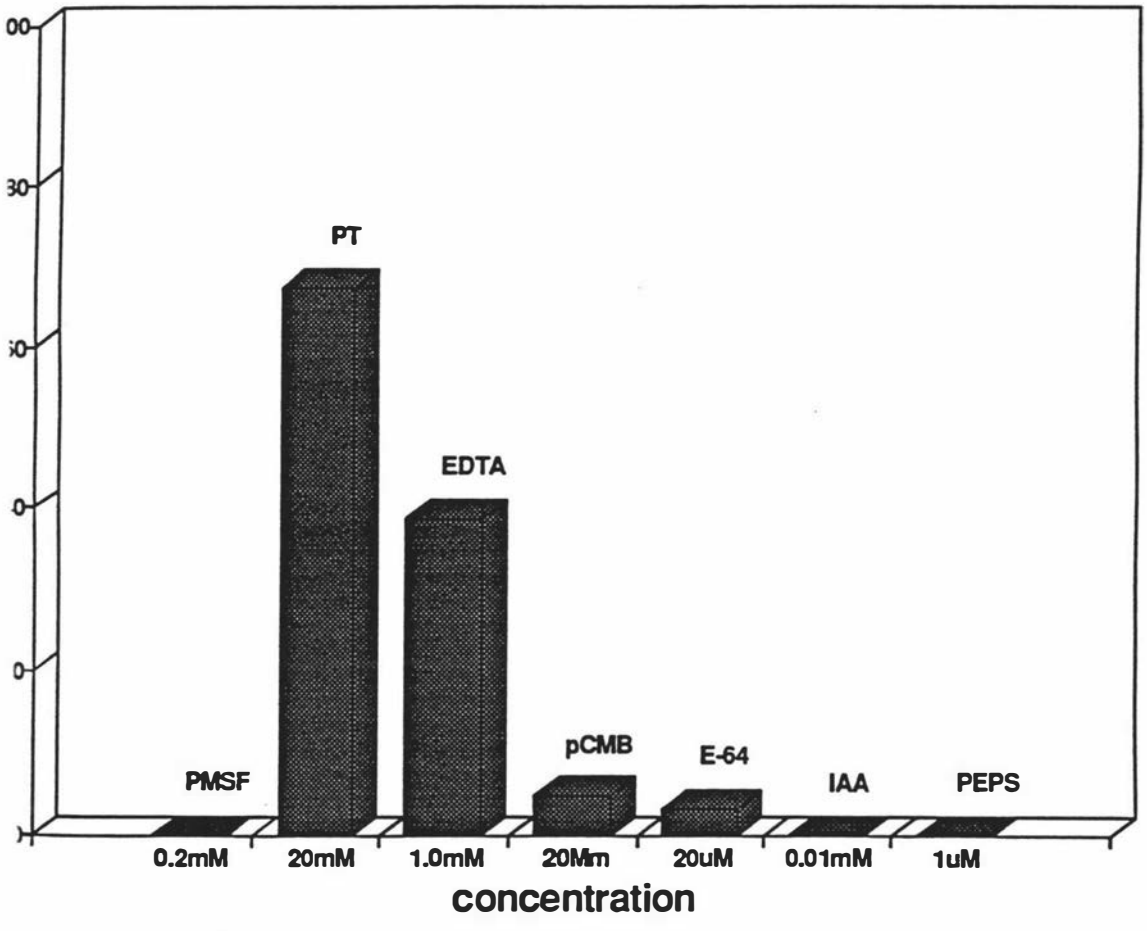


Fig.3-30: Inhibition of elastase activity in *M. canis* filtrate by various inhibitors.

For abbreviations refer to Fig. 3-29.



3.4.5.2 *M. cookei.*

Table 3-24: Effect of inhibitors on *M. cookei* crude filtrate activity.

Inhibitor	Concentration	Inhibition	
		% relative to solvent controls	
		Azocoll	Elastin
α_1-P	100µg/ml	88.5	ND
PMSF	0.4mM	96.0	0.6
PT	20mM	9.6	76.0
EDTA	1.0mM	16.0	41.9
$_p$CMB	20mM	0.4	0.0
E-64	10µM	0.0	1.5
IAA	0.02mM	0.0	0.0
PEPS	1µM	3.3	0.0

ND = not determined.

For *M. cookei* using azocoll (Table 3-24), both PMSF (96%) and α_1-Proteinase (88.5%) showed strong inhibition of azocollytic activity. There was an absence of inhibition with E-64 and IAA. PT, $_p$CMB and PEPS produced very little inhibition. For elastin, strong inhibition was detected with PT (76%) and EDTA (41.9%) with the others showing little or no inhibition.

Fig.3-31: Inhibition of proteinase (azocollytic) activity in *M. cookei* filtrate by various inhibitors.

For abbreviations refer to Fig. 3-29.

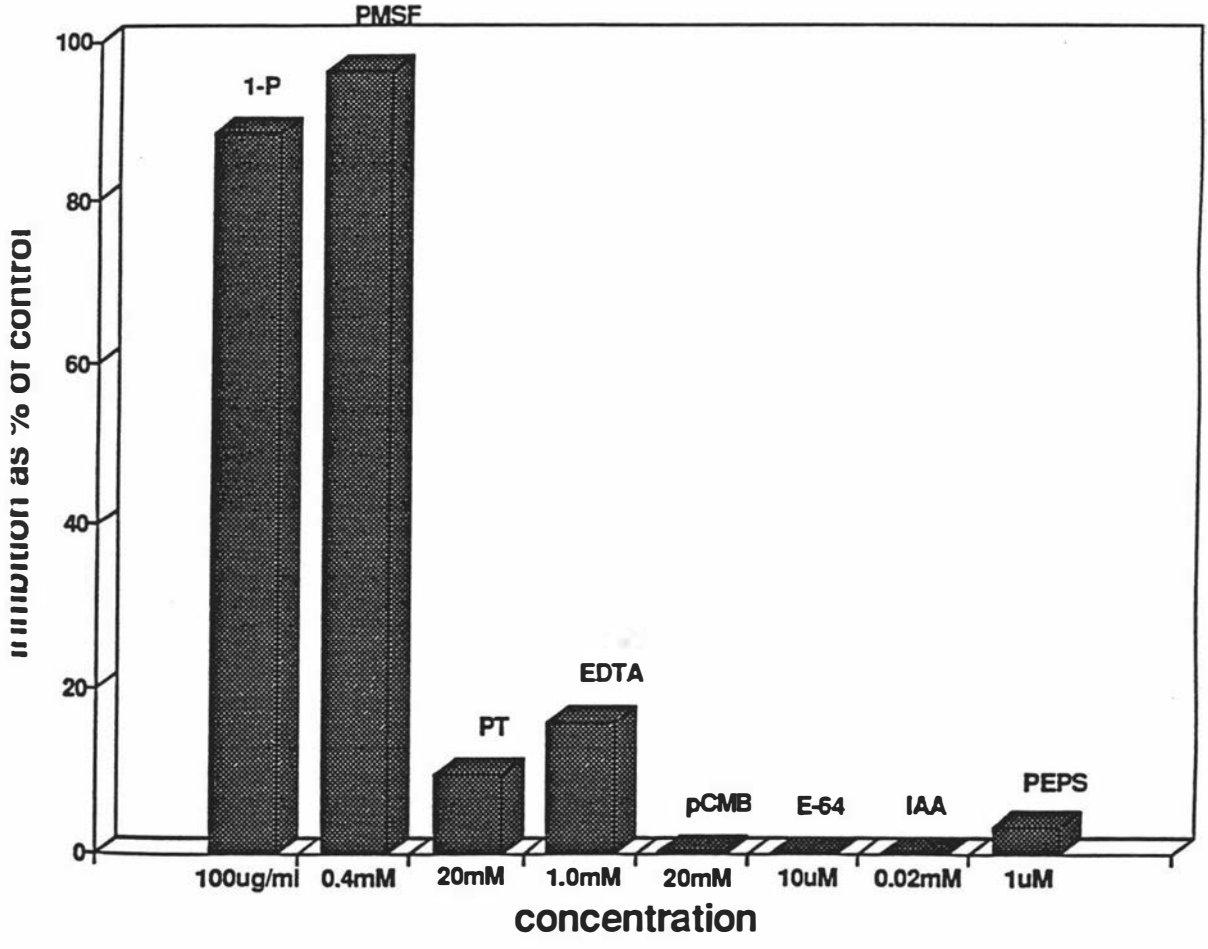
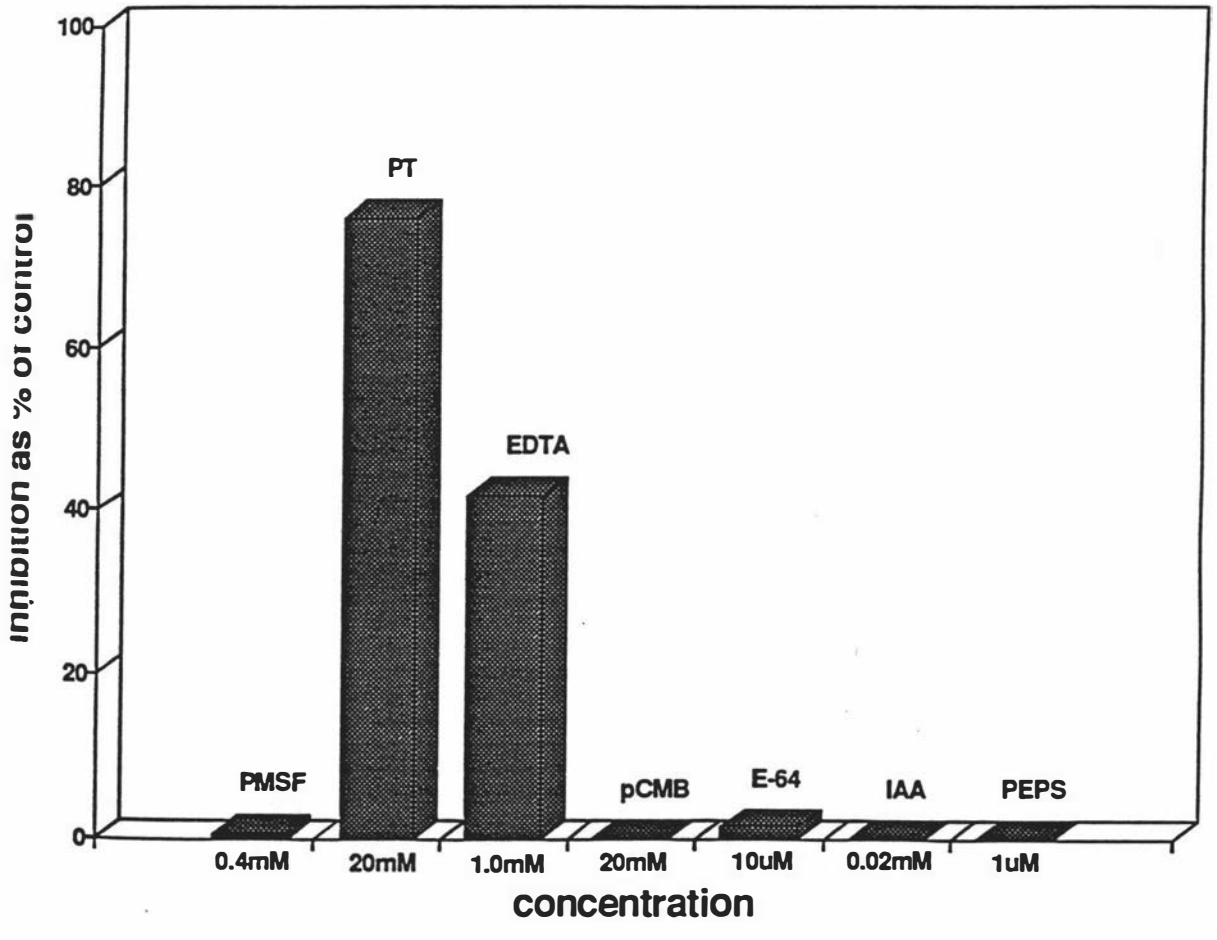


Fig.3-32: Inhibition of elastase activity in *M. cookei* filtrate by various inhibitors.

For abbreviations refer to Fig. 3-29.



3.5 ANALYSIS OF PROTEINASES BY SDS-SUBSTRATE COPOLYMERISED GELS.

Following extraction of enzymes from shake and stationary cultures in Sabouraud broth as described in Section 2.3.3, proteinase expression (gelatinase and elastase) was compared by the substrate/SDS-PAGE techniques (Section 2.3). Since the proteins were separated under SDS-denaturing conditions (Section 2.3), the molecular weight (M_r) of each enzyme was estimated from their positions relative to standards of known M_r. The M_r standards (Bio-Rad) in Kilobases (Kb) were; myosin (200 000), galactosidase (116 250), phosphorylase (97 400), bovine serum albumin (66 200), hen egg white albumin (45 000), bovine carbonic anhydrase (31 000), soy trypsin inhibitor (21 500) and lysozyme (14 500).

3.5.1 Gelatinases of *M. canis*.

The six main proteinase enzymes (gelatinases) which were detected from *M. canis* isolates corresponded approximately to M_rs of 122 KDa, 64 KDa, 62 KDa, 44 KDa, 36 KDa and 28 KDa (Tables 3-25, 3-26, 3-27, 3-28, Figs.3-33, 3-34).

3.5.2 Gel Scanning.

Scanning of stained polyacrylamide gels was done to further confirm the proteinase bands.

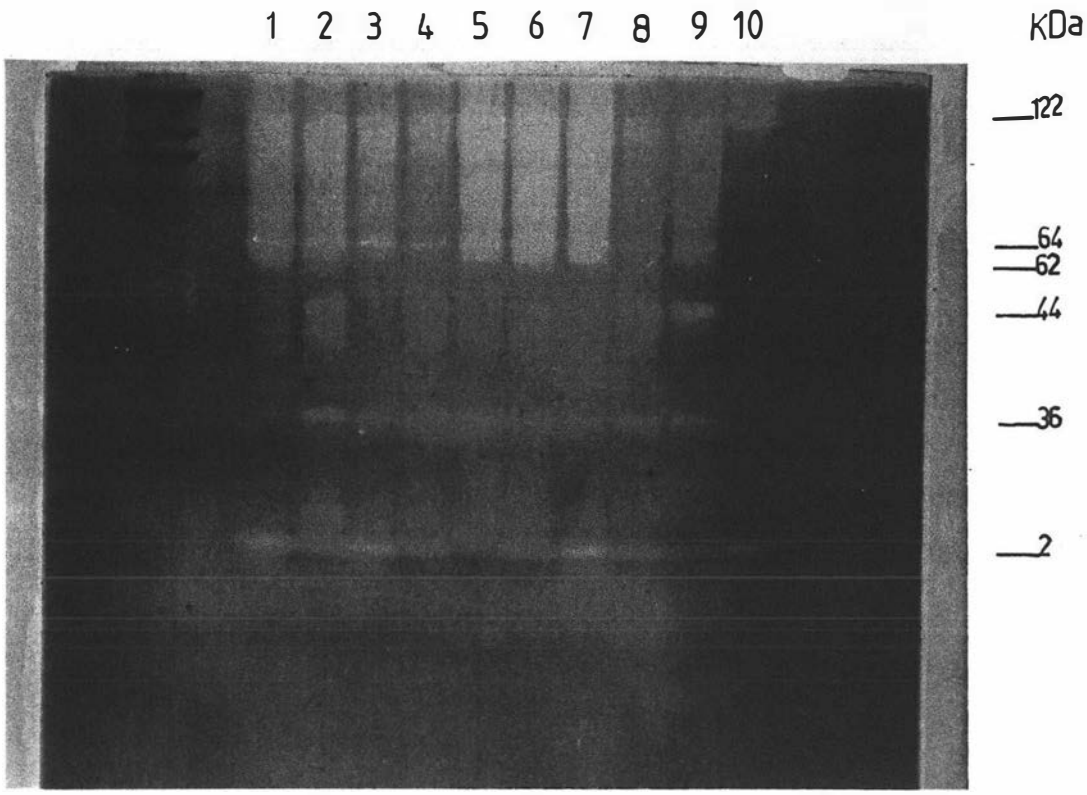
Fig.3-33: A 12% gelatin/SDS-PAGE of continuous shake culture filtrates of *M. canis* isolates.

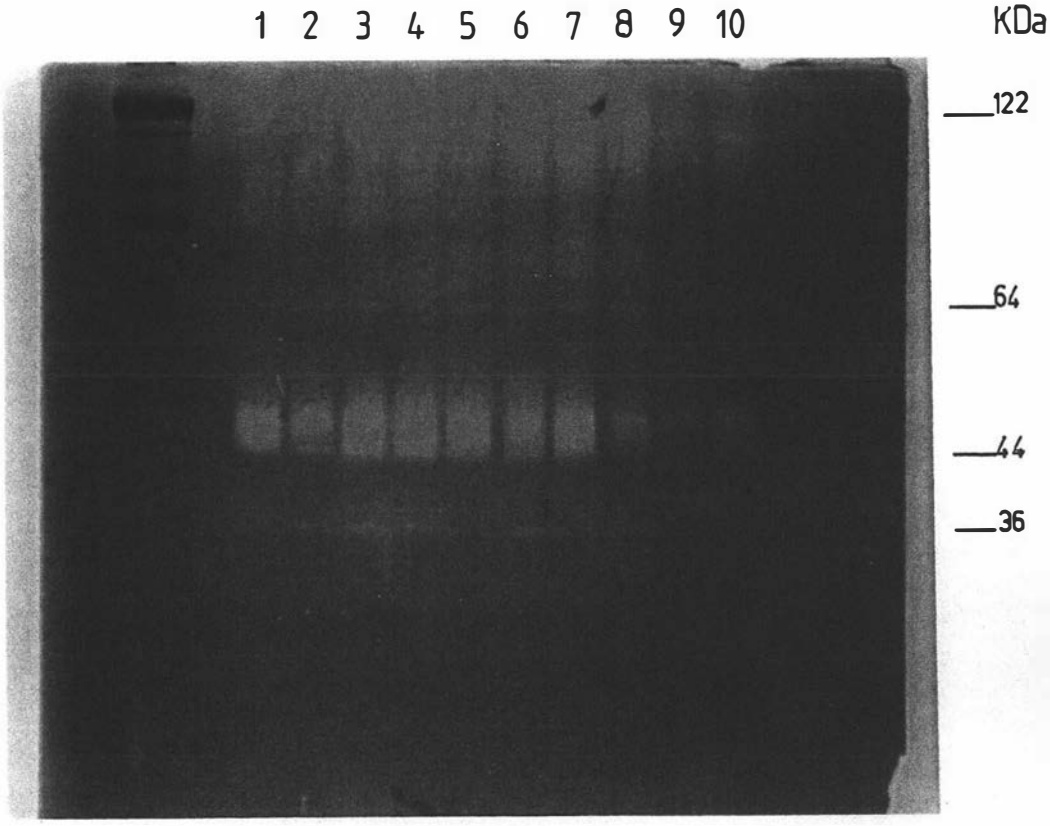
Lane 1=PN21, lane 2=PN22, Lane 3=PN23, Lane 4=PN24, Lane 5=PN25, Lane 6=PN26, Lane 7=PN27, Lane 8=PN28, Lane 9=PN29, Lane 10=PN30A.

Gelatinolysis in the upper part of the electrophoretic tracks may be due to incomplete SDS-inactivation of the enzymes, resulting in proteolysis during electrophoresis. This was in spite of carrying out the electrophoresis at 4°C to reduce enzyme activity. Gelatinolysis during electrophoresis in upper part of the gel has also been reported by other workers (Heussen and Dowdle, 1980; Apodaca and McKerrow, 1990).

Fig.3-34: A 10% gelatin/SDS-PAGE of stationary culture filtrates of *M. canis* isolates.

Lane 1=PN21, lane 2=PN22, Lane 3=PN23, Lane 4=PN24, Lane 5=PN25, Lane 6=PN26, Lane 7=PN27, Lane 8=PN28, Lane 9=PN29, Lane 10=PN30A.


1 2 3 4 5 6 7 8 9 10
KDa
122
64
62
44
36
2


1 2 3 4 5 6 7 8 9 10
KDa
122
64
44
36

3.5.1.1 *M. canis* from Clinical Cases Grown In Shake Culture.

Table 3-25: Proteinases of *M. canis* from clinical cases grown in shake culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)						nº
			122	64	62	44	36	28	
M. canis	PN05	cat	+	+	+	+	+	+	6
"	PN07	cat	+	+	+	-	+	+	5
"	PN08	kitten	+	+	+	+	+	+	6
"	WG09	cat	+	+	+	-	+	+	5
"	PN29	cat	+	+	+	+	+	-	5
"	PN44	dog	+	+	+	+	+	+	6
"	PN06	dog	+	+	+	-	+	+	5
"	WG10	human	+	+	+	+	+	+	6
"	WG30A	human	+	-	-	-	-	+	2
"	WG30B	human	+	+	+	+	+	-	5
"	WG31	human	+	+	+	+	+	+	6
"	WG32	human	+	+	+	+	+	+	6
"	WG33	human	+	+	+	+	+	+	6
"	WG34	human	+	+	+	+	+	+	6
"	WG35	human	+	+	+	+	+	+	6
"	AK45	human	-	+	-	+	+	-	3
"	AK46	human	-	+	-	+	+	+	4
"	AK47	human	-	+	+	-	+	-	3
"	AK48	human	+	+	+	+	+	+	6
"	AK49	human	-	+	+	-	+	-	3
"	AK50	human	-	-	-	-	-	+	1
Others	RV87	ITM	-	+	-	-	+	-	2
"	RV88	ITM	-	+	-	-	+	-	2
"	PN21	blkt	+	+	+	-	-	+	4
"	? 36	N	+	+	+	+	+	+	6

AK = Auckland; PN = Palmerston North; WG = Wellington

N = unknown; NA = not applicable

The proteinases detected in clinical isolates (Table 3-25) grown in shake culture ranged from one proteinase (AK50) to six proteinases (the highest) detected in isolates PN05, PN08, PN44, WG10, WG31,WG32, WG33, WG34, WG35, AK48 and ?36.

3.5.1.2 *M. canis* from Carriers Grown In Shake Culture.

Table 3-26: Proteinases of *M. canis* from carriers grown in shake culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)						nº
			122	64	62	44	36	28	
M. canis	PN01	cat	+	+	-	+	+	+	5
"	PN02	cat	+	+	+	+	+	+	6
"	PN03	cat	+	+	+	+	+	+	6
"	PN04	cat	+	+	+	+	+	+	6
"	PN11	cat	+	+	+	+	+	+	6
"	PN12	cat	+	+	+	+	+	+	6
"	PN13	cat	+	+	+	+	+	+	6
"	PN14	cat	+	+	+	+	+	+	6
"	PN15	cat	+	+	+	+	+	-	5
"	PN16	cat	+	+	+	+	+	+	6
"	PN17	cat	+	+	+	+	+	+	6
"	PN18	cat	+	+	+	+	+	+	6
"	PN19	cat	+	+	+	+	+	+	6
"	PN20	cat	+	+	+	+	+	+	6
"	PN22	cat	+	+	+	+	+	+	6
"	PN23	cat	+	+	+	-	+	+	5
"	PN24	cat	+	+	+	+	+	+	6
"	PN25	cat	+	+	+	+	+	-	5
"	PN26	cat	+	+	+	+	+	+	6
"	PN28	cat	+	+	+	+	+	+	6
"	PN37	cat	+	+	+	+	+	+	6

Table 3-26 contd.

"	PN38	cat	+	+	+	-	-	+	4
"	PN39A	cat	-	+	+	+	+	+	5
"	PN39B	cat	+	+	-	+	+	-	4
"	PN40	cat	-	+	+	+	+	+	5
"	PN41	cat	+	+	+	+	+	+	6
"	PN42	cat	+	+	+	+	+	+	6
"	PN43	cat	-	+	+	+	+	+	5
"	PN27	dog	+	+	+	-	-	+	4

The proteinases (Table 3-26) detected in isolates from carriers grown in stationary cultures ranged from four proteinases (PN38, PN39B and PN27) to six proteinases (most isolates). The remaining isolates expressed five proteinases.

3.5.1.3 *M. canis* from Clinical Cases Grown in Stationary Culture.

Table 3-27: Proteinases of *M. canis* from clinical cases grown in stationary culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)						nº
			122	64	62	44	36	28	
M. canis	PN05	cat	+	+	-	+	+	-	4
"	PN07	cat	+	+	+	+	+	-	5
"	PN08	kitn	-	+	-	+	+	-	3
"	WG09	cat	-	+	-	+	+	-	3
"	PN29	cat	-	-	-	+	+	-	2
"	PN44	dog	-	+	-	+	+	-	3
"	PN06	dog	+	-	-	-	+	+	3

Table 3-27 contd.

"	WG10	human	-	-	-	+	-	-	1
"	WG30A	human	+	+	-	+	+	-	4
"	WG30B	human	-	+	-	+	-	-	2
"	WG31	human	-	-	-	+	+	-	2
"	WG32	human	-	+	-	+	+	-	3
"	WG33	human	-	+	-	+	+	-	3
"	WG34	human	-	+	-	+	+	-	3
"	WG35	human	-	-	-	+	+	-	2
"	AK45	human	-	+	-	+	+	-	3
"	AK46	human	-	-	-	+	-	-	1
"	AK47	human	-	+	-	+	-	-	2
"	AK48	human	-	+	-	+	+	-	3
"	AK49	human	-	+	-	+	+	-	3
"	AK50	human	-	-	-	-	-	+	1
Others	RV87	ITM	-	-	-	+	-	-	1
"	RV88	ITM	-	+	-	+	+	-	3
"	PN21	blkt	-	-	-	+	+	-	2
"	? 36	N	-	-	-	+	-	-	1

The proteinases detected in isolates from clinical cases (Table 3-27) grown in stationary culture ranged from one proteinase (WG10, AK46, AK50, RV87 and ?36) to five proteinases (PN07). The remaining isolates expressed either two, three or four proteinases.

3.5.1.4 *M. canis* from Carriers Grown in Stationary Culture.

Table 3-28: Proteinases of *M. canis* from carriers grown in stationary culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)						nº
			122	64	62	44	36	28	
M. canis	PN01	cat	+	+	+	+	+	-	5
"	PN02	cat	-	-	-	+	-	-	1
"	PN03	cat	+	+	-	+	+	-	4
"	PN04	cat	+	+	+	+	+	-	5
"	PN11	cat	-	+	-	+	+	-	3
"	PN12	cat	-	-	-	+	-	-	1
"	PN13	cat	-	-	-	+	-	-	1
"	PN14	cat	-	+	-	+	+	-	3
"	PN15	cat	-	+	-	+	+	-	3
"	PN16	cat	-	-	-	+	+	-	2
"	PN17	cat	-	+	-	+	+	+	4
"	PN18	cat	-	-	-	+	+	-	2
"	PN19	cat	-	-	-	+	+	+	3
"	PN20	cat	-	-	-	+	+	-	2
"	PN22	cat	-	+	-	+	+	-	3
"	PN23	cat	+	+	-	+	+	-	4
"	PN24	cat	+	-	-	+	+	-	3
"	PN25	cat	+	+	-	+	+	-	4
"	PN26	cat	+	+	-	+	+	-	4
"	PN28	cat	+	+	-	+	+	-	4
"	PN37	cat	-	+	-	+	+	-	3
"	PN38	cat	-	+	-	+	+	-	3
"	PN39A	cat	-	+	-	+	+	-	3
"	PN39B	cat	-	-	-	-	+	-	1

Table 3-28 contd.

"	PN40	cat	-	+	-	+	+	-	3
"	PN41	cat	-	+	-	+	+	-	3
"	PN42	cat	-	+	-	+	+	-	3
"	PN43	cat	-	+	-	+	+	-	3
"	PN27	dog	+	+	-	+	+	-	4

The proteinases detected in isolates from carriers grown in stationary culture ranged from one proteinase (PN02, PN12, PN13 and PN39B) to five proteinases (PN01 and PN04). The remaining isolates expressed either two, three or four proteinases.

3.5.1.5 Comparison of Culture Methods.

The shake cultures (Tables 3-25, 3-26) of *M. canis* showed a higher number of proteinase enzymes than the stationary cultures (Tables 3-27, 23-28). Furthermore, the proteinases from shake cultures gave clearer, well resolved sharp bands (Fig.3-33), compared to the ones from the stationary cultures (Fig.3-34) which were difficult to interpret.

Table 3-29: Comparison of shake and stationary cultures of 50 *M. canis* from clinical cases and carriers.

M_r (x10^3)	shake	stationary	P-value
122	42	13	0.0001
64	48	34	0.0002
62	44	03	0.0001
44	40	47	0.0377
36	46	42	0.1399
28	43	04	0.0001

When the two culture methods are compared using analysis of variance (ANOVA), the enzyme expression of M_rs 122 KDa, 62 KDa and 28 KDa

(P=0.0001) and 64 KDa (P=0.0002) was significantly greater in shake culture than stationary culture. The expression of M_rs 44 KDa was marginally greater (P=0.0377) in shake culture compared to stationary culture while 36 KDa showed no significant difference (P=0.1399).

When clinical isolates were compared by ANOVA, M_rs 122 KDa, 62 KDa and 28 KDa were again significantly more highly expressed (P=0.0001). The other proteinases of M_rs 64 KDa, 44 KDa and 36 KDa were not highly expressed. The same M_rs of 122 KDa, 62 KDa and 28 KDa were also significantly more highly expressed (P=0.0001) in isolates from carriers and in addition, 64 KDa M_r (P=0.0008).

Table 3-30: Significance levels for different M_r enzymes between shake and stationary cultures of clinical and carrier isolates of *M. canis*.

	Clinical (21)			Carriers (29)		
M_r (x10^3)	shake	station.	P-value	shake	station.	P-value
122	16	04	0.0001	26	09	0.0001
64	19	14	0.0623	29	20	0.0008
62	17	01	0.0001	27	02	0.0001
44	14	19	0.0623	26	28	0.3084
36	19	16	0.2240	27	26	0.3974
28	16	02	0.0001	27	02	0.0001

station. = stationary

3.5.2 Gelatinases of *M. cookei*.

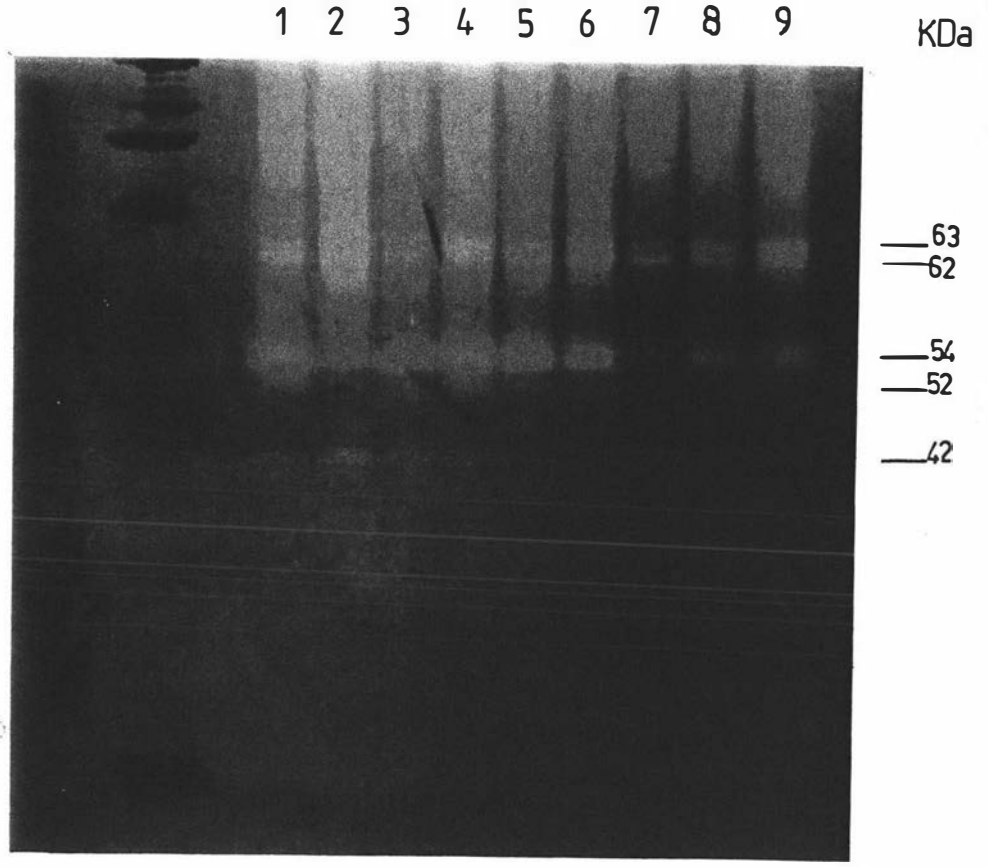
The seven main proteinases which were detected from *M. cookei* isolates corresponded approximately to M_rs of 67 KDa, 64 KDa, 63 KDa, 62 KDa, 54 KDa, 52 KDa and 42 KDa (Tables 3-31, 3-32, Figs. 3-35, 3-36).

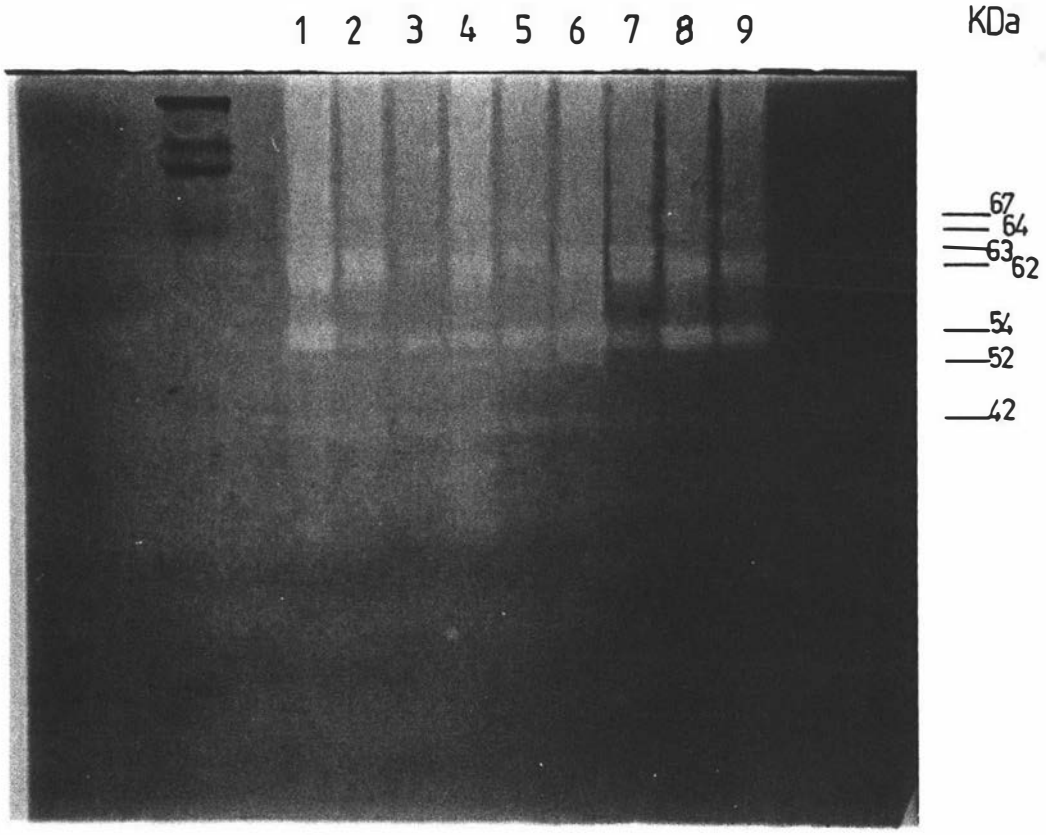
Fig.3-35: A 12% gelatin/SDS-PAGE of continuous shake culture filtrates of *M. cookei* isolates.

Lane 1=H08, Lane 2=H219, Lane 3=H223, Lane 4=H228,
Lane 5=H30, Lane 6=A32, Lane 7=S33, Lane 8=A39,
Lane 9=A81

Fig.3-36: A 10% gelatin/SDS-PAGE of stationary culture filtrates of *M. cookei* isolates.

Lane 1=H08, Lane 2=H219, Lane 3=H223, Lane 4=H228,
Lane 5=H30, Lane 6=A32, Lane 7=S33, Lane 8=A39,
Lane 9=A81


1 2 3 4 5 6 7 8 9
KDa
63
62
54
52
42


1 2 3 4 5 6 7 8 9
KDa
67
64
63
62
54
52
42

3.5.2.1 *M. cookei* Grown in Shake Culture.

Table 3-31: Proteinases of *M. cookei* grown in shake culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)					nº
			63	62	54	52	42	
M.cookei	H08	soil	+	+	+	-	-	3
"	H30	soil	+	+	+	+	+	5
"	A32	soil	+	+	+	+	+	5
"	S33	soil	+	+	+	-	+	4
"	A39	soil	+	+	+	-	-	3
"	A81	soil	+	+	+	-	+	4
"	A107	soil	+	+	+	+	+	5
"	A125	soil	-	-	+	-	-	1
"	A152	soil	+	+	+	+	+	5
"	A153	soil	+	+	+	+	+	5
"	H154	soil	+	+	+	+	+	5
"	H173	soil	+	+	+	-	+	4
"	H192	soil	+	+	+	-	+	4
"	H219	soil	+	-	+	-	+	3
"	H223	soil	+	+	+	-	+	4
"	H228	soil	+	+	+	+	+	5
"	C551	cat	+	+	+	-	+	4

A = Isolates from soil moistened with water containing antibiotics.
H = Isolates from soil moistened with water only.
S = Isolate from the teaching culture collection.

(Other isolates examined:
T.mentagrophytes = 180, 84 + 39 KDa
Diheterospora 28A = 180, 129 KDa
" 32H = 0)

The proteinases detected in *M. cookei* isolates grown in shake culture (Table 3-31) ranged from one proteinase (A125) to five proteinases (H30, A32, A107, A152, A153 and H154). The remaining isolates expressed three or four proteinases.

3.5.2.2 *M. cookei* Grown in Stationary Culture.

Table 3-32: Proteinases of *M. cookei* grown in stationary culture, as detected by gelatin/SDS-PAGE.

Species	Isolate code	Source	Proteinase ca. M_r (x10^3)							nº
			67	64	63	62	54	52	42	
M.cookei	H08	soil	+	+	+	-	+	+	+	6
"	H30	soil	+	+	+	+	+	+	+	7
"	A32	soil	+	+	+	-	+	+	+	6
"	S33	soil	-	-	+	+	+	-	+	4
"	A39	soil	+	+	-	+	+	+	+	5
"	A81	soil	+	+	-	+	+	+	+	6
"	A107	soil	-	-	+	+	+	-	-	3
"	A125	soil	-	-	+	-	+	+	+	4
"	A152	soil	+	+	+	+	+	+	+	7
"	A153	soil	-	-	+	+	+	+	+	5
"	H154	soil	-	-	+	+	+	+	+	5
"	H173	soil	-	-	+	+	+	+	+	5
"	H192	soil	-	-	+	+	+	+	+	5
"	H219	soil	-	-	+	-	+	+	+	4
"	H223	soil	-	-	+	+	+	+	+	5
"	H228	soil	+	+	+	-	+	+	+	6
"	C551	cat	-	-	+	+	+	+	+	5

(Other isolates examined:

T.mentagrophytes = 180, 45 KDa

Diheterospora 28A = 0

Diheterospora 32H = 191, 117 KDa)

The proteinases detected in *M. cookei* isolates grown in stationary culture (Table 3-32) ranged from three proteinases (A107) to seven proteinases (H30, A152). The remaining isolates expressed either four, five or six proteinases.

3.5.2.3 Comparison of Culture Methods.

In contrast to *M. canis*, *M. cookei* showed a higher expression of proteinase enzymes in stationary cultures (Table 3-32) than in shake cultures (Table 3-31).

Table 3-33: Proteinases detected in shake and stationary cultures of *M. cookei*.

M_r (x10^3)	*M. cookei* (17 isolates)		
	shake	stationary	P-value
67	00	07	
64	00	07	
63	16	15	0.5595
62	15	12	0.2150
54	17	17	0.0000
52	07	15	0.0031
42	14	16	0.3014

When the two culture methods are compared by ANOVA, the M_r 52 KDa was significantly more highly expressed (P=0.0031) in stationary culture. The M_r 67 KDa and 64 KDa were only expressed in stationary culture. The remaining M_rs 63 KDa (P=0.5595), 62 KDa (P=0.2150) and 42 KDa (P=0.3014) were not significantly different between the two culture methods.

3.5.3 Elastases Detected in *M. canis* and *M. cookei* Filtrates.

Elastases were detected in shake cultures of *M. canis* but not in stationary cultures. For *M. cookei*, no elastases were detected in either shake and stationary cultures using elastin/SDS-PAGE. The M_rs of the two elastases detected in *M. canis* filtrates were 64 KDa and 62 KDa, common to all isolates.

3.6 MULTILOCUS ENZYME ELECTROPHORESIS.

3.6.1 Basic data.

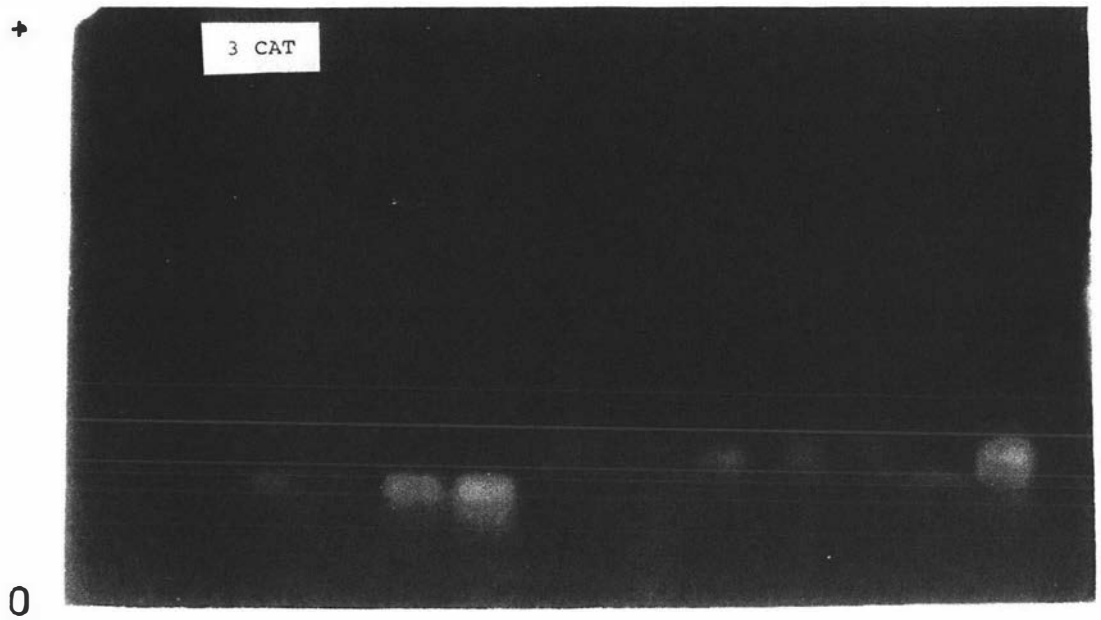
Isozyme banding patterns were recorded according to their relative mobilities (Rf.), Table 3-34. Each *band* was interpreted as an **electromorph** while the isozyme *patterns* for each enzyme were referred to as **phenotypes**. For comparing electrophoretic mobilities of the eight enzymes between gels the following isolates were used as internal standards; H08 for CAT and MDH, H153 for EST, PN13 for LAP and GPI, H173 for G6P and PER, and PN23 for PEP (Figs.3-45, 3-46, 3-47).

Fig.3-37: Electromorph profiles of catalase (CAT).

Lane 1=PN11, Lane 2=PN12, Lane 3=PN13, Lane 4=PN14,
Lane 5=PN15, Lane 6=A125, Lane 7=A152, Lane 8=A153,
Lane 9=A154, Lane 10=A155 Lane 11=H08.

Fig.3-38: Electromorph profiles of esterase (EST).

Lane 1=PN06, Lane 2=PN07, Lane 3=PN08, Lane 4=WG09,
Lane 5=WG10, Lane 6=A32, Lane 7=S33, Lane 8=A39,
Lane 9=A81, Lane 10=A107 Lane 11=H153.


+
3 CAT
0
1 2 3 4 5 6 7 8 9 10 11

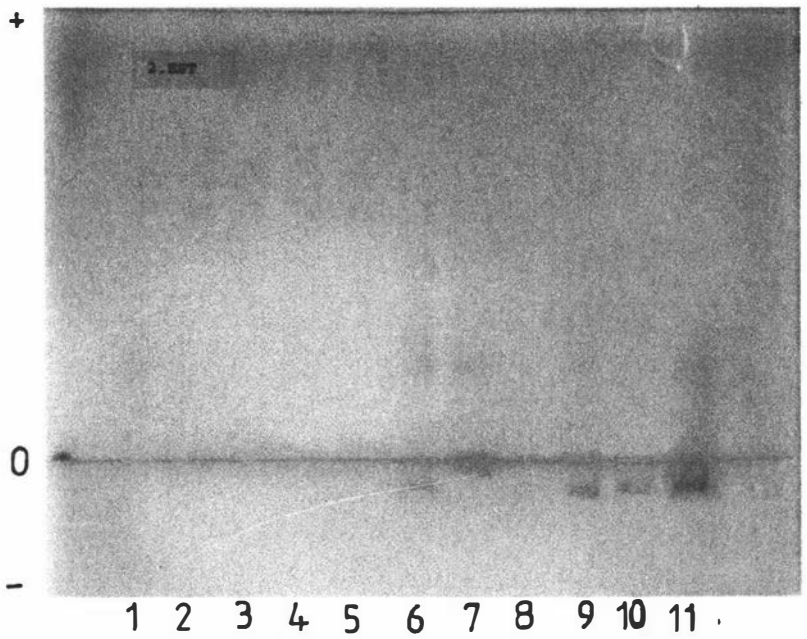
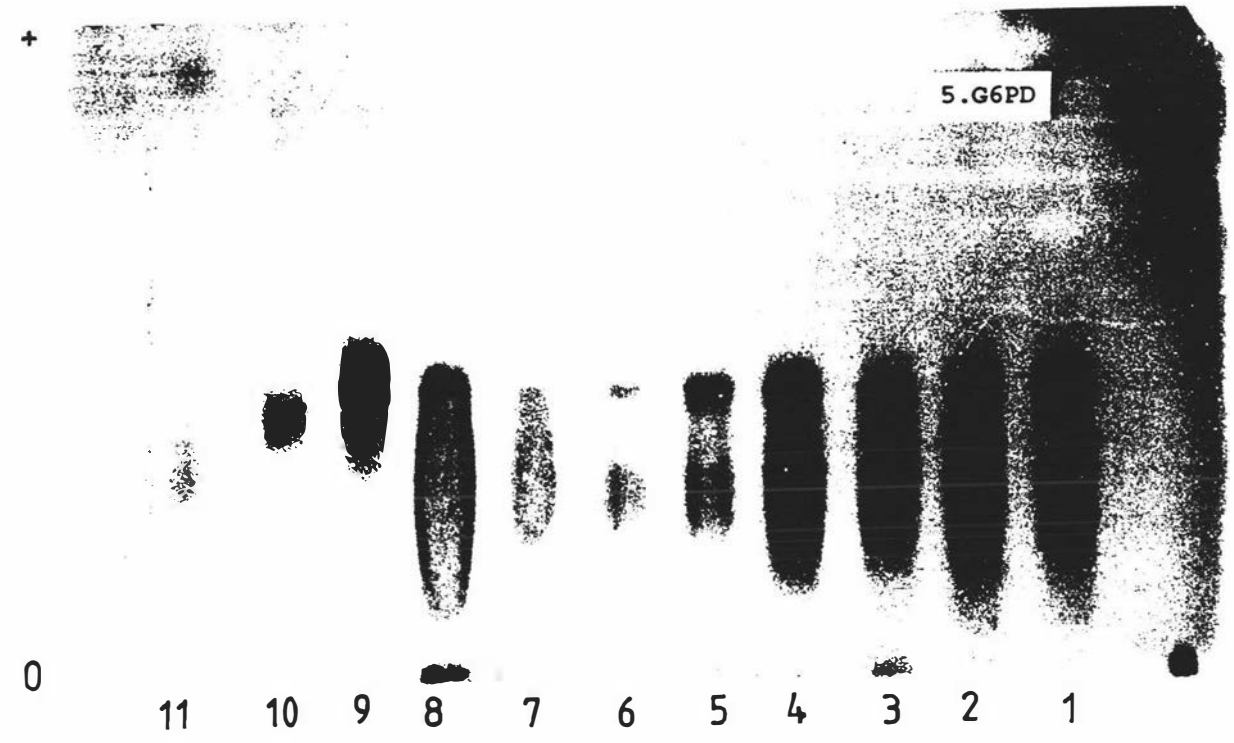

+
0
-
1 2 3 4 5 6 7 8 9 10 11

Fig.3-39: Electromorph profiles of glucose-6-phosphate dehydrogenase (G6P).

Lane 1=PN21, Lane 2=PN22, Lane 3=PN23, Lane 4=PN24,
Lane 5=PN25, Lane 6=PN26, Lane 7=RV87, Lane 8=RV88,
Lane 9=*T. mentagrophytes*, Lane 10=*Paecilomyces*
Lane 10=H153, Lane 11=H173 .

Fig.3-40: Electromorph profiles of glucose-6-phosphate isomerase (GPI).

Lane 1=PN01, Lane 2=PN02, Lane 3=PN03, Lane 4=PN04,
Lane 5=PN05, Lane 6=H08, Lane 7=H219, Lane 8=H223,
Lane 9=H228, Lane 10=H30, Lane 11=PN13.


+
5.G6PD
0
11
10
9
8
7
6
5
4
3
2
1

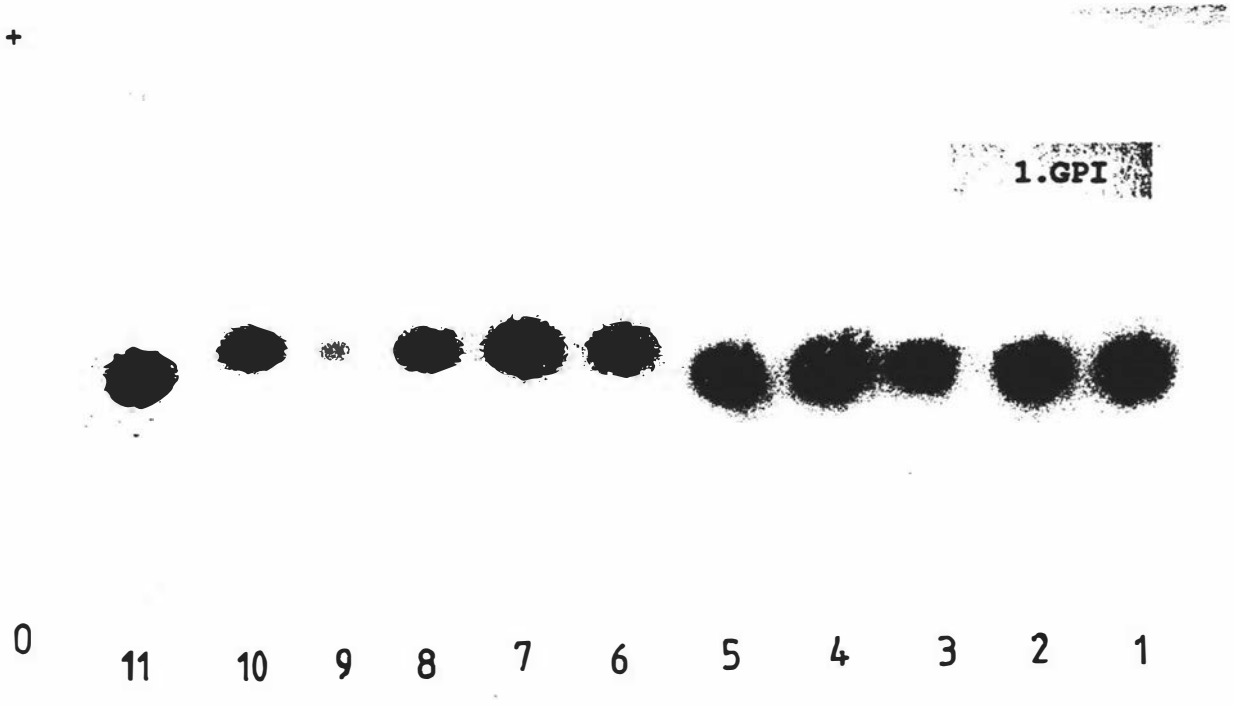
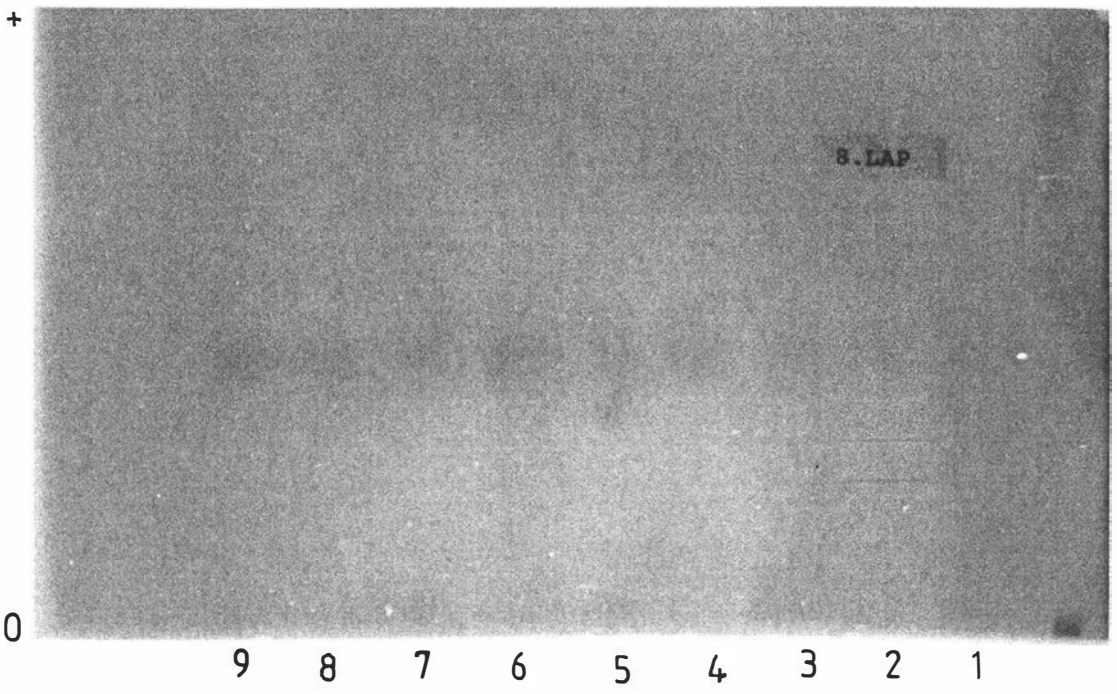

+
1.GPI
0
11
10
9
8
7
6
5
4
3
2
1

Fig.3-41: Electromorph profiles of leucine aminopeptidase (LAP).

Lane 1=PN43, Lane 2=PN44, Lane 3=AK45, Lane 4=AK46,,
Lane 5=AK47, Lane 6=AK48, Lane 7=AK49, Lane 8=AK50,
Lane 9=PN13.

Fig.3-42: Electromorph profiles of malate dehydrogenase (MDH).

Lane 1=PN16, Lane 2=PN17, Lane 3=PN18, Lane 4=PN19,
Lane 5=PN20, Lane 6=A173, Lane 7=H192, Lane 8=C551,
Lane 9=AD28, Lane 10=HD32 Lane 11=H08.


+
8. LAP
0
9 8 7 6 5 4 3 2 1

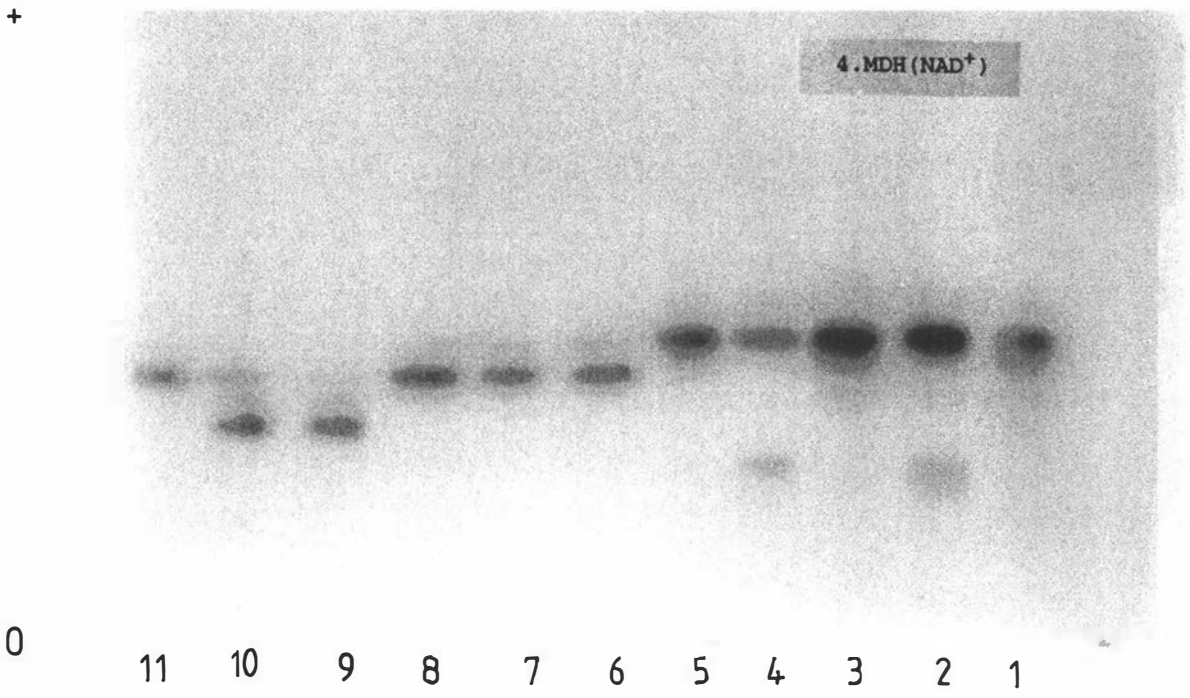
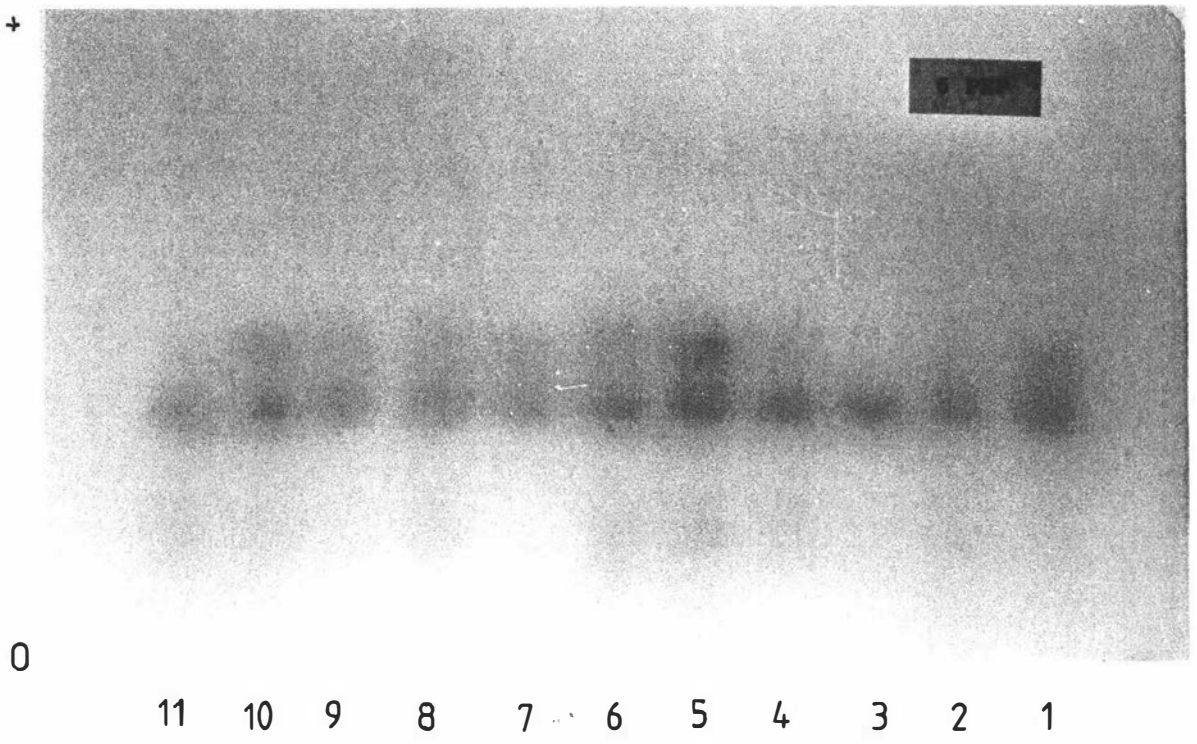

+
4. MDH (NAD+)
0
11 10 9 8 7 6 5 4 3 2 1

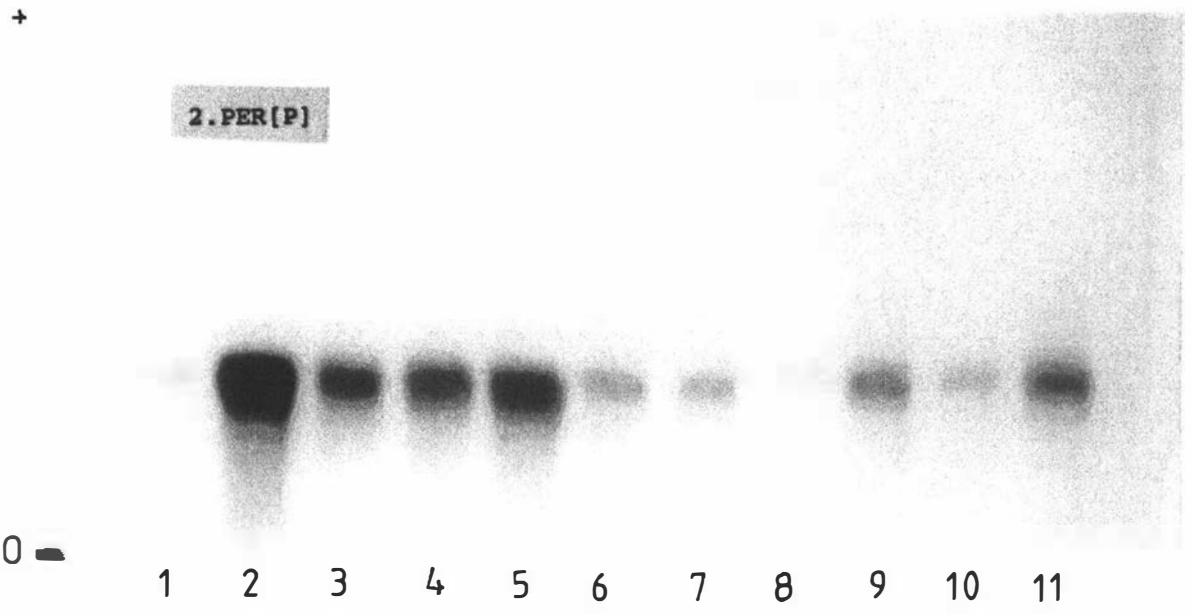
Fig.3-43: Electromorph profiles of peptidase (PEP).

Lane 1=PN27, Lane 2=PN28, Lane 3=PN29, Lane 4=WG30A,
Lane 5=WG30B, Lane 6=WG31, Lane 7=WG32, Lane 8=WG33,
Lane 9=WG34, Lane 10=WG35 Lane 11=PN23.

Fig.3-44: Electromorph profiles of peroxidase (PER).

Lane 1=PN06, Lane 2=PN07, Lane 3=PN08, Lane 4=WG09,
Lane 5=WG10, Lane 6=A32, Lane 7=S33, Lane 8=A39,
Lane 9=A81, Lane 10=A107 Lane 11=H173.


+
0
11 10 9 8 7 6 5 4 3 2 1


+
2.PER[P]
0
1 2 3 4 5 6 7 8 9 10 11

(a) Electromorphs.

Each electromorph is calculated as a percentage of isolates with the band per total number of isolates. The percentage can be equal to or less than 100%, or 0% for absence of a band (Table 3-34).

Table 3-34: Comparison of electromorphs of 72 *Microsporum* isolates and 2 *Diheterospora* isolates.

Enzyme system	% of isolates with electromorph present.			
	*Rf.	*M. canis* n=54	*M. cookei* n=18	*Diheterospora* n=2
CAT	0.32	0.0	67.0	0.0
	0.27	0.0	0.0	100.0
	0.22	76.0	0.0	0.0
EST +	0.65	96.3	0.0	0.0
	0.58	75.9	44.0	0.0
	0.49	7.4	0.0	0.0
	0.46	0.0	6.0	100.0
	0.24	0.0	28.0	100.0
	0.06	0.0	0.0	100.0
-	0.11	0.0	72.0	0.0
G6P	0.51	87.0	100.0	0.0
	0.42	54.0	6.0	0.0
	0.33	0.0	0.0	100.0
	0.04	17.0	0.0	0.0
GPI	0.56	0.0	0.0	100.0
	0.51	0.0	100.0	0.0
	0.46	100.0	0.0	0.0

- = refers to the electromorph which migrated to the cathode.
The rest of the electromorphs for the isozymes migrated to the anode.

Table 3-34 Contd.

LAP	0.50	0.0	0.0	100.0
	0.48	100.0	0.0	100.0
	0.35	0.0	100.0	0.0
MDH	0.54	7.4	0.0	0.0
	0.49	7.4	0.0	0.0
	0.46	100.0	0.0	0.0
	0.41	0.0	100.0	100.0
	0.36	0.0	0.0	100.0
	0.27	77.8	0.0	0.0
PEP	0.53	70.0	72.0	0.0
	0.43	100.0	100.0	0.0
	0.26	15.0	7.0	0.0
PER	0.33	0.0	0.0	100.0
	0.31	78.0	100.0	0.0

$$^{*}\text{Rf(relative mobility)} = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

In Table 3-34, when electromorphs are allocated 100% this indicates that all isolates had the same relative mobility for that electromorph. For example, the Rf 0.46 band with *M. canis* for MDH means that, that MDH electromorph was present in all isolates. Electromorphs with less than 100% means that some isolates lacked the electromorph.

(b) Electrophoretic phenotypes.

Table 3-35: Comparison of electrophoretic phenotypes of *Microsporum* and *Diheterospora* spp..

Phenotype	Percentage of isolates with phenotype present.		
	M. canis n=54	*M. cookei* n=18	*Diheterospora* n=2
CAT			
1	24.0	33.0	0.0
2	76.0	0.0	0.0
3	0.0	67.0	0.0
4	0.0	0.0	100.0
EST			
1	4.0	17.0	0.0
2	0.0	11.0	0.0
3	0.0	11.0	0.0
4	0.0	28.0	0.0
5	0.0	22.0	0.0
6	0.0	6.0	0.0
7	0.0	6.0	0.0
8	11.0	0.0	0.0
9	70.0	0.0	0.0
10	15.0	0.0	0.0
11	0.0	0.0	100.0

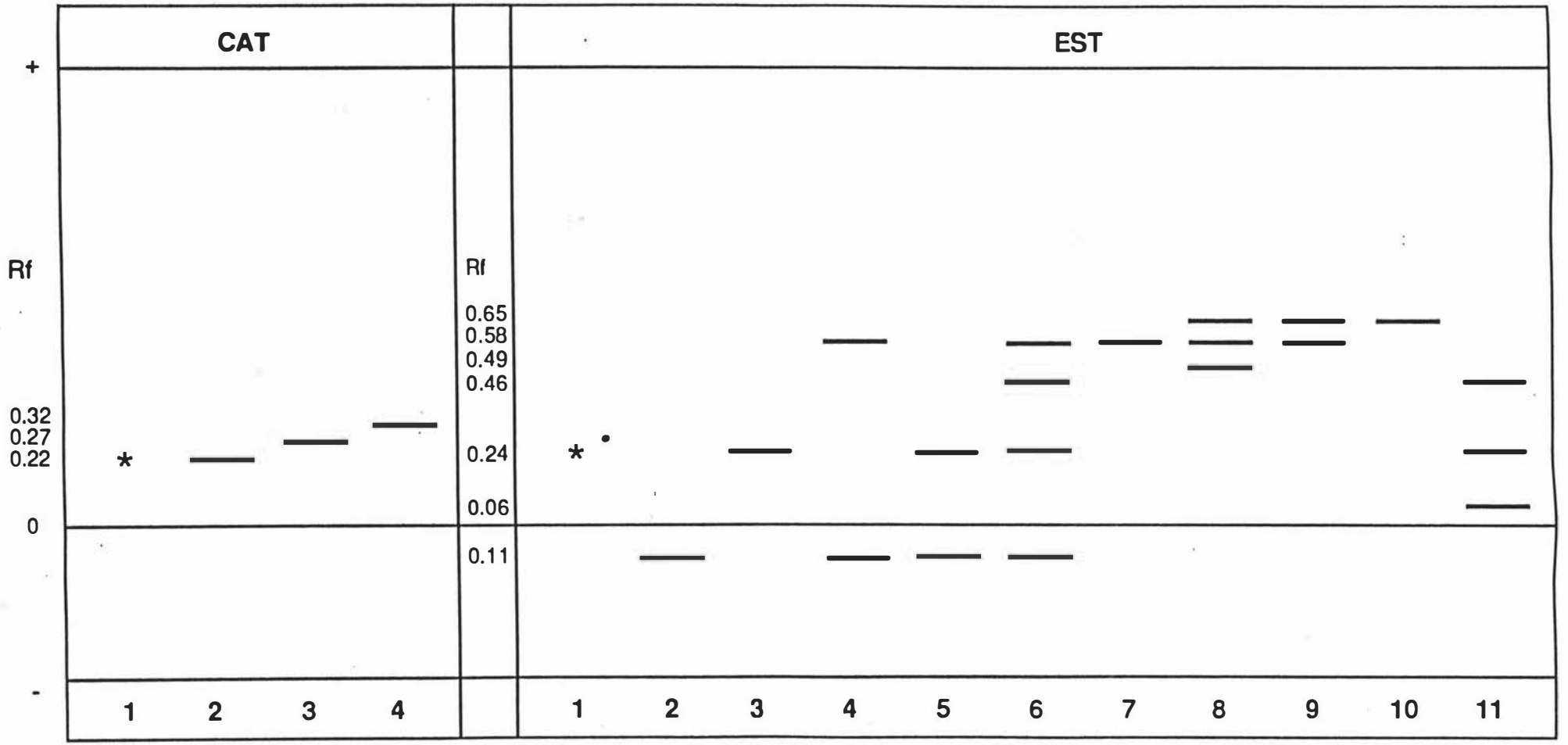
Table 3-35 Contd.

G6P			
1	11.0	0.0	0.0
2	17.0	0.0	0.0
3	31.0	22.0	0.0
4	41.0	78.0	0.0
5	0.0	0.0	100.0
GPI			
1	100.0	0.0	0.0
2	0.0	100.0	0.0
3	0.0	0.0	100.0
LAP			
1	0.0	100.0	0.0
2	100.0	0.0	0.0
3	0.0	0.0	100.0
MDH			
1	7.0	0.0	0.0
2	70.0	0.0	0.0
3	22.0	0.0	0.0
4	0.0	100.0	0.0
5	0.0	0.0	100.0
PEP			
1	0.0	0.0	100.0
2	26.0	11.0	0.0
3	50.0	56.0	0.0
4	24.0	33.0	0.0
PER			
1	22.0	0.0	0.0
2	78.0	100.0	0.0
3	0.0	0.0	100.0

The isozyme patterns were referred to as phenotypes. When calculating phenotypic frequency, the percentage for single-banded isozymes tends to be the same as when the Rf values of electromorphs are used to calculate the frequency. However, for multi-banded phenotypes, the Rf values are only useful to indicate relative mobility for the different electromorphs of an enzyme but are not used for calculating phenotypic frequency. In this study, the calculated frequencies (Section 2.5) (Table 3-35) are based on the phenotypes in Figs.3-45, 3-46, 3-47. In these figures phenotype 1 for the enzymes CAT, EST, G6P, PEP and PER indicate the absence of enzyme activity e.g. for CAT phenotype 1, 24% of *M. canis* isolates did not show this electromorph. Both *M. canis* and *M. cookei* isolates revealed different phenotypes whereas the two *Diheterospora* isolates had the same phenotypes for all the eight enzymes.

Fig.3-45: Summary of isozyme phenotypes of catalase and esterase.

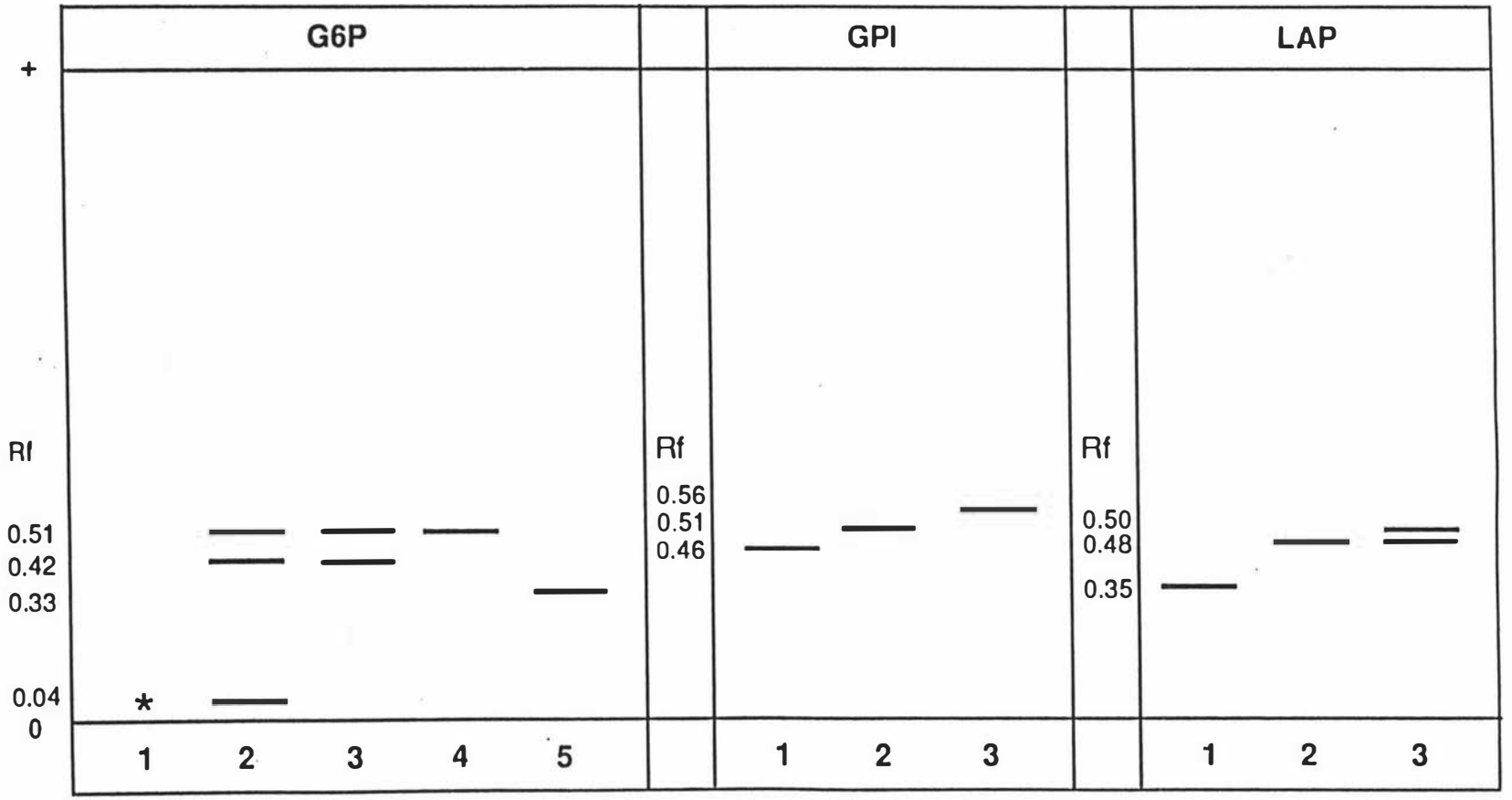
* = indicates absence of phenotype.



1 = *M. canis* & *M. cookei*
2 = *M. canis*
3 = *M. cookei*
4 = Diheterospora

1 = *M. canis* & *M. cookei*
2 to 7 = *M. cookei*
8 to 10 = *M. canis*
11 = Diheterospora

Fig.3-46: Summary of isozyme phenotypes of glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase and leucine aminopeptidase.



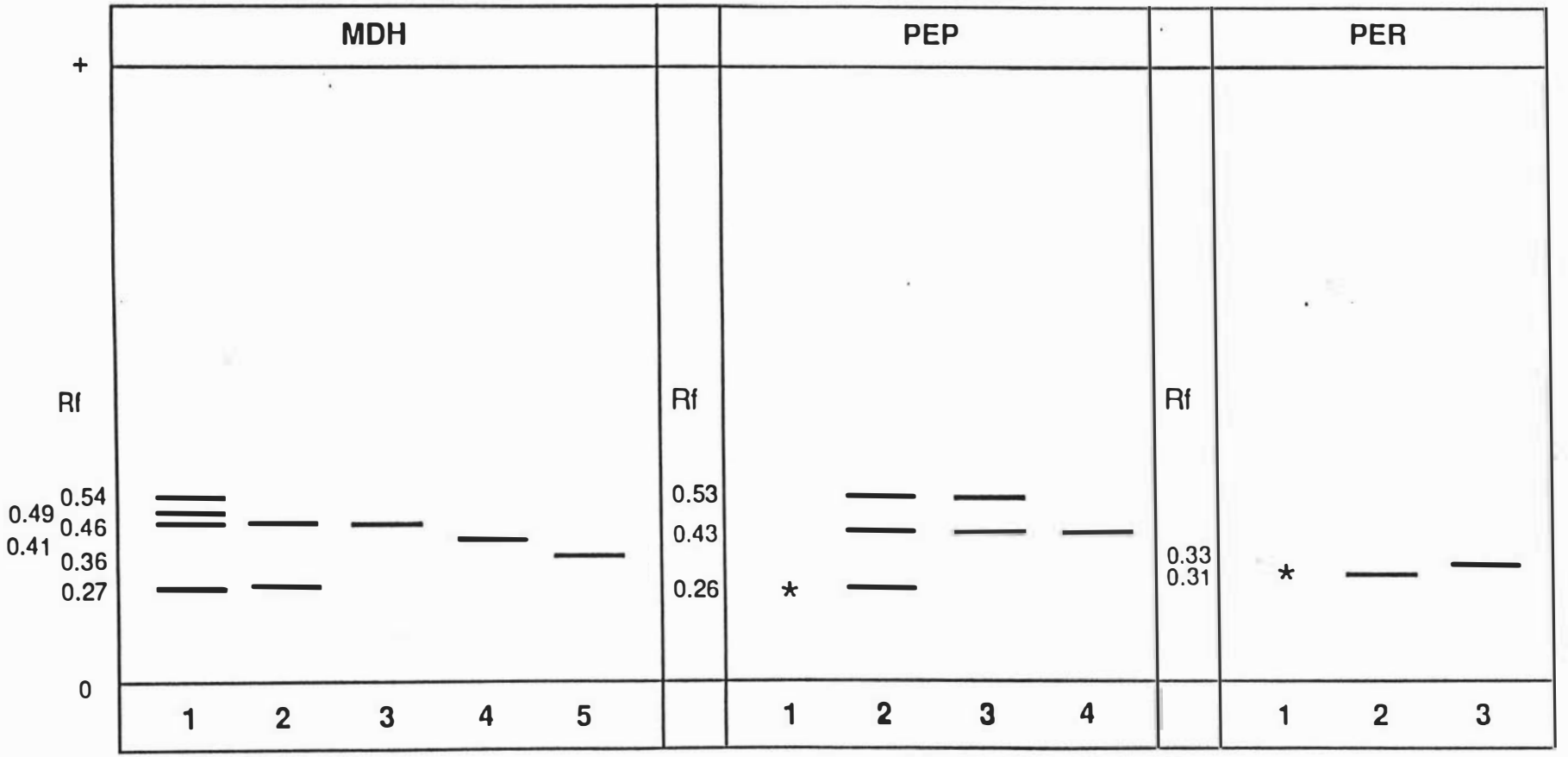
1 to 4 = *M. canis* & *M. cookei*
5 = Diheterospora

1 = *M. canis*
2 = *M. cookei*
3 = Diheterospora

1 = *M. cookei*
2 = *M. canis*
3 = Diheterospora

Fig.3-47: Summary of isozyme phenotypes of malate dehydrogenase, peptidase and peroxidase.

* = indicates absence of phenotype.



1 to 3 = *M. canis*
4 = *M. cookei*
5 = Diheterospora

1 = Diheterospora
2 to 4 = *M. canis* & *M. cookei*

1 & 2 = *M. canis* & *M. cookei*
3 = Diheterospora

3.6.2 Phenotypic Diversity.

From the calculated frequency data, the Shannon-Wiener diversity measure was calculated for each of the eight enzyme systems as described in Section 2.5.1, using the formula:

$$H = - \sum p_i \, \mathrm{Log}_2 \, p_i$$

where:

H = phenotypic diversity

p_i = the frequency of the ith phenotype in the population

The calculated Shannon-Wiener diversity values for the eight enzymes were normalised by $H = H/H_{max}$ (Sheldon, 1969) in which H is the usual Shannon-Wiener diversity measure over phenotypes, and H_{max} is ln(N), the maximum diversity for the sample of size N (Section 2.5.1).

3.6.2.1 Overall Diversity.

In Table 3-36, the normalised mean phenotypic diversity of *M.cookei* is 11.11% less than *M. canis*. The enzyme systems, GPI and LAP showed no phenotypic diversity for *M. canis* and *M. cookei*, while PER enzyme system showed no phenotypic diversity for *M. cookei* only. *Diheterospora* had no phenotypic diversity for all eight enzymes.

Table 3-36: Phenotypic diversity of eight enzyme systems of *Microsporum* and *Diheterospora* spp..

Enzyme system	*M. canis* n=54	*M. cookei* n=18	*Diheterospora* n=2
CAT	0.400	0.529	0.000
EST	0.657	1.513	0.000
G6P	0.923	0.440	0.000
GPI	0.000	0.000	0.000
LAP	0.000	0.000	0.000
MDH	0.558	0.000	0.000
PEP	0.754	0.778	0.000
PER	0.383	0.000	0.000
H	0.459	0.408	0.000

3.6.2.2 Diversity of *Microsporum* spp. from clinical cases, carriers and soil.

In Table 3-37, the enzyme systems of CAT, G6P, MDH and PEP from clinical cases all had a diversity measure of $H > 0.5$ while EST and PER H was less than 0.5. The isozymes EST, G6P and PEP from carriers had a diversity measure of $H > 0.5$ and CAT, MDH and PER had $H < 0.5$. With isolates from clinical cases and carriers the mean diversity was similar but, in the case of soils (*M. cookei*), there was a lower mean diversity.

Table 3-37: Phenotypic diversity of isolates of *Microsporum* spp. isolated from clinical cases, carriers and soils.

Enzyme system	Clinical n=21	Carriers n=29	Soils n=18
CAT	0.503	0.350	0.526
EST	0.150	0.764	1.269
G6P	0.998	0.870	0.226
GPI	0.000	0.000	0.000
LAP	0.000	0.000	0.000
MDH	0.598	0.454	0.378
PEP	0.793	0.701	0.747
PER	0.481	0.350	0.000
H	0.440	0.436	0.393

3.6.2.3 Diversity of *M. canis* isolated from Humans and Cats.

Isolates from humans and cats (Table 3-38) had a diversity measure of $H > 0.5$ for G6P and PEP, with CAT and PER having $H < 0.5$. GPI and LAP showed no diversity for both humans and cats. The mean diversity was very similar.

Table 3-38: Phenotypic diversity in isolates of *M. canis* spp. isolated from humans and cats.

Enzyme system	Humans n=14	Cats n=33
CAT	0.285	0.331
EST	0.208	0.734
G6P	1.016	0.871
GPI	0.000	0.000
LAP	0.000	0.000
MDH	0.652	0.458
PEP	0.849	0.693
PER	0.285	0.331
H	0.412	0.427

3.6.2.4 Diversity of *M. canis* between regions.

The mean phenotypic diversity for each of the three regions, Palmerston North, Wellington and Auckland (Table 3-39), compared to the overall mean phenotypic diversity (Table 3-36, $H = 0.463$), shows a mild degree of inter-regional differentiation. Palmerston North and Auckland isolates were similar while Wellington isolates were different from the other two regions.

Table 3-39: Mean phenotypic diversity in isolates of *M. canis* isolated from different geographical regions.

Enzyme system	Palm.Nth n=36	Wellington n=09	Auckland n=06
CAT	0.352	0.640	0.278
EST	0.725	0.000	0.366
G6P	0.890	0.789	0.813
GPI	0.000	0.000	0.000
LAP	0.000	0.000	0.000
MDH	0.457	0.324	0.813
PEP	0.732	0.640	0.813
PER	0.312	0.640	0.278
H	0.434	0.379	0.420

3.6.2.5 Apportionment of Diversity.

Total diversity (H_T) of the *M. canis* isolates from Auckland, Palmerston North and Wellington was apportioned by calculating the mean phenotypic diversity for each region (sub-population) for each of the four enzyme systems (excluding monomorphic enzymes, Table 4-1) from which the overall mean was calculated for the within population diversity.

This allows us to subdivide total phenotypic diversity (H_T) into diversity within populations (H_S) and diversity among populations (D_{ST}) using the the method of Nei (1975), described in Section 2.5.2.:

$$H_T = H_S + D_{ST}$$

and differentiation among populations is calculated as:

$$G_{ST} = D_{ST}/H_T$$

where G_{ST} can vary between 0 ($H_S = H_T$) and 1 (H_S=0, that is the population is fixed for different phenotypes).

Table 3-40: Phenotypic diversity of *M. canis* within and between Auckland, Palmerston North and Wellington for each enzyme system.

Enzyme System	H_T	H_S	D_{ST}	G_{ST}
EST	0.657	0.364	0.293	0.446
G6P	0.923	0.831	0.092	0.100
MDH	0.558	0.531	0.027	0.048
PEP	0.754	0.728	0.026	0.034
H	0.723	0.614	0.109	0.157

All monomorphic enzymes were excluded from the analysis

H_T = Total diversity for the populations
H_S = diversity within populations
D_{ST}= diversity among populations
G_{ST}= interpopulation differentiation

The enzymes which were monomorphic (Table 4-1) were excluded from the analysis of interpopulation diversity between the three geographical regions. Table 3-40 demonstrates a considerable variation among isozymes for total levels of phenotypic diversity (H_T), with high diversity residing within populations (H_S=0.614) and a low diversity among populations (D_{ST}=0.109). Interpopulation differentiation was moderate (G_{ST}=0.157).

3.6.2.6 Diversity among *M. cookei* "+" and "-" mating strains.

In Table 3-41, the enzyme systems CAT, G6P, MDH, LAP and PER exhibited no diversity for the "+" mating type. EST (H = 1.431) had the highest phenotypic diversity with G6P and PEP having the same diversity measure of H = 0.644 for the "+" mating type. Similarly the "-" mating strains had no diversity at three enzyme systems GPI, MDH and PEP. The other five enzyme systems showed variation ranging from H = 0.235 for PER to H = 1.366 for EST.

Table 3-41: Phenotypic diversity in "+" and "-" mating strains of *M. cookei.*

Enzyme system	(+) n=06	(-) n=12
CAT	0.000	0.292
EST	1.431	1.366
G6P	0.644	0.474
GPI	0.000	0.000
MDH	0.000	0.000
PEP	0.644	0.000
LAP	0.000	0.869
PER	0.000	0.235
H	0.340	0.400

For "+" and "-" isolates refer to Table 3-10.

The "+" and "-" (Table 3-41) isolates had very similar phenotypic diversity.

3.6.3 Phenotypic Identity.

3.6.3.1 Relatedness Between the Sepcies.

Phenotypic similarities between pairs of the two *Microsporum* species and the *Diheterospora* "control" (populations) were calculated using Hedrick's identity measure (*I*). Phenotypic identity was calculated for each enzyme system, and a mean identity calculated from these values (Section 2.5.3).

$$I_{x.y} = \frac{\sum P_{j.x} P_{j.y}}{\frac{1}{2}\left(\sum P^2_{j.x} + \sum P^2_{j.y}\right)}$$

$P_{j.x} + P_{j.y}$ = is the frequency of the jth phenotype in populations x and y.

Table 3-42: Estimation of the degree of similarity between the populations of *Microsporum* spp. and *Diheterospora* using Hedrick's identity measure (*I*).

Species	*M. canis*	*M. cookei*	*Diheterospora*
M. canis	1.000	0.779	0.000
M. cookei		1.000	0.000
Diheterospora			1.000

Values represent the means of the identity values calculated separately for each enzyme system.

Based on Hedrick's identity measure the two *Microsporum* species can be considered to be closely related, but neither species is closely related to *Diheterospora*. (The no identity ($I = 0$) in Table 3-42 could be interpreted to indicate a lack of close identity to *Microsporum* spp. because of the very few isolates of *Diheterospora*).

3.6.3.2 Relatedness of *M. canis* from the Three Regions.

Using Hedrick's identity measure (*I*), isolates from Palmerston North are more closely related to Auckland, as are isolates from Wellington. The Palmerston North and Wellington isolates have the lowest phenotypic identity.

Table 3-43: Estimation of the degree of similarity between the geographical regions using Hedrick's identity measure (*I*).

	Palm. Nth	Wellington	Auckland
Palm. Nth	1.000	0.794	0.901
Wellington		1.000	0.874
Auckland			1.000

3.6.4 Estimation of phenotypic similarity using the cluster method.

To generate a dendrogram showing phenotypic similarity, an electromorphic matrix (Table 3-44) containing the isozyme phenotypes of all isolates was analysed by cluster analysis. Normalised percent disagreement (PTC) was used as a distance measure and the dendrogram computed by the average-linkage method of clustering (UPGMA) (Sneath and Sokal, 1973, Section 2.5.3). The calculations were performed by the statistical package, SYSTAT (Wilkinson, 1989).

Table 3-44: Electromorph profiles of 76 isolates of *Microsporum* and *Diheterospora*.

Isolate code	S.	Inf.	Phenotype Nº for each enzyme system							
			CAT	EST	G6P	GPI	LAP	MDH	PEP	PER
PN01	cat	-	2	9	4	1	2	2	3	2
PN02	cat	-	2	9	4	1	2	2	2	2
PN03	cat	-	2	9	4	1	2	3	3	2
PN04	cat	-	2	9	4	1	2	2	4	2
PN05	cat	+	2	9	4	1	2	2	3	2
PN06	dog	+	1	9	4	1	2	2	3	2
PN07	cat	+	2	9	4	1	2	2	3	2
PN08	ktn	+	2	9	4	1	2	2	2	2
WG09	cat	+	2	9	4	1	2	2	3	2
WG10	hmn	+	2	9	4	1	2	2	3	2
PN11	cat	-	1	10	4	1	2	2	3	1
PN12	cat	-	2	9	4	1	2	2	3	2
PN13	cat	-	1	1	1	1	2	2	3	1
PN14	cat	-	2	10	4	1	2	3	3	2
PN15	cat	-	2	10	4	1	2	3	3	2
PN16	cat	-	2	10	4	1	2	3	4	2
PN17	cat	-	2	10	4	1	2	2	3	1
PN18	cat	-	2	1	1	1	2	3	3	2
PN19	cat	-	1	10	4	1	2	2	3	1
PN20	cat	-	2	10	1	1	2	3	3	2
PN21	bkt	NA	2	10	3	1	2	2	2	2
PN22	cat	-	2	9	3	1	2	2	4	2
PN23	cat	-	1	9	2	1	2	2	2	1
PN24	cat	-	2	9	3	1	2	2	3	2
PN25	cat	-	2	9	3	1	2	2	3	2
PN26	cat	-	2	9	3	1	2	2	3	2
RV87	ITM	NA	2	8	3	1	2	1	4	2

S = source; Inf = infection; N = unknown; NA = not applicable;
ITM = tester strains from the Institute for Tropical Medicine;
hmn = human; bkt = small pieces of blanket (rugs).

Table 3-44 contd.

RV88	ITM	NA	2	8	2	1	2	1	2	2
PN27	dog	-	2	9	3	1	2	2	2	2
PN28	cat	-	2	9	2	1	2	2	2	2
PN29	cat	+	2	9	1	1	2	3	4	2
WG30A	hmn	+	1	9	2	1	2	2	2	1
WG30B	hmn	+	1	9	2	1	2	2	2	1
WG31	hmn	+	2	9	2	1	2	1	2	2
WG32	hmn	+	1	9	2	1	2	2	3	1
WG33	hmn	+	2	9	3	1	2	2	2	2
WG34	hmn	+	1	9	2	1	2	2	3	1
WG35	hmn	+	2	9	2	1	2	2	2	2
?36	ukn	N	2	8	3	1	2	2	4	2
PN37	cat	-	1	9	3	1	2	2	4	2
PN38	cat	-	2	9	3	1	2	2	3	2
PN39A	cat	-	2	8	3	1	2	2	3	2
PN39B	cat	-	2	8	3	1	2	3	4	2
PN40	cat	-	2	9	4	1	2	2	3	2
PN41	cat	-	2	9	4	1	2	2	4	2
PN42	cat	-	2	9	3	1	2	2	4	2
PN43	cat	-	2	9	4	1	2	3	3	2
PN44	dog	+	2	9	1	1	2	3	3	2
AK45	hmn	+	2	8	3	1	2	2	2	2
AK46	hmn	+	1	9	3	1	2	2	2	1
AK47	hmn	+	1	9	1	1	2	3	4	1
AK48	hmn	+	2	9	3	1	2	1	3	2
AK49	hmn	+	2	9	4	1	2	3	4	2
AK50	hmn	+	1	9	4	1	2	2	4	1
H08	soil		1	4	4	2	1	4	4	2
H30	soil		3	5	3	2	1	4	4	2
A32	soil		3	2	4	2	1	4	3	2
S33	soil		3	5	4	2	1	4	3	2
A39	soil		1	3	4	2	1	4	4	2

Table 3-44 contd.

A81	soil	3	1	4	2	1	4	3	2
A107	soil	3	6	3	2	1	4	3	2
A125	soil	3	7	4	2	1	4	3	2
A152	soil	1	2	4	2	1	4	3	2
A153	soil	3	4	4	2	1	4	3	2
H154	soil	3	1	3	2	1	4	4	2
A155	soil	3	4	4	2	1	4	3	2
H173	soil	3	4	4	2	1	4	2	2
H192	soil	1	4	4	2	1	4	3	2
H219	soil	3	5	3	2	1	4	3	2
H223	soil	3	5	4	2	1	4	4	2
H228	soil	1	1	4	2	1	4	4	2
C551	cat	1	3	4	2	1	4	2	2
AD28	soil	4	11	5	3	3	5	1	3
HD32	soil	4	11	5	3	3	5	1	3

Based on the isozyme data in Table 3-44, *M. canis* showed variation among EST, G6P, MDH and PEP. GPI and LAP had the same phenotype for all the *M. canis* isolates. Similarly, *M. cookei* showed variation at three enzyme systems EST, G6P and PEP but had no variation at GPI, LAP and MDH. For both *M. canis* and *M. cookei* at two enzyme systems CAT and PER some isolates showed the absence of a band.

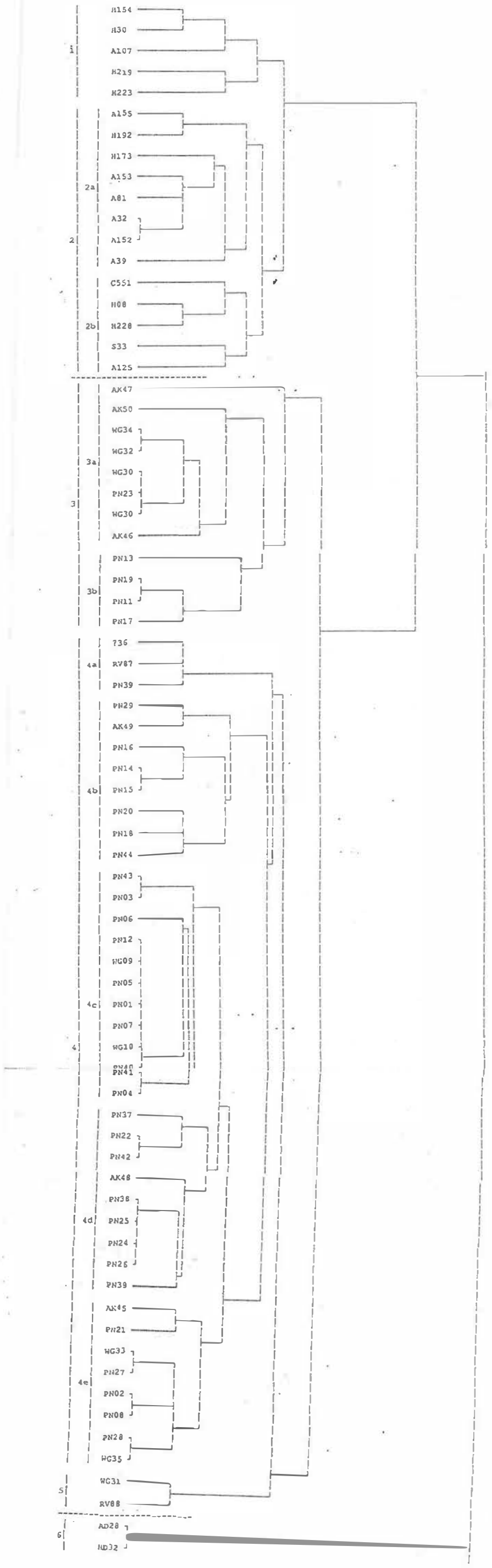
Fig.3-48: Cluster analysis performed on the results of isozyme electrophoresis in relation to isolate. (PN,Palmerston North; AK, Auckland; WG, Wellington) Clusters 1-2, *M. cookei*; Cluster 3-5, *M. canis*; Cluster 6, *Diheterospora*.

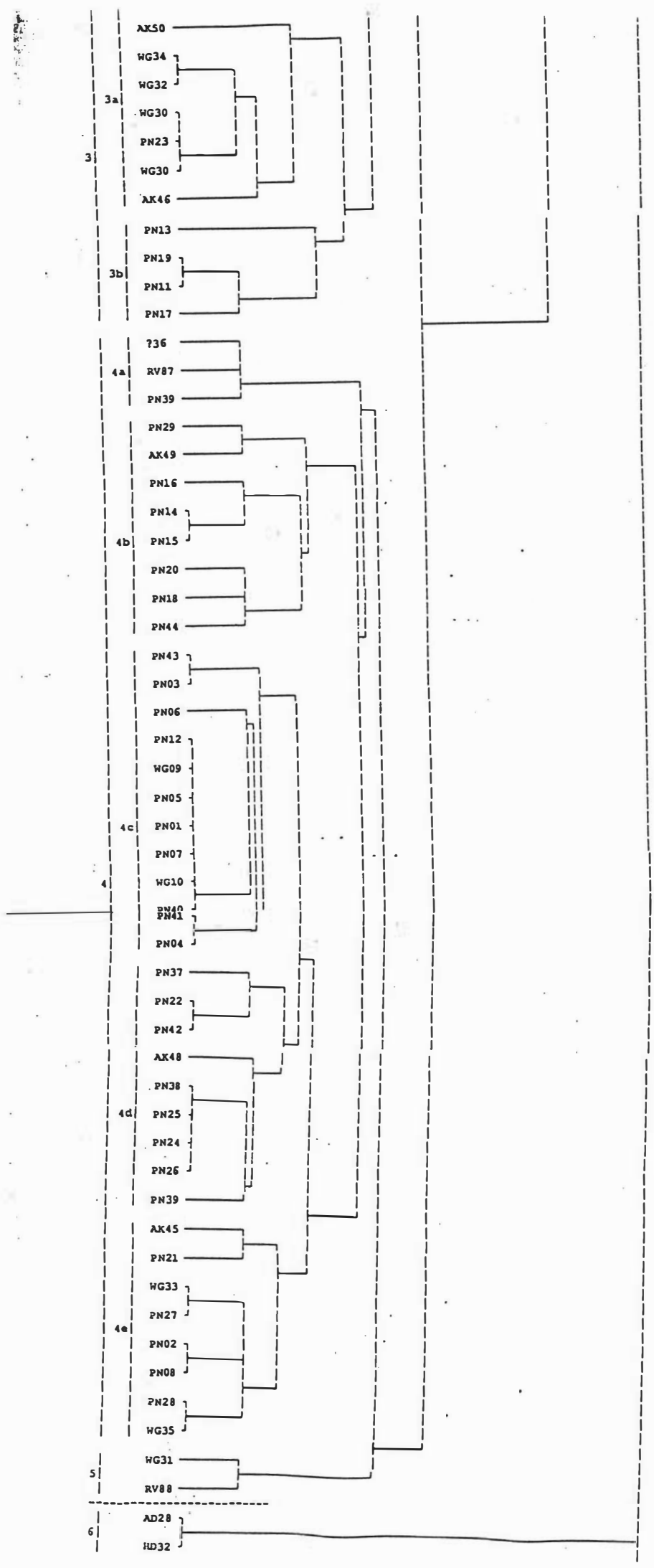
Distance metric is normalized percent disagreement
average linkage method

Source

Distances

0.0 50.0 100.0




AK50
WG34
WG32
3a
WG30
PN23
3
WG30
AK46
PN13
PN19
3b
PN11
PN17
?36
4a
RV87
PN39
PN29
AK49
PN16
PN14
4b
PN15
PN20
PN18
PN44
PN43
PN03
PN06
PN12
WG09
PN05
4c
PN01
PN07
4
WG10
PN40
PN41
PN04
PN37
PN22
PN42
AK48
PN38
4d
PN25
PN24
PN26
PN39
AK45
PN21
WG33
PN27
4e
PN02
PN08
PN28
WG35
WG31
5
RV88
AD28
6
HD32

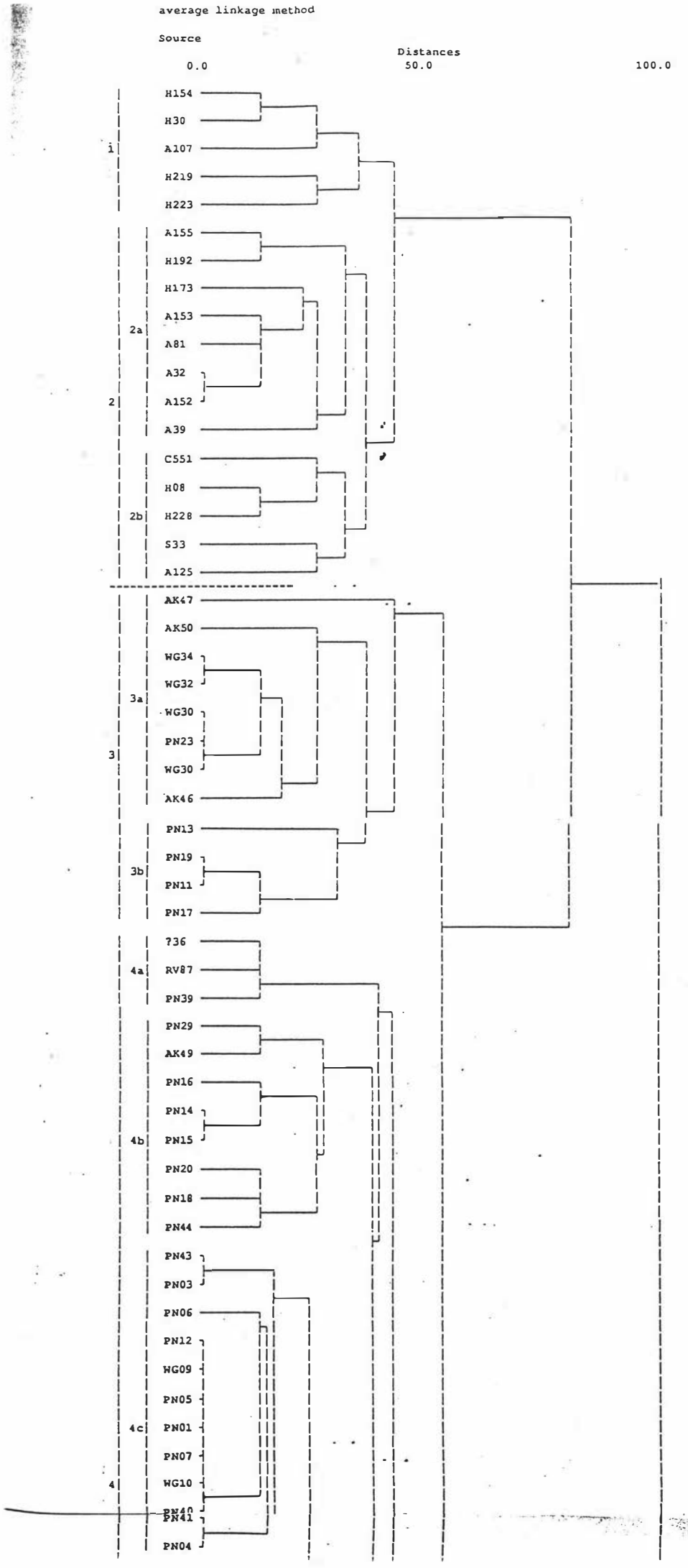
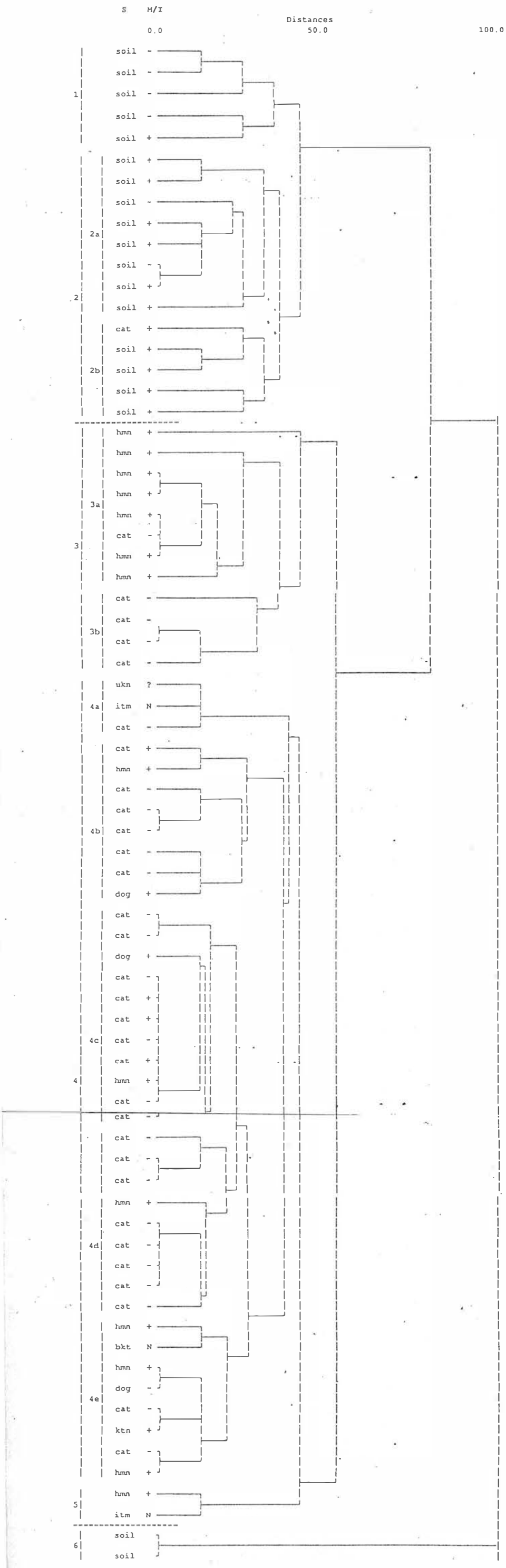
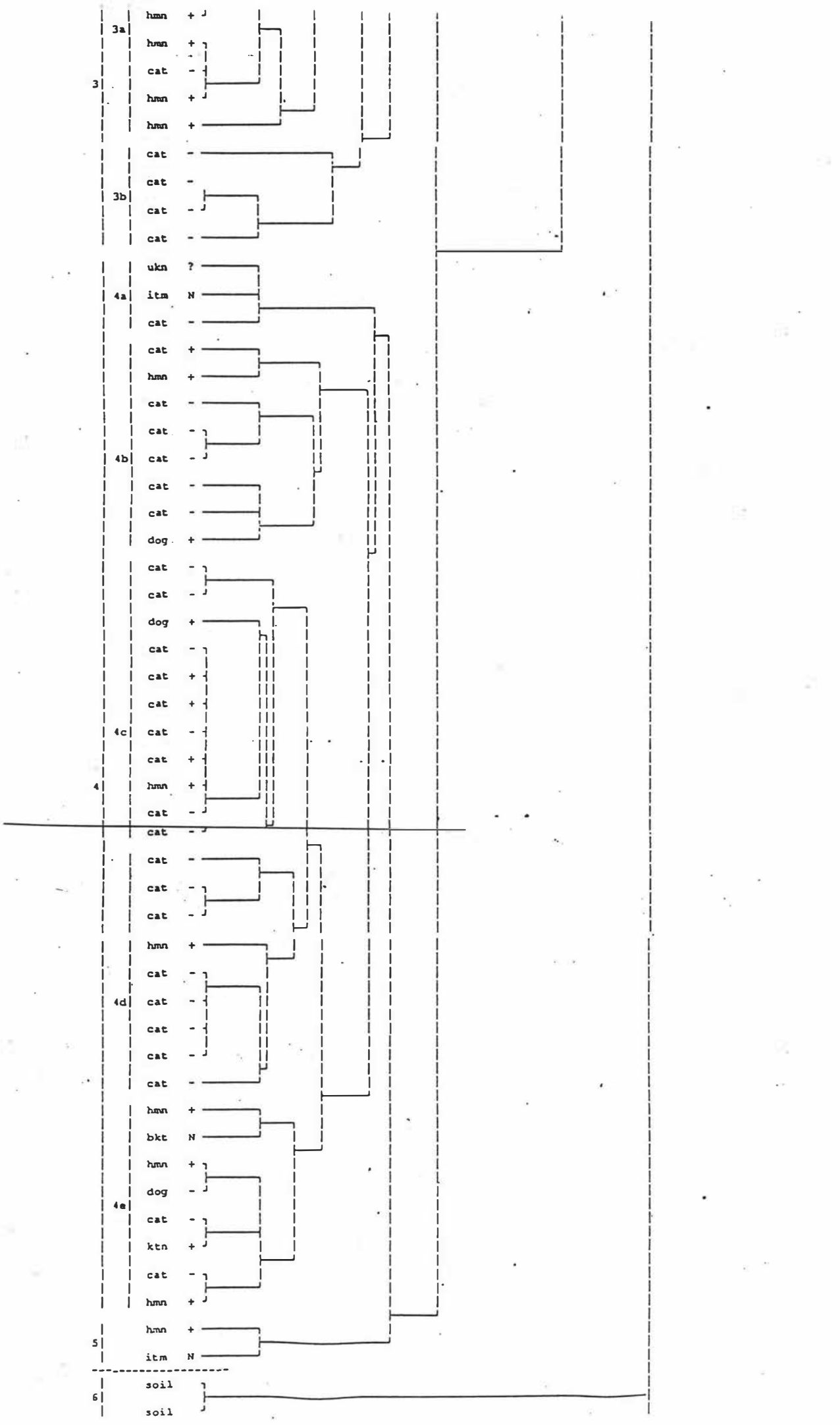
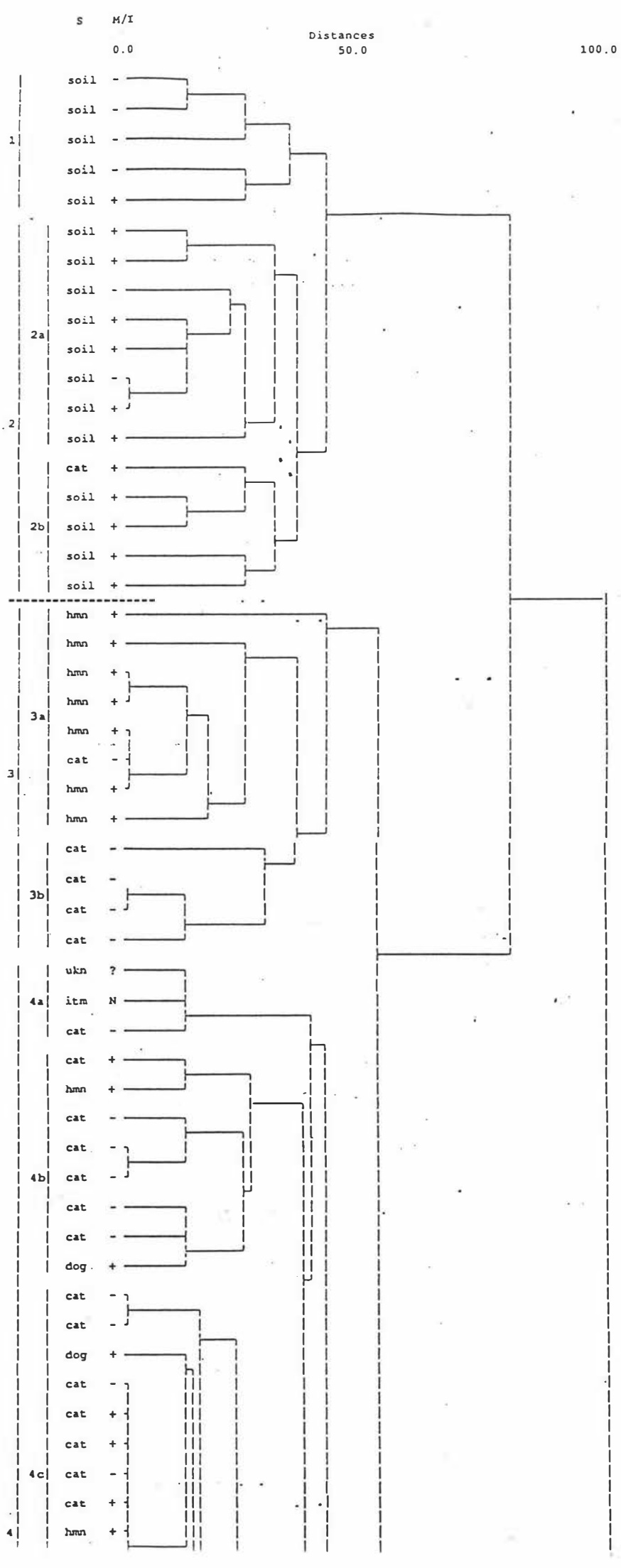

average linkage method
Source
Distances
0.0
50.0
100.0
H154
H30
A107
H219
H223
A155
H192
H173
A153
A81
A32
A152
A39
C551
H08
H228
S33
A125
AK47
AK50
WG34
WG32
WG30
PN23
WG30
AK46
PN13
PN19
PN11
PN17
?36
RV87
PN39
PN29
AK49
PN16
PN14
PN15
PN20
PN18
PN44
PN43
PN03
PN06
PN12
WG09
PN05
PN01
PN07
WG10
PN40
PN41
PN04
1
2
2a
2b
3
3a
3b
4
4a
4b
4c

Fig.3-49: Cluster analysis performed on the results of isozyme electrophoresis. (S, Source; M, mating type (-/+ for *M. cookei*) and I, Infection (-/+ for *M. canis*); ITM, tester strain from the Institute of Tropical Medicine; ukn, source unknown; bkt, "blanket") Clusters 1-2, *M. cookei*; Clusters 3-5, *M. canis*; Clusters 6, *Diheterospora*.

Distance metric is normalized percent disagreement
average linkage method




3
3a
hmn +
hmn +
cat -
hmn +
hmn +
3b
cat -
cat -
cat -
cat -
4
4a
ukn ?
itm N
cat -
4b
cat +
hmn +
cat -
cat -
cat -
cat -
dog +
4c
cat -
cat -
dog +
cat -
cat +
cat +
cat -
cat +
hmn +
cat -
cat -
4d
cat -
cat -
cat -
cat -
cat -
hmn +
cat -
cat -
cat -
cat -
cat -
4e
hmn +
bkt N
hmn +
dog -
cat -
ktn +
cat -
hmn +
5
hmn +
itm N
6
soil
soil


S M/I
Distances
0.0
50.0
100.0
1
soil -
soil -
soil -
soil -
soil +
2a
soil +
soil +
soil -
soil +
soil +
soil -
soil +
2
soil +
2b
cat +
soil +
soil +
soil +
soil +
3
3a
hmn +
hmn +
hmn +
hmn +
hmn +
cat -
hmn +
hmn +
3b
cat -
cat -
cat -
cat -
cat -
4
4a
ukn ?
itm N
cat -
4b
cat +
hmn +
cat -
cat -
cat -
cat -
cat -
dog +
4c
cat -
cat -
dog +
cat -
cat +
cat +
cat -
cat +
hmn +

CHAPTER 4

DISCUSSION

The Soil as a Natural Reservoir of Pathogenic Fungi.

Central to the study and control of mycotic diseases is the acquisition and utilisation of knowledge concerning the natural habitat of the aetiologic agents (Ajello, 1980). Such information is of great value in determining the prevalence of pathogenic fungi in the environment in any particular area. It allows the delineation of endemic regions, the tracing and discovering of the point of outbreaks, and the development of control measures. Fresh isolates for taxonomic, genetic and other studies should always be obtained.

During the present study, the investigation of the soil was by the hair-baiting technique (Vanbreuseghem, 1952b) which selectively allows the growth of keratinophilic fungi at the expense of other soil fungi. The studies were particularly aimed at the isolation of *M. cookei*, a non-pathogenic geophile morphologically similar to *M. canis*, for comparative enzyme and epidemiological studies. A total of 16 isolations of *M. cookei* was achieved from the 236 soil samples examined. Using the Stockdale technique and all possible mating combinations, five of the isolates were found to be of the "-" mating type, and 11 were of the "+" mating type. The "-" mating types were not fertile with each other and neither were any of the "+" mating types. Padhye and Carmichael (1971) also found predominantly *M. cookei* of the "+" mating type and that fertility in *M. cookei*, just like in *A. uncinatum* is not an all-or-none phenomenon, there were both strong and weak reactors existing in nature.

All the keratinolytic fungi isolated in this study are usually considered as non-pathogens, with the exception of the *M. gypseum* complex. *M. gypseum* has been reported to cause infections in man, cats, dogs and horses (Vanbreuseghem *et al.*, 1978; Demange *et al.*, 1992). For the period 1987 to 1992, *M. gypseum* isolations in New Zealand have been reported to range from 1.0% to 1.8% of the total dermatophytes from skin

samples and was the only geophilic dermatophyte isolated (Mycoses Newsletter, CDCNZ, 1991 to 1992). However, *M. gypseum* was very rare in the soils sampled, even though some of the soils came from paddocks and school grounds. This is a similar observation to that made by Marples (1965) in her study of New Zealand soils. Marples suggested that the general absence of *M. gypseum* could be due to competition for the keratin bait by *T. ajelloi*, which is more prevalent in New Zealand soils.

Since *T. ajelloi* was the most frequently isolated keratinolytic fungus, its distribution in relation to soil pH was assessed and *M. cookei* included for comparison. Both were most often found in soils within the pH range of 5.1 to 7.0 (83.8%, *T. ajelloi* and all *M. cookei* isolates). Other workers have found *T. ajelloi* to be widely distributed in the environment, and it is a true soil inhabitant, like *Ctenomyces serratus* (Pugh, 1964). Marples (1965) proposed that *T. ajelloi* 's distribution was related to the soil pH and temperature, being found in alkaline soils and cool to temperate climates. The distribution of keratinolytic fungi in general however, seems to be affected by the distribution and availability of keratinous substrates in the environment (Böhme and Ziegler, 1969; De Vroey, 1984). For instance, *Arthroderma curreyi* survives in the close vicinity of keratinous substrates (Baxter, 1969). In this study, *A. curreyi* was isolated from the hair of a cat and a dog.

There were no isolations of any anthropophilic nor zoophilic dermatophytes from soil, although *T. mentagrophytes* has been isolated from the soil by other workers (Lurie and Borok, 1955; Baxter, 1969). The apparent absence of anthropophilic and zoophilic fungi in soil has been suggested to be due to their inherent inability to survive in such an environment (Ajello and Cheng, 1967). For example, studies by Grin and Ozegovic (1963) demonstrated that anthropophiles and zoophiles which are highly adapted to a parasitic life on the keratinous structures of man and animals were lysed by a variety of microorganisms when placed in the soil. *M. gypseum*, *T. ajelloi* and *T. terrestre* were resistant to lysis.

It was observed that the use of "keratin-in-contact with soil" (Griffin, 1960b) produced a characteristic fungal flora. The environment of

"keratin-in-contact with soil" was first occupied by highly competitive saprophytic fungi with the ability to use the less complex nutrient substrates. Moulds such as *Fusarium* and *Penicillium* and a number of the Mucorales, which are fast growing and nutritionally less exacting, are good examples (English, 1965). Besides the above fungi, others which appeared as part of the soil environment can be regarded as less competitive saprophytic fungi, able to utilise the most resistant part of the substrate. The keratinolytic fungi such as *M. cookei*, *M. gypseum*, *T. ajelloi* and *T. terrestre* isolated in this study are very characteristic of this group. Other studies conducted in Australasia on the isolation of keratinophilic fungi from the soil have reported similar types of fungi, for example *Chrysosporium*, *Cladosporium*, *Diheterospora*, *Fusarium*, *Gliocladium*, *Microsporum*, *Mucor*, *Paecilomyces*, *Penicillium*, *Trichophyton* and *Verticillium* (Ajello and Alpert, 1972; Marchisio *et al.*, 1991; Soon, 1991).

The rare isolations of e.g. *Cladosporium*, *Rhizopus*, *Mucor* and *Trichothecium* were possibly of little significance as "keratinophilic". Perhaps the definition of "keratinophilic" used here was too broad. However, some of these rarely encountered genera e.g. *Scopulariopsis*, do have some ability to digest keratin (Baxter, 1968, English, 1965) and may be known "opportunistic" nail and skin invaders (Evans and Gentles, 1985).

Although the primary aim of the keratin-baiting technique was to isolate keratinolytic spp., for the purposes of this discussion, some comments on the isolation of other potentially pathogenic fungi will now be included, as most of the fungi causing human infections are acquired from the environment and the soil is a known source of most of the pathogenic fungi (Dei Cas and Vernes, 1986). In the broad studies reported here, it was found that keratinophiles (Section 3.1) in general were very abundant in soils. In particular, *Cunninghamella*, *Diheterospora*, *Fusarium*, *Gliocladium* and *Paecilomyces* (Table 3-2) were very frequently isolated. Many of these moulds found in the soil have a common biological role as agents of decomposition of the foliage and dead material of plants (Male, 1990), as well as the destruction of keratinous substrates. The acquisition of infections by man caused by

these species can therefore be considered as an "accident" as these fungi do not need to colonise man or other animals for their survival. Even without causing disease they are able to survive in the soil indefinitely.

Some of the fungi isolated have been reported as pathogens in compromised patients in New Zealand, particularly the genera *Fusarium* and *Paecilomyces* (CDCNZ, 1991,1992). *Fusarium* spp. were most common in soils from recreational parks but had a similar frequency in soils from cleared areas and school grounds. *Paecilomyces*, on the other hand, was more prevalent in soils from school grounds, roadsides and recreational parks. Soil therefore, can be considered a potential source of infections by the fungus. In contrast, another genus often associated with infections of compromised patients, *Aspergillus*, was not found in the soils sampled by the hair baiting technique. A study by Griffin (1960b) in Australia also reported that *Aspergillus* was extremely scarce from the soils he surveyed.

One species isolated from soil and meeting the definition of "keratinophile" used here was *Conidiobolus coronatus* (*Entomophthora coronatus*). *C. coronatus* usually occurs as a saprophyte on plant debris. A disease (entomophthoromycosis) caused by the fungus is more common in tropical and subtropical areas of the world (King, 1979; Costa *et al.*, 1991). At the moment, there is no known report of the infection in New Zealand. The only other report of this fungus in this part of the world is by Griffin (1960b) from a soil sample in Australia, although Baxter (personal communication) has noted it a number of times on keratin baits.

Small Animal Reservoirs of Potentially Pathogenic Fungi.

Studies by Marples (1956) and many others world-wide have provided evidence over the years that cats and dogs are a major reservoir of human infections caused by *M. canis*. For this study therefore, the investigation of the status of animals for potentially pathogenic fungi, particularly *M. canis* was confined to cats and dogs.

In the study, 18.5% of cats and 5.1% of dogs were either carriers of or where infected with *M. canis*. Similar results have been reported from other investigations of small animals as carriers of keratinophilic fungi, e.g. 36% of cats and 7.5% of dogs were found to be positive for *M. canis* by Baxter (1973). Zaror *et al.* (1985) in Chile reported 88.46% of cats and 7.93% of dogs positive for *M. canis*, while Kaplan and Ivens (1961) in the United States found 29.5% of cats and 12.8% of dogs positive for *M. canis*. A *t*-test performed by SAS (SAS Institute Inc., 1988) showed a significant difference ($P \leq 0.05$) between the two animals as carriers of *M. canis* (and other fungi), cats being the favoured host.

The present study and other studies over the years (Marples, 1956, 1959; Smith *et al.*, 1969; Baxter, 1973; Woodgyer, 1977; Carman *et al.*, 1979) highlight the importance of cats as reservoirs of *M. canis* in New Zealand. The cat is considered by some investigators to be the natural host of this fungus (Male *et al.*, 1980).

The *M. canis* isolates in this study were all of the "-" mating type. Studies from other parts of the world have also reported only the "-" mating type isolated from either actual infections or carriers (Weitzman and Padhye, 1978). Whether this is due to a "high virulence" (or selective advantage) of the "-" mating type in infecting the host is not clear. Kwon-Chung (1974) suggested that the "-" mating type may have a selective advantage over the "+" mating type in actual pathogenicity. However, the factors favouring the "-" mating type were not defined.

If the hypothesis of Person (1968; cited by Dei Cas and Vernes, 1986), regarding parasitism is evoked to explain this observation it is possible to imagine the existence of "specific genetic interrelationships" between genes in the host governing resistance to the parasite and genes in the parasite which govern its virulence to the host. Accordingly, selection may favour mutations in the host which place the parasite, in this case the "+" mating type, at a disadvantage. The selective pressure may have lead to the "-" mating type becoming progressively more virulent as a result of selection of genes of greater virulence.

Most isolations from cats examined by the hair brush technique were of keratinolytic fungi (Table 3-7). Among these *Chrysosporium* spp. were

the most common, followed by *Microsporum* spp. and lastly, *Trichophyton* spp.. *Chrysosporium* spp. were also very common on dogs. Its pathogenicity still remains uncertain (Chabasse *et al.*, 1989). Of the *Microsporum* spp., *M. canis* constituted the majority of the isolates, with only a single isolate of the geophile *M. cookei* from a cat. Of the *Trichophyton* spp., *T. terrestre* was the most frequent, with *T. mentagrophytes var. mentagrophytes* as the second most common. *T. ajelloi* was infrequent and there were two isolates of *Arthroderma curreyi.*

Other fungi of medical importance which were isolated from cats and dogs were *Alternaria*, *Aspergillus*, *Cladosporium*, *Mucor*, *Penicillium* and *Paecilomyces*. As has been said, these can be responsible for opportunistic infections especially in debilitated and compromised hosts. Some of the fungi, such as *Alternaria* and *Cladosporium* are also responsible for rhinitis, asthma and extrinsic allergic alveolitis. These fungi are both common in air by day in dry weather (Lacey, 1990).

The high incidence of *M. canis* of 18.5% on cats and 5.1% on dogs with and without clinical symptoms emphasizes the potential for the presence of the fungus in the home environment. In New Zealand, females get infected by *M. canis* more than males (CDCNZ, 1992) possibly because they tend to make more frequent close contact with cats. La Touche (1955) and McAleer (1980) in their studies found that the major factor in the spread of tinea was the opportunity of direct contact with animals which were either carriers or infected. Children were the most susceptible to infection and acquired the infection through direct contact with the animals. This situation leads to transmission of the parasite to other members of the family resulting in intrafamilial infections (Lunder, 1992), particularly of siblings and mothers.

The other mode of transmission to man is indirect through infective propagules originating from active lesions of infected animals which enter the human environment, through contact with contaminated fomites such as furniture, clothing and floors. (Male, 1980; De Vroey, 1984). This is well illustrated in this study by the isolation of *M. canis* from a "blanket" used for cats. Infected hair and scales are known to remain infective for months, in one case they stayed viable for 1½ years (Male, 1980). In New

Zealand, studies by Smith *et al.* (1969) have shown that zoophilic species, with the exception of *M. canis*, are of less importance to human populations. This has lead to the suggestion by some authors (Smith *et al.*, 1969; Baxter, 1973; CDCNZ, 1991, 1992) that the dynamics of human infections caused by *M. canis* in New Zealand tends to reflect the incidence of infections in the feline populations. This is well illustrated by the corresponding high incidence of *M. canis* infections in humans compared to other dermatophytes reported from CDCNZ. Over the years 1987 to 1992, *M. canis* was by far the most frequent zoophilic dermatophyte isolated.

The lack of isolation of *M. canis* directly from the soil in this and other studies (Hill, 1959; Georg, 1959; Marchisio *et al.*, 1991) suggests that *M. canis* has lost its ability for saprophytic life in the soil.

In summary, the inter human, human-animal and human-environment relationships constitute the prime factors that can explain the presence or the absence of infection (Mantovani, 1982). The increasing number of pet animals such as cats and dogs means a frequent source of infective particles in the home environment and therefore the creation of a permanent source of exposure.

Dermatophytes and other disease-producing fungi depend on a favourable environment for their continued existence as pathogens. However, the environment is continuously changing and the reaction of the fungus determines its ability to survive. The environment can be studied at two levels, that of the substrate with which the pathogen is in contact and, at a broader level, changes in the substrate and/or environment which allow dispersal of the fungus to occur (English, 1980).

The seasonal distribution of *M. canis* isolations from animals in this study was 22.2% for the "summer" from November to April and 77.8% for the "winter" months. Isolations were compared to the average meteorological readings (Table 3-9) using statistical analysis. There was a strong positive correlation between *M. canis* and relative humidity, the higher the relative humidity, the more isolations being made. But there was a negative correlation between *M. canis* and temperature. This has been

interpreted to mean that relative humidity and temperature have an effect on the seasonal distribution of *M. canis*, since there was an increase in *M. canis* with decreasing temperatures and increasing relative humidity. Other studies (Marples, 1951; Smith *et al.*, 1969; McAleer, 1980) have also reported an increase of *M. canis* in the feline population in winter. The increase in clinical cases of human ringworm at this time has been attributed to an increase in the kitten population. Kittens are born at the end of December and January, and sometimes in April and early May (Smith *et al.*, 1969). The young animals, which are more susceptible to infection, therefore become more abundant at the times when temperatures are cool and seem to favour the spread of the fungus from animal to animal. In this study *M .canis* distribution peaked in May-June, thus a combination of the animal (kitten) reservoir and optimum temperature for spread of the fungus could be postulated.

Biochemical Assays.

The biochemical assays performed in this study were primarily aimed at determining if relative enzyme activity as revealed by a comparison of the collected *M. canis* and *M. cookei* strains might serve as an indicator of virulence. The underlying assumption made in determining enzymatic activity by biochemical assays is that the quantitative variation in for example, proteolytic and/or elastinolytic activity is responsible for differences in virulence between strains (Gadasi and Kobiler, 1983; Eakin *et al.*, 1993). The identification of such virulence factors has been dependent on, and limited by, the ability to mimic host environmental factors in the laboratory. It is to be hoped that the expression of most virulence factors is regulated by environmental conditions *in vitro* that presumably reflect similar cues present in the host tissue.

Enzymes as virulence factors are hypothesized to act (a) when the pathogen comes into contact with the target cells and/or (b) at a distance as diffusible soluble product(s) of the pathogen, which may enhance its survival in tissues by chemically or physically altering the immediate environment, or by directly digesting host proteins.

The gelatin plate hydrolysis used in this study as a general indicator of proteinase expression by the *Microsporum* isolates, was not of much diagnostic value in relation to the concept of pathogenicity. The values obtained expressing relative proteolytic activity for *M. canis* isolates from clinical cases and carriers were very similar (see Table 3-12). Indeed, the non-pathogenic *M. cookei* showed much higher activity. Thus this measure could just indicate that *M. cookei* is better able to utilise proteinaceous substrates *in vitro*.

One factor which can influence expression of enzymes is the method of culture. In the case of dermatophytes, shake cultures are useful for such studies as the fungus produces hyphae and arthrospores resembling the parasitic life phase of the fungi found in scales, hair and nails (Raubitschek, 1955). This is the growth type referred to as "pseudo-parasitic" (Evron-Maoz and Raubitschek, 1960). Stationary culture on the other hand, produces hyphae and macro- and microconidia as found in

the saprophytic phase of dermatophytes, in which quite different factors could be involved in keratin breakdown.

The enzyme assays for proteinase (using azocoll) and elastase (using elastin powder) had a good reproducibility, but keratinase (using dyed wool) had large variations from one assay to another. This may be due to the uneven content of the dye in wool. Nevertheless, this problem was partly overcome by the statistical methods employed. The weighted mean for the three enzymes was employed for comparison based on the contrasting treatments of shake and stationary culture. Proteinase activity for *M. canis* isolates as a group for each treatment showed a very highly significant (P = 0.0016) difference in expression in shake compared to stationary cultures (Table 3-14, 3-15; Fig.3-18). Elastase activity on the other hand was very significantly higher (P = 0.0001) in stationary than shake cultures (Table 3-14, 3-15; Fig.3-21). This suggests that although elastase may be produced during infection it is not equally as important as proteolytic activity during pathogenesis. But there was no significant difference (P = 0.4324) observed for keratinase production between the shake and stationary cultures.

The correlation coefficient (*r*) of *M. canis* in shake cultures for proteinase and elastase expression were significantly negatively correlated (r = -0.505). This may mean that when the expression of one increases the other enzyme decreases. It should be noted, however that correlation does not necessarily mean causality. The correlations for the other enzymes (keratinase and proteinase; keratinase and elastase) were insignificantly very low (Table 3-17). In stationary cultures, keratinase and proteinase (r = 0.068) were very weakly correlated and so were proteinase and elastase expressions (r = 0.006), both were not significant. For *M. cookei* shake cultures, all the correlations (proteinase and elastase (r = -0.344); keratinase and proteinase (r = -0.204) and keratinase and elastase (r = -0.145)) were negative and non significant. Therefore, there was no coordinated relationship in the expression of the three enzymes.

Proteolytic activities of the individual isolates varied widely for *M. canis*. Nevertheless, for stationary cultures, isolates from clinical cases had a slightly higher baseline proteinase activity compared to isolates from

carriers (Fig.3-20). For all the isolates, there was also a consistently higher expression of proteinase activity in shake cultures than stationary cultures. These observations also emphasized the differences in enzyme expression between the two growth phases, "pseudo-parasitic" and saprophytic. Similar results have been reported for differences in enzyme expression between the mycelial (saprophytic) and yeast (parasitic) phases of *Paracoccidioides brasiliensis* (Bedoya-Escobar *et al.*, 1993). The proteinase-elastinolytic activity was high in the mycelial phase and absent in the yeast phase.

The expression of elastinolytic activity of *M. canis* was detected in both isolates from clinical cases and carriers. For stationary cultures, isolates from clinical cases had a slightly higher baseline elastase activity than the carriers (Fig.3-22). Overall and in contrast to proteinase production, the isolates showed a significantly lower expression of elastases in shake culture as discussed above. This result is again in good agreement with Bedoya-Escobar *et al.* (1993). The reduced expression of elastase in shake culture may mean that elastases probably do not play as critical a role as proteinases in the pathogenesis of dermatophytoses caused by *M. canis*. Other workers (Denning *et al.*, 1993) have also expressed doubts about the importance of elastase as a virulence factor. In stained histological sections of invasive pulmonary aspergillosis, they found no disruption of elastin in the immediate proximity to hyphae (Denning *et al.*, 1992). However, this hypothesis requires further *in vitro* and *in vivo* studies such as use of cell monolayers and animal inoculation experiments.

For keratinase production, some isolates showed higher expression in shake culture than in stationary culture (Fig.3-24). But there was no significant difference between the two series. This may seem to support the hypothesis of O'Sullivan and Mathison (1971) that keratolysis probably does not play a critical role once the mycelium is established in the epidermis. Additionally there was no significant levels of keratinase detectable in cultures of most isolates and keratin assays had a poor reproducibility. Other authors have reported similar observations and suggested a restricted substrate specificity as a possible reason, or the removal of some accessory proteins capable of splitting disulfide bonds present in keratinized proteins (Sanyal *et al.*, 1985; Kunert, 1992). This

theory has been partly supported by Kunert's studies which seemed to suggest that proteolytic enzymes are more active in the presence of reducing agents and of sulphite in particular. He has proposed the reduction of sulfide as the reason, since the reduction of 20% of disulphide bonds resulted in enhanced wool hydrolysis.

The results with *M. cookei* for proteinase expression was also significantly higher (P = 0.0161) in shake than in stationary cultures. Nevertheless, the results of these enzyme assays did not compare well with gelatin/SDS-PAGE, unlike those with *M. canis*. Shake cultures which showed high proteolytic activity using enzyme assays produced fewer proteinase bands on gelatin/SDS-PAGE. On the other hand, stationary cultures with low proteolytic activity produced more proteinase bands compared to shake cultures. But elastase expression was statistically (P=0.1377) similar between the two treatments. For keratinase, there was a complete lack of expression of this enzyme in stationary culture.

It should be noted that in this study, the capacity of a dermatophyte to produce enzymes (proteinase, elastase and keratinase) was estimated under controlled conditions of laboratory culture. It is quite likely that some strains may be able to produce greater or lesser quantities of the enzyme(s) under the growth conditions available on the skin of a patient. For example, some workers have reported leucyl-aminopeptidase, proteinase and keratinase activity of some strains of dermatophytes are markedly enhanced when they are grown on keratin *in vitro* as compared to the activity demonstrated during growth on ordinary media (Male and Holubar, 1968). Gadasi and Kobiler (1983) demonstrated significant differences in content of proteolytic enzymes in *Entamoeba histolytica* trophozoites of two strains known to differ in their virulence, but differences were dependent on the substrate used to demonstrate them. For instance, differences were found with azocoll and azocasein as substrates, probably attributable to specific enzymes e.g. collagenase which recognises azocoll but not azocasein, as azocoll is a collagen substrate with an azo dye attached to it. The bond between collagen and the azo dye is also recognised by other proteolytic enzymes (Moore, 1969).

Thus the difficulty in using biochemical assays *in vitro* to correlate pathogenicity is that pathogenicity occurs only *in vivo*, under nutritional

and environmental conditions which may be different from those provided in laboratory cultures. For this reason, Mahan *et al.*, (1993) have developed a genetic system, termed *in vivo* expression technology (IVET), that does not rely on reproduction of the environmental signals but depends on the induction of genes in the host. This technique uses an avirulent *Salmonella* strain which lacks *purA*. Functional copies from other bacteria joined to *lacZ* (a gene that makes an enzyme easily detectable by a colour assay) are cut and inserted in front of the two gene combination to make DNA constructs. These are transformed into mutant bacteria lacking *purA* and used to infect mice. Some of the bacteria that survive after a few days in mice are considered to contain host specific *Salmonella* gene fragments which allowed them to survive in the host.

Similarly, dermatophytes may not express some of the virulent genes *in vitro* and may only be turned on *in vivo*. Some enzyme assays therefore may be useful only in as far as they allow the detection of enzyme production by an organism, but may not necessarily relate to the organism's ability to cause disease.

Characterisation of Proteinase Enzymes using Inhibitors.

The first indication for multiple proteinases in both *M. canis* and *M. cookei* filtrates came from the use of specific inhibitors. For both *M. canis* (Table 3-23) and *M. cookei* (Table 3-24), there was inhibition of azocollytic activity by α_1-proteinase and phenylmethylsulfonyl fluoride (PMSF) inhibitors, indicating the presence of serine-catalysed proteinase activity. Proteinases from other dermatophytes have also been reported to be sensitive especially to PMSF and α_1-Proteinase (Meevootisom and Niederpruem, 1979; Sanyal *et al.*, 1985; Asahi *et al.*, 1985). The production of a serine proteinase by *M. canis* has also been reported by Takiuchi *et al.* (1982, 1984). They estimated it to be a 45 000-M_r proteinase. The antiserum raised against it cross-reacted with material in the culture filtrates of *M. gypseum*, *T. mentagrophytes* and *T. rubrum*. This result means that structurally similar proteinases may be expressed by some or most of the dermatophytes. A similar finding was reported for elastinolytic serine proteinase from *Aspergillus flavus* which had antibodies immunologically related to *A. fumigatus* (Kolattukudy *et al.*,

1993). These findings together emphasize that structurally similar enzymes may be expressed by closely related species.

M. canis culture filtrate had no sensitivity to 1,10-phenanthroline (PT) but to ethylenediaminetetraacetate (EDTA), while *M. cookei* was only slightly inhibited by the two metallo-proteinase inhibitors (see Table 3-24; Fig.3-31). In fact, *M. canis* filtrate increased in activity in the presence of PT. The opposite effects of EDTA and PT may be attributed to an activation or a stabilisation of some members of other enzyme classes (serine, cysteine or aspartic) by metal ions (Dunn, 1989). That is why the diagnosis of metallo-proteinases is best determined on their insensitivity to the three other classes of inhibitors plus the observation of inhibition by the chelating agent. At present it is not clear why increased *M. canis* enzyme activity was observed with PT, as this did not occur with EDTA.

Studies with cysteine inhibitors showed inhibition of azocollytic activity of *M. canis* and not *M. cookei* using three different cysteine inhibitors (Tables 3-23, 3-24). There was significant inhibition by *p*-chloromercuribenzoic acid (*p*CMB) (73.1%) while the other cysteine inhibitors, L-trans-epoxysuccinyl leucylamido (4-guanidino)-butane (E-64) and iodoacetic acid (IAA) showed less inhibition (31.2% and 31.6% respectively). This suggested the presence of a cysteine proteinase(s) in *M. canis* filtrate. Chattaway *et al.* (1956, 1963) reported strong inhibition of peptidases of *T.verrucosum var discoides* and *M. canis* by *p*CMB and 65% inhibition of endogenous respiration in *M. canis* using IAA. The production of cysteine proteinase by a *Micropsorum* spp. has also been reported by Roberts and Doetsch (1967), although the fungus was not identified to a species level. In addition, *M. fulvum* (*Nannizzia fulva*) has been reported to produce a cysteine elastinolytic proteinase which was inhibited by *p*CMB, urea and IAA (Werb *et al.*, 1982). However, there is no information on the structural similarity of the cysteine proteinases from different dermatophytes.

Some inhibition was observed with *M. canis* filtrate with pepstatin, which suggested aspartic proteinase(s). However, there was no inhibition by *p*CMB, IAA, E64 and pepstatin with *M. cookei* filtrate, indicating an absence of extracellular cysteine and aspartic enzymes.

From the data presented in this study, it is suggested that *M. canis* produces one or more serine proteinase(s), one or more cysteine proteinase(s) and possibly an aspartic proteinase(s). *M. cookei* produces only serine proteinase(s). *M. canis*, therefore, expressed a range of proteinase types, all which have been implicated in the pathogenicity of a wide range of microorganisms, including *Trypanosoma*, *Aspergillus* and *Serratia*. However, purification and characterisation of the enzymes with the used methods and additional ones would further define the proteinases produced especially by *M. canis* and whether EDTA inhibition was due to the presence of a metallo-proteinase(s). The presence of more than one type of proteinase has been reported in a number of other species with at least two and sometimes three types of proteinases produced. Multiple forms (isoforms) of a proteinase of the same activity but of different M_r have been reported to be produced by the same organism (North, 1982).

Inhibition of *M. canis* and *M. cookei* elastase enzyme(s) occurred only with the two chelating agents, EDTA and PT. There was no inhibition of the elastase(s) by the three other classes of inhibitors (Table 3-23, 3-24). Therefore, both *M. canis* and *M. cookei* express only metallo-elastinolytic proteinase(s). This finding is supported by the fact that microbial elastases are usually metallo-proteinases (Mckerrow *et al.*, 1985). Nevertheless, the function of fungal elastases is not well known, although one of their characteristics is a broad substrate specificity. They have the ability to degrade fibronectin, laminin, gamma globulins, $\alpha_1P_1$keratin and type IV collagen (Apodaca and Mckerrow, 1990). Elastin is thus only one substrate elastase enzymes can degrade.

Characterisation of proteinases and elastases would allow rational design of synthetic chemotherapeutic agents. For example, synthetic peptidase inhibitors have been reported to lyse *T. cruzi* trypomastigotes *in vitro*.

Multiple Proteinase Expression.

Extracellular enzyme expression in stationary and shake cultures was determined using Sabouraud broth as medium. Although there were some variations in the number of enzymes expressed by the different isolates under the same treatment, in general, six proteinases of different molecular weights (M_r) were detected with *M. canis* (122 KDa, 64 KDa, 62 KDa, 44 KDa, 36 KDa, and 28 KDa). Of these, M_r 122 KDa, 62 KDa and 28 KDa were very highly expressed (P=0.0001) in shake but not in stationary cultures. To determine if the degree of aeration might be involved in these differences of enzyme expression, the oxygen content of the Sabouraud broth was measured in the two cultural methods. There was no significant difference (X^2 =8.95; P≥0.05) in oxygen concentration of Sabouraud broth in the two cultural methods.

The proteinases of M_r 64 KDa, 44 KDa, and 36 KDa, expressed in both shake and stationary cultures, can be considered to be constitutively expressed since they are expressed in both the saprophytic and "pseudo-parasitic" forms. The M_r 64 KDa proteinase was more highly expressed in shake culture, thus this proteinase may be expressed constitutively at low levels and only peaks when induced by a change in morphology, from the saprophytic to the parasitic form.

In contrast, those proteinases of M_r 122 KDa, 62 KDa, and 28 KDa could well be induced by a change in morphology from saprophytic to parasitic form. It is suggested that the additional proteinases produced in shake culture may play a critical role in the pathogenicity of *M. canis*.

These proteinases may possibly be the ones which provoke inflammatory responses. Studies by Minocha *et al.* (1972) have shown a correlation of high proteolytic activity and the more acute inflammatory infections of *T. mentagrophytes*. When keratolysis is considered as distinct from proteolysis of the less hardened epidermal proteins, keratolysis probably plays a less critical role once the mycelium is established in the epidermis (O'Sullivan and Mathison, 1971). Maeda and Molla (1989), studying the role of proteinases in the pathogenicity of bacterial pathogens, including *Serratia marcescens* 56 KDa, 60 KDa, and 73 KDa proteinases and *Pseudomonas aeruginosa* alkaline proteinase and

elastase (and also a proteinase from *Aspergillus melleus*), have suggested the involvement of the activation of the Hageman factor and/or prekallikrein of the complement system resulting in enhanced vascular permeability. All the proteinases are reported to degrade immunoglobulins like IgG and IgA and cause destruction of structural matrices like fibronectin. The bacterial proteinases are also reported to inactivate *in vitro* the complement system, e.g. C3 and C5 in human serum.

Takiuchi *et al.* (1982, 1984) using stationary culture, detected a 45 KDa extracellular proteinase using SDS-PAGE of *M. canis* filtrates. This may correspond to the 44 KDa detected in this study. Studies by O'Sullivan and Mathison (1971) using shake cultures found that *M. canis* synthesized a complex of proteolytic enzymes with pH optima at pH 6.6, 8.0, and 9.5 to 10.0. Production of multiple proteinases has also been reported in other dermatophytes, such as *T. rubrum* and *T. mentagrophytes* (Meevootisom and Niederpruem, 1979; Sanyal *et al.*, 1985; Asahi *et al.*, 1985; Apodaca and McKerrow, 1989a, 1989b, 1990).

A common class of proteinases reported from a wide range of pathogenic organisms which have been subjected to detailed characterisation are serine-proteinases (North, 1982). Most of the serine proteinases are generally of a low M_r, in a range of 18.5 to 35 KDa and usually around 25 KDa, although larger enzymes have also been reported (North, 1982). The proteinase of M_r 28 KDa expressed by *M. canis* is within this M_r range. Assuming serine proteinases are critical in fungal pathogenesis, their small size would allow them to diffuse into the lower epidermis, and dermis to cause inflammation. This view is supported by Minocha *et al.* (1972) who observed dermo-epidermal separation and spongiosis when fungal extracts were injected intradermally into excised human skin. They suggested that the changes were due to proteolytic enzymes. Peptidases and aminopeptidases identified in the present studies with starch gels probably cleave the peptides generated by proteinases into amino acids. Daniels (1953) had demonstrated an accumulation of amino acids when *M. canis* was cultured on human hair as a nutrient source.

With the elastin/SDS-PAGE, two elastase enzymes were detected having the same M_rs as two proteinases (64 KDa and 62 KDa). This finding

suggests that the two enzymes have a substrate specificity for both gelatin and elastin. A similar result was reported by Apodaca and McKerrow (1990) for *T. rubrum*.

However, postulated roles for secreted proteinases do not prove their association with virulence. The detection of the enzymatic activity in most pathogenic microorganisms, including fungi, has been obtained *in vitro*, so that the role of these enzymes *in vivo* remains hypothetical. The detection of serine proteinase(s) in infected tissues (Olgerts *et al.*, 1979; Becker *et al.*, 1988; Reichard *et al.*, 1990; Gessner and Mortensen, 1990) only provides circumstantial evidence that they may play a pathogenic role. Further evidence for relevance *in vivo* must be demonstrated by showing that the putative determinant is biologically effective in animal tests by protecting animals against the disease with antibodies to it and by showing that strains lacking it are less virulent (Smith, 1988). Studies of Purnell and Martin (1971) and Purnell (1978) were among the earlier ones to suggest an association between virulence and the enzyme alkaline phosphatase. Mutants defective in alkaline phophastase were found avirulent compared to the wild-type. The virulence was assayed by the morbidity of mice inoculated intravenously. A separate study by Grappel and Blank (1972) found that both active and inactivated keratinases were able to elicit delayed type cutaneous hypersensitivity reactions in guinea pigs. In addition the α-globulin fraction of sera contained an inhibitor of the keratinases. The implication of these findings is that keratinases and other enzymes produced during infection may play a role in the invasiveness of the fungus and in hypersensitivity reactions associated with dermatophytosis. Curson (1978) reported similar findings for two phospholipases produced by amebae. What is required is *in vivo* confirmation of the role of these enzymes in pathogenicity.

Studies on the importance of transformation to a parasitic morphology by dermatophytes were carried out by Rippon and Scherr (1959). They induced two dermatophytes, *T. rubrum* and *M. audouinii* both *in vitro* and *in vivo* to assume a yeast morphology consistent with dimorphic pathogenic fungi. This alteration resulted in increased pathogenicity as manifested by the invasion of deep tissue. However, it was not investigated whether there was an associated increase in enzyme

expression, especially of proteinases. In studies by Medoff *et al.* (1986) and Maresca and Kobayashi (1989) on *Histoplasma capsulatum*, a dimorphic fungus which requires -SH compounds to transform from the mycelial (saprophytic) to yeast (parasitic) phase, it was shown that in the presence of an -SH blocking agent (p-chloromercuryphenylsulfonic acid, PCMS) which inhibits the formation of the yeast phase, the fungus remains in the mycelial form and is non-pathogenic. However, there were no differences in virulence of normal and PCMS treated yeast cells in mice (Medoff *et al.*,1987). This finding has provided direct evidence that transformation of *Histoplasma* to yeast is a necessary prerequisite for infection. Furthermore, in the early stages of infection there is an increased expression of heat shock proteins (hsp), thought to play a role in thermoadaptation, along with the acquisition of the capacity to invade.

Since dermatophytes require a morphological change from conidial to hyphal for infection, increased proteinase expression would probably occur *in vivo*, as observed in this study. These changes would allow the adaptation of the dermatophyte and initiation of infection of their host. But further studies are needed to provide unequivocal evidence for the role of enzymes in the virulence of the dermatophytes. A suggested line of investigation would be to develop an *in vitro* epidermal keratinocyte culture. This would allow additional studies on whether the highly expressed proteinases detected in the "pseudo-parasitic" morphology are important in pathogenicity as defined by their cytopathic effect(s) on cell monolayers. This could be a preliminary study leading towards further studies to confirm the role of proteinases as virulence determinants in experimental animals. Another method could be the use of mutants defective in some or all of the usually highly expressed proteinases.

In contrast to *M. canis*, *M. cookei*, a nonpathogen, expressed seven different M_r proteinases (67 KDa, 64 KDa, 63 KDa, 62 KDa, 54 KDa, 52 KDa, and 42 KDa) in stationary cultures. Shake culture had five different M_r proteinases of 63 KDa, 62 KDa, 54 KDa, 52 KDa and 42 KDa. The enzyme expression detected for *M. canis* and *M. cookei* may be a function of their natural ecological roles. The proteinases for *M. cookei* were very close in M_r compared to those of *M. canis* which ranged from 122 KDa to 28 KDa. The higher M_r proteinases expressed by *M. canis* may be those involved in infection and digestion of keratin. Apodaca and

McKerrow (1989a, 1989b, 1990) had also found high M_r proteinases to be very good at degrading keratin and elastin. However the ability of *M. cookei* to express a range of proteinases may mean that its lack of pathogenicity may be due to other factors, such as inability to tolerate high temperatures (weak thermotolerance, Lorincz and Sun, 1963) as compared to *M. canis*. Some subcutaneous mycoses, such as sporotrichosis and chromoblastomycosis are known to be sensitive to increase in temperature, which influences their deep fungus pathology (Dei Cas and Vernes, 1986).

Baxter (1968) compared non-pathogenic *T. ajelloi*, "trained" to grow at 35°C to others maintained at 25°C for their ability to infect guinea pigs. Only strains able to grow at 35°C were able to produce low grade scaling with slight erythematous reaction within five days but this only lasted for eight days. This illustrates that the ability to grow at or near the host's skin temperature can be an important factor in the pathogenicity of a microorganism.

According to Smyth (1972), the "ideal" parasite must be capable of (a) recognising its habitat to allow the metabolic and morphologic transformations which characterise the parasitic stage to occur, (b) fixing and maintaining its position, (c) adapting to the physicochemical conditions of the host, (d) using most nutrient materials at a level generally permitting host survival, (e) "mimicking" the antigens of the host to minimise host immune reaction and finally (f) synchronising its development with the host.

Similarly, when pathogenicity is defined as an expression of a two component system, the host-parasite relationship, such a definition emphasizes the host as an environment for the parasite (Garber, 1956). Pathogenicity therefore, with respect to the parasite, is the capacity to use the host environment as a growth medium and to overcome the defence mechanisms of the host (Garber, 1956). With dermatophytes in general and *M. canis* and *M. cookei* in particular, nutritional inhibition is less likely because of the available keratinised tissue for growth. This means that host defence mechanisms may be a more important factor. For example, host factors affecting the host-parasite relationship in dermatophyte infections include the effect of higher temperature and

long-chain unsaturated fatty acids secreted by sebaceous glands after puberty, which have fungistatic and/or fungicidal properties (Hajini *et al.*, 1970; Dei Cas and Vernes, 1986; El-Naghy *et al.*, 1990). Other methods which may offer a selective advantage to the host include inhibition by serine proteinase inhibitors (serpins) of exogenous proteinases postulated to act as virulence factors produced by infectious agents upon infection (Hill and Hastie, 1987), although these serpin inhibitors are known to be inactivated by certain proteinases as well (Maeda and Molla, 1989)

Phenotypic ("Genetic") Variability of *Microsporum* Species.

Molecular genetic studies of *Microsporum* spp. and its relatives are practically non-existent. Among the reasons are (a) the genetics of the group is not well characterised and (b) there is a lack of suitable methods and scoreable markers for assaying genetic variability in natural populations. This has made research on both pathogenicity and epidemiology of *M. canis* and other dermatophytes more challenging.

For this study multilocus enzyme electrophoresis was used for indexing allelic variation among the different isolates. Isozyme analysis offers a relatively independent and selectively neutral means of identifying population levels of genetic variation (Ayala, 1984; Milchelmore and Hulbert, 1987). However, genetic analysis of variation requires Mendelian ratio studies on parents and segregating generations (Sigh and Jain, 1971; Ayala, 1984). Although the sexual states of *M. canis* and *M. cookei* are known, difficulties in producing fertile ascospores (Padhye and Carmichael, 1971; Takashio, 1981) has meant that the genetic basis for the electrophoretic banding patterns are as yet not known. Because it has not yet been demonstrated that the phenotypes observed on gels for the genus *Microsporum* represent allelic forms as isozymes coded by particular loci, the observed patterns have been referred to as phenotypes.

The use of gel electrophoresis to measure genetic variation is subject to several potential sources of bias. One important difficulty is that not all allelic variation is detectable by electrophoresis, that is, enzymes with the same electrophoretic mobilities may have different amino acid sequences (Lewontin and Hubby, 1966a). Therefore, the amount of genetic variation tends to be underestimated (Ramshaw *et al.*, 1979; Ayala, 1984), although it is not yet possible to indicate by how much. Furthermore, only structural genes coding soluble proteins can be studied while regulatory genes or genes coding for non-soluble proteins may not be detectable (Ayala, 1975). Another obstacle is that although protocols exist for detection of over 57 specific enzymes in diverse eukaryotes (Vallejos, 1983), fewer than fifteen enzyme systems are usually found to be useful in any one species, and most studies have employed far fewer (Milchelmore and Hulbert, 1987).

Since there was no prior knowledge of detectable isozymes of *Microsporum* spp., preliminary studies were focused on screening 28 different enzymes. Eight were found to be suitable (Table 2-3). All the enzymes in Table 2-3 gave well resolved isozyme patterns with the exception of EST and G6P. EST and G6P produced isozyme patterns which needed particular care in interpretation and determination of electromorphs.

The eight enzyme systems for the *Microsporum* spp. examined can be classified into two groups (a) polymorphic and (b) monomorphic. An enzyme is considered polymorphic if a protein exhibited more than one electromorph or isoform. Monomorphic proteins did not show any variation within the same species. Using the above criteria the eight enzyme systems were divided as shown in Table 4-1.

Table 4-1: Classification of the eight enzymes.

	Polymorphic	Monomorphic
A)	1. MDH	1. GPI
	2. PEP	2. LAP
B)	3. EST	3. CAT
	4. G6P	4. PER

See Table 2-3 for abbreviations.

The enzymes in (A) were detected in all isolates, while in (B) there was absence of enzyme activity in a few isolates. Of the eight enzyme systems, 50% were polymorphic for *M. canis*, and 37.5% for *M. cookei*. The MDH enzyme system was polymorphic in *M. canis*, but monomorphic in *M. cookei*.

In addition to the eight enzymes, glutamate dehydrogenase, although detected in both *Microsporum* spp., was not included in this study because its relative mobility (Rf) for both spp. was the same. Alkaline phosphatase activity proved difficult to resolve for both spp. even after

trials using seven different buffers (see appendix D). Reddy and Threlkeld (1971) also reported to have tried unsuccessfully to resolve acid phosphatase into clear isozyme bands in *Neurospora*. Acid phosphatase in this study, was only visualised from the extracts of *Diheterospora* species. Diaphorase and superoxide dismutase were very poorly stained and the migration patterns were not readable.

A number of reasons may be advanced to explain the lack of enzyme activity for the remaining enzymes. One explanation could be that the enzymes are not synthesized by the fungi examined in this study, or the levels of enzyme production were too low to be detected. Another, perhaps more plausible, reason would be the use of electrophoretic or staining buffers which may denature or inactivate the enzyme(s).

The enzymes of CAT, LAP, PER and GPI did not show electrophoretic variation between isolates within the same species but showed variation between species. These four enzyme systems, showed one band per isolate (Figs.3-37, 3-40, 3-41, 3-44, 3-45, 3-46, 3-47), consistent with a haploid organism with a single locus. For the remaining enzyme systems, both *M. canis* and *M. cookei* exhibited some unusual biochemical features. For MDH, PEP, EST and G6P one, two or three additional bands were observed in different isolates. For a few isolates MDH exhibited four bands. This observed banding pattern suggested that there are multiple copies of the loci producing different isozymes. Similar observations have been reported in other haploid fungi (Kurjeza and Garber, 1973; Spieth, 1975; Leuchtmann and Clay, 1990). Leuchtmann and Clay (1990) suggested that multiple copies may result from heterokaryosis or aneuploidy. In *Avena* populations, genome duplications have been suggested to be a significant factor in the evolution of enzyme diversity (Sigh and Jain, 1971). Mutations in the duplicated loci may then provide a source of genetic diversity comparable with diploid species with more than one allele (Kurjeza and Garber, 1973). The complex banding pattern of EST may be due to either multiple copies of the locus with different isozymes or lack of substrate specificity leading to detection of several bands in the isolates (Nealson and Garber, 1967; Reddy and Threlkeld, 1971). According to Reddy and Threlkeld (1972), esterases form a complex family of enzymes whose precise classification is made difficult through overlapping substrate specificities. *M. cookei* exhibited a more complex banding pattern with EST than *M. canis* (Fig.3-38, 3-45).

Of the triple bands detected in G6P, the middle band was less well resolved, appearing diffuse (Fig.3-39). The band near the origin did not migrate very far from the origin by the end of the electrophoretic run. Some isolates showed absence of enzyme activity with G6P, which is unexpected for an enzyme involved in the glycolytic pathway. A possible explanation could be the inability of the isozymes to recognise artificial substrates, or a low level of G6P enzyme production by the isolates. The other enzymes in which a few isolates showed absence of enzyme activity were CAT, PER and EST. Other studies have reported similar observations (Selander *et al.*, 1985).

The analysis of isozyme data using the normalised Shannon-Wiener diversity measure revealed that *M. canis* has a higher mean phenotypic diversity (H = 0.459) than *M. cookei* (H = 0.408). This is in spite of *M. canis* being only of the "-" mating type while *M. cookei* had both "+" and "-" mating types (Tables 3-10 and 3-11). This result was rather unexpected as low variation has been suggested to be a feature of asexual populations (Maynard-Smith, 1971). However, the low level of fertility found among *M. cookei*, with some isolates being completely infertile (Table 3-11), may explain the low mean phenotypic diversity among *M. cookei* isolates. Tibayrenc *et al.* (1991) have also pointed out that the demonstration of sexual reproduction in laboratory cultures or even in nature simply shows that the potential for sexual reproduction has not been lost, but even so might not occur.

The normalised Shannon-Wiener mean diversity measure of clinical and carrier isolates of *M.canis* was very similar. This suggests that *M. canis* from carriers may have the potential to cause infections. Probably differences in host defence or lack of e.g. trauma may be an important factor in resistance to *M. canis* infections.

The small variation among the regional populations of *M. canis* is not surprising in view of a complete absence of a "+" mating type among the isolates, the existence of one mating type and therefore only the anamorphic phase limiting their capacity to vary genetically.

Since *M. canis* exists in nature as an asexual anamorphic form, gene shuffling would seem to be inhibited with a likely existence of a

uniparental lineage(s). Since evolutionary convergence to the same multilocus phenotype is highly improbable, isolates with identical or similar multilocus phenotypes are considered members of the same cell line (Selander *et al.*, 1985). However, this does not mean that the cell lines (Fig.3-48, 3-49) identified by a limited number of enzyme markers are genetically homogenous. More detailed genetic studies may reveal additional variability among the isolates. Maynard-Smith (1971) has suggested that lack of genetic recombination may not be detrimental to a population or species if (a) the offspring are consistently dispersed into an environment similar to that of the parent, (b) there is intense selection pressure, and (c) populations are small.

M. canis occurs in nature on discrete hosts. Apart from the infective propagules, the pathogen lives primarily on the host. This may provide a relatively uniform environment for the fungus for several generations thus meeting Maynard-Smith's first criterion. Secondly, host specialisation of *M. canis* for cats and to a lesser extent dogs and humans suggests that host-pathogen interactions exert strong selective pressure on the fungus. Since *M. canis* is predominantly haploid, any genetic differences would be expected to be immediately expressed, increasing the selective pressures. Thirdly, the dependence of the fungus on an uncertain vector is likely to produce a population size that is intermittently low, acting as a bottle neck, leading to a reduction in effective population size. Therefore, it seems that all the three criteria are met.

Multilocus diversity as measured by the Shannon-Wiener diversity measure although widely used has only a moderate discriminant ability and in addition is sensitive to sample size (Magurran, 1988). When comparing regional populations this statistic is, therefore, affected by both the number of phenotypes observed within each region as well as equitability (evenness of distribution) of the phenotypic frequencies (Gaur *et al.*, 1981). It is indicated to be more sensitive to the number of phenotypes than the evenness of distribution. That is, more phenotypes with a skewed distribution appear to give higher diversity than fewer phenotypes with an equitable distribution (Goodwin *et al.*, 1993). The number of phenotypes detected will be a function of the sample, and since the sample sizes varied among the three regions, the diversity values were normalised by $H = H/Hmax$ (Sheldon, 1969) in which H is

the usual Shannon-Wiener diversity measure over phenotypes, and Hmax is ln(N); the maximum diversity for a sample of size N. The normalised statistic has been found to be relatively stable and has provided the best measure of diversity when sample sizes vary (Sheldon, 1969; Goodwin *et al.*, 1992).

According to Nei (1977), the apportionment of genetic variability in terms of the Shannon diversity measure (Lewontin, 1972) is similar to Nei's (1972, 1978) gene diversity analysis except that Nei's measure can be related to the number of codon differences per locus when the genetics of the organism is known. However, the estimation of codon differences by statistical methods (Nei, 1972) may not reflect the true substitution at the gene level. It may either underestimate or over estimate the amount of substitution occurring.

The apportionment of total diversity (H_T) of *M. canis* from Auckland, Palmerston North and Wellington was determined using the diversity analysis of Nei (1973, 1975, 1977). Nei modified the *F*-statistics developed by Wright (1951) to give a means of analysing population structure, that is, of partitioning the genetic variation of the total population into components. G_{st} (which is a similar statistic as F_{st}, Nei, 1973, 1975, 1977) as a measure of subpopulation differentiation, becomes a function of past and present subpopulation size and intermigration. Therefore, where differentiation is due purely to sampling drift, the value of G_{st} should be the same for all phenotypes at all enzyme systems, or where the genetics of the organisms is known, for all alleles at all loci, since being in the same organism, they were subject to the same evolutionary population history (Wright, 1978).

From the results in Table 3-40, *M. canis* contains a substantial phenotypic variability, which for the most part exists within local populations and accounts for 84.9% of the total mean phenotypic diversity. The interpopulation differentiation on the other hand is moderate, accounting for 21.7%, while the diversity among populations is 15.1%. Spieth (1975) reported a similar result with *Neurospora* spp., of high variation within populations and with little dissimilarity between populations. Riba *et al.* (1986) and St Leger *et al.* (1992a) using *Metarhizium anisophiliae*, an entomopathogenic fungus with no known

sexual stage, found a substantial interisolate variability. Other workers using *Rhynchosporium secalis*, an imperfect fungus, have also reported a high amount of variation within populations as defined by geographical areas (Goodwin *et al.*, 1993). Spieth (1975) suggested that the haplophase selection in *Neurospora* spp. ought to dominate the behaviour of some, if not many enzyme loci. He proposed a model based on environmental heterogeneity which evokes gene flow among neighbouring populations that occupy shifting multiple adaptive niches.

The mechanisms that generate and maintain these phenotypic diversity structures for *M. canis* are not yet known. However, in general, population structure results from complex interactions between intrinsic factors, primarily gene flow, genetic drift and natural selection. These promote or constrain genetic similarity within populations and act in different ways: gene flow, by facilitating the spread of genetic variants, genetic drift, by random fixation or eliminating genetic variants (i.e. similarity by descent) and natural selection, by favouring genetic variants that are better adapted to the ecological niches. In population theory, intermigration is recognised as an important force in maintaining homogeneity among populations of a species (Lewontin, 1974).

The electrophoretic data can also be used to calculate phenotypic identity (*I*) between pairs of closely related species. Hendrick's identity measure was used to compare interspecies identity. The phenotypic identity between *M. canis* and *M. cookei* was 0.779 ($I = 0.779$). This seems a reasonable estimate of their identity, as for example, Hamrick *et al.* (1986) estimate of identity ranged from $I = 0.576$ to $I = 0.792$ for *Histoplasma capsulatum* from different sites using isozyme data.

The recovery of isolates of the same electrophoretic phenotypes from the three different geographical regions suggests that the phenotypic structure of *M. canis* may comprise cell lines. This means that for *M. canis* there must be a means of dispersal over wide areas. Since *M. canis* can be transmitted from animal to human and vice versa and human to human, the strong possibility is that humans, animals, especially pet animals, and contaminated fomites may act as agents of dispersal. For some fungi (Spieth, 1975; St Leger, 1992a) and bacteria (Selander *et al.*, 1985) wind dispersal has been suggested. To what

extent this may occur for *M. canis* is not yet known, although *M. canis* spores have been isolated from air in enclosed surroundings (Friedman *et al.*, 1960; Uscavage and Kral, 1961; Midgley *et al.*, 1972; Smith, 1982).

Relationships Among Electrophoretic Phenotypes.

The dendrograms generated (Figs.3-48, 3-49) summarise the phenotypic relationships. At normalised percent disagreement (PTC) of about 50%, there are three main clusters of *M. canis* isolates (clusters 3,4, and 5) and two of *M. cookei* (clusters 1 and 2), each represented by subclusters within each cluster of electrophoretic phenotypes. The clustering based on isozymes was in good agreement with the taxonomy based on morphology of *M. canis*, *M. cookei* and *Diheterospora* (cluster 6). Each of the three species were clustered separately from each other based on isozyme data. The use of genetic similarities in fungal studies is still in its infancy (St Leger, 1992b), therefore, there are no guidelines for levels of similarities at which varietal distinctions can be made.

M. cookei was represented by clusters between H154 to A125 (Fig.3-48). Most electrophoretic phenotypes belonged to cluster 2 bounded by A155 and A125 which is divided into two subgroups (or subclusters 2a and 2b). Cluster 1 is bounded by H154 and H223. Of the "-" mating types (H154, H30, A107, H219, H173 and A32), four had similar electrophoretic phenotypes except isolates H173 and A32 which were more closely related and therefore grouped in the same subcluster (2a) while H154, H30, A107 and H219 were grouped in cluster 1.

Analysis of *M. canis* revealed three main divisions (i) cluster 3 (ii) cluster 4 and (iii) cluster 5. The two *Diheterospora* isolates had a single line for 28AD and 32HD, cluster 6.

Clinical Distribution.

Cluster 3 of the *M. canis* isolates comprised a subcluster 3a with mostly clinical isolates from infected human cases (Fig.3-49). The grouping of this subcluster was found to be statistically significant using a *Z*-test ($Z = 2.731 > 1.96$; $P \leq 0.05$). This indicates a nonrandom association, which

suggests that the isolates may have some isozyme (biochemical) characteristics which allow them to be grouped together (? existence of special virulence factor(s)). Cluster 4, subcluster 4c had a number of clinical isolates, but this was not significant ($Z = 1.405 < 1.96$; $P \geq 0.05$). This may be a different cell line of clinical isolates.

Distribution According to Host.

The isolates from clinical cases grouped in the subcluster 3a were mainly from humans, the others from infected cats and dog were scattered in the subclusters 4b and 4c. Although the clustering seems to indicate isolates "specialised" for human infection, the other clinical isolates outside cluster 3 means that infection can also be caused by other outside isolates from outside the cluster 3. Since the majority of isolates from cats were from carriers, it is not clear from this study whether significant clustering based on clinical and carrier status is a common phenomenon with *M. canis*.

Geographical Distribution.

The isolates in cluster 3 represent all the three geographical regions, which implies some form of dispersal or intermigration operating to allow presence of the same or closely related phenotypes in the three regions. Cluster 4 is mostly of isolates from Palmerston North (PN) with a few from Auckland (AK) and Wellington (WG). This suggests some inter-geographical migration of especially the clinical isolates. Similarly, cluster 5 had one isolate from Wellington grouped together with the tester strain RV88 obtained from the Prince Leopold Institute for Tropical Medicine. The clustering this tester strain (RV88) is interesting in that although it is distinctly different from the New Zealand isolates, with only the one similar isolate (WG31) the other tester strain (RV87) is however, similar to the New Zealand isolates. The emphasizes the possibility of some isolates being clones.

CONCLUSION

The findings reported in this thesis confirm the importance of cats and to a lesser extent dogs and contaminated fomites as important reservoirs not only of *M. canis* but also of other potentially pathogenic fungi. A variety of potential opportunistic pathogens e.g. *Fusarium*, *Mucor*, *Penicillium* and *Paecilomyces* were isolated from both cats and dogs and also from soil. One noteworthy fungus isolated from the soil was *Conidiobolus coronatus* which can cause entomophthoromycosis.

To minimise human infections by pathogenic and potentially pathogenic fungi, it would be necessary to raise awareness and educate the public on the role of cats, dogs and soil as reservoirs of these fungi. This is especially in view of a growing passion for animals by people.

The enzyme assays conducted showed that although proteolytic activity was variable among the *M. canis* isolates, there was consistently higher proteinase expression in shake cultures compared to stationary cultures. Similarly, keratinase activities of isolates was greater in shake cultures. However, elastase activity was higher in stationary cultures. *M. cookei* isolates also had higher proteinase expression in shake cultures but showed no significant difference for elastase activity between the two methods. It is postulated that shake cultures of *M. canis* more closely resemble the *in vivo* growth of the fungus and thus the differences in enzyme expression are important in our understanding of differences in mechanisms of pathogenicity.

Enzyme inhibitors studies revealed that both *M. canis* and *M. cookei* expressed serine-catalysed proteinase(s), and metallo-elastinolytic proteinase(s) but aspartic-, cysteine- and metallo-catalysed proteinase(s) were expressed only by *M. canis*. Perhaps, these proteinases act as virulence factors in the hydrolysis of skin proteins.

Gelatin/SDS-PAGE also detected more proteinases in shake cultures than stationary cultures of *M. canis* isolates. Of the six proteinases detected, those of M_r 122 KDa, 62 KDa and 28 KDa were very highly

expressed (P=0.0001) in shake cultures. *M. cookei* expressed more proteinases in stationary cultures than shake cultures. The enzyme expression by these two *Microsporum* spp. may be a reflection of their differing ecological niches. It is proposed that the highly expressed proteinases of M_r 122 KDa, 62 KDa and 28 KDa (probably 62 KDa) may play an important role in the pathogenicity of dermatophytoses caused by *M. canis*.

An understanding of the biological and biochemical mechanisms of disease may suggest the types of molecules needed for drugs. For rational design of chemotherapeutic agents, proteinase inhibitors can be developed by (a) characterisation of specificities of proteinases and (b) synthesizing peptides with similar features but with a hydrolyzable amide bond replaced with a non reactive "isostere". This approach has been used for renin inhibitors and for inhibitors for HIV-1 proteinase (Kunitz, 1992). One can go further and add specific moieties, such as chloromethyl ketones or phosphonates capable of forming transition-state analog complexes with the enzyme. Example are the *Schistosoma mansoni* cercarial elastase (Cohen *et al.*, 1991) and carboxypeptidase A (Kaplan *et al.*, 1991).

In the population studies based on isozyme patterns reported, *M. canis* isolates had a higher diversity than those of *M. cookei*. The detection of mostly weakly fertile and unfertile isolates among *M. cookei* may mean low levels of sexual reproduction in nature and therefore low phenotypic variation. The apportionment of total diversity of *M. canis* according to geographical regions revealed a substantial phenotypic variability within local populations and a moderate interpopulation differentiation.

The main deficiencies with respect to the study of the populations are small sample sizes from Auckland and Wellington, very few isolates from dogs, a slightly higher number of isolates from carriers and a lack of knowledge about the genetics of a number of bands. But it is possible to draw broad tentative conclusions, (a) there is a high level of variation within populations (as defined by geographical regions) and (b) there is a lack of sharp difference between the regions.

With the help of cluster analysis on the isozyme data using normalised percent disagreement as a distance measure and a dendrogram computed by the average-linkage method of clustering, there was a significant subclustering of human isolates (subcluster 3a) while other isolates from infected sources were scattered throughout the subclusters of cluster 4.

When the data for the clinical isolates was examined, they had in common EST phenotype 9. Although EST was present among some isolates from carriers, it was more commonly associated with clinical isolates, which also had a low phenotypic diversity at EST (H =0.150) compared to carriers with a higher diversity (H = 0.764). The low EST diversity among clinical isolates means the isolates have an EST which is closely related. If EST is important in pathogenicity, it could be used in the breakdown of the inhibitory fatty acids found in sebum and therefore offer clinical isolates an advantage in the process of pathogenesis. The fact that esterases have overlapping substrate specificities would make them good candidates in overcoming this defence barrier of the host leading to infection. This aspect would be worthy of further investigation.

APPENDIX A

Microsporum canis isolated and submitted from different sources.

Nº	Host	Description	Source	Isolate
9735	kitten	female	SPCA	*M.canis*
331	cat	male, adult	SPCA	*M.canis*
4261	cat	Infected	Vet. Clinic	*M.canis*
4487	cat	-	Vet. Clinic	*M.canis*
457	cat	female, stray	SPCA	*M.canis*
551	cat	female, 1yr	SPCA	*M.canis* (*M.cookei*)
511	cat	male, 6mths	SPCA	*M.canis*
4484	dog	-	Vet. Clinic	*M.canis*
767	cat	1 yr	SPCA	*M.canis*
768	cat	1yr	SPCA	*M.canis*
733	cat	male	SPCA	*M.canis*
795	cat	female	SPCA	*M.canis*
730	cat	stray	SPCA	*M.canis*
729	cat	adult	SPCA	*M.canis*
790	cat	female	SPCA	*M.canis*
794	cat	female, 8yrs	SPCA	*M.canis*
745	cat	adult, stray	SPCA	*M.canis*
761	cat	stray	SPCA	*M.canis*
797	cat	male, 5mths	SPCA	*M.canis*
Blkt2	-		SPCA	*M.canis*
852	cat	male	SPCA	*M.canis*
854	cat	female, 8mths	SPCA	*M.canis*
839	cat	adult, stray	SPCA	*M.canis*
862	cat	male, 6mths	SPCA	*M.canis*
855	cat	female	SPCA	*M.canis*

APPENDIX A Contd.

2	dog	-	SPCA	*M.canis*
mb0014	cat	+ve ectothrix infection	Hkwhitu Clinic	*M.canis*
mb28	cat	-	SPCA	*M.canis*
989	cat	male, 8wks	SPCA	*M.canis*
983	cat	stray, adult	SPCA	*M.canis*
991	cat	adult, stray	SPCA	*M.canis*
993a+b	cat	male, adult	SPCA	*M.canis*
1001	cat	female, adult	SPCA	*M.canis*
1002	cat	stray, 1yr	SPCA	*M.canis*
995	cat	adult	SPCA	*M.canis*
397437	human	leg infection	Wgtn	*M.canis*
397437	human	face infection	Wgtn	*M.canis*
385195	human	face infection	Wgtn	*M.canis*
x5	human	infection	Wgtn	*M.canis*
x2	human	infection	Wgtn	*M.canis*
?(mb35)	human?	source unkwn	Wgtn	*M.canis*
5014	dog	-	Vet. Clinic	*M.canis*
85154	cat	-	CDCNZ Wgtn	*M.canis*
90359	girl	2yrs	Waipukurau	*M.canis*
295	human		Akld hosp.	*M.canis*
773	human		Akld hosp.	*M.canis*
399svb	human		Akld hosp.	*M.canis*
628	human		Akld hosp.	*M.canis*
760	human		Akld hosp.	*M.canis*
211	human		Akld hosp.	*M.canis*

Akld hosp. = Auckland hospital, Wgtn = Wellington, Vet. Clinic = Veterinary Faculty Clinic, Massey University, CDCNZ = Communicable Disease Center, New Zealand, SPCA = Society for the Prevention of Cruelty to Animals, 993a = no pigment (cream white), 993b = yellow pigment was produced.

PPENDIX A Contd.

) *M. cookei* Isolates from Different Sites in Palmerston North.

Nº	Location	Land use	Stock	pH	Isolate
8	Fielding rd	cleared grd	-	6.2	A= *M. cookei*
29	Square	flower/gdn	-	5.6	
30	Milverton	roadside	-	6.3	H= *M. cookei*
32	Fergusson st	park		5.9	A= *M. cookei*
39	Freyberg	school	-	6.2	A= *M. cookei*
81	Albert st	park	-	6.1	A= *M. cookei*
107	Fergusson st	school		5.9	A= *M. cookei*
125	Stacks/Mana place	residential empty plot	-	6.7	A= *M. cookei*
152	Cuba st	A+Pshowgrds	-	6.3	A= *M. cookei*
153	Cuba st	A+Pshowgrds	-	5.1	H= *M. cookei*
154	Pascal st	empty plot	-	5.6	H= *M. cookei*
155	Pascal st	empty plot	-	6.0	A= *M. cookei*
173	Botanical rd	school	-	5.3	H= *M. cookei*
192	Chippendale Crescent	park	-	6.7	H= *M. cookei*
219	Park rd	park	-		H= *M. cookei*
223	Kimbolton rd	roadside	-		H= *M. cookei*
228	Oroua river	riverbank	-		H= *M. cookei*

e:- S33 (*M. cookei*) was from Massey University Departmental Culture Collection.

A = isolates obtained from antibiotic moistened soils.

H = isolates obtained from water only moistened soils.

APPENDIX B

Microsporum spp. deposited in Microbiology & Genetics Departmental Cultures Collection.
(Freeze-dried in 20% skim milk).

Microsporum spp.	Original Code	Laboratory Code	Culture Collection Nº
Nannizzia otae(+)	RV42487	RV87	836
Nannizzia otae(-)	RV42488	RV88	820
Arthroderma simii(+)	MY03684	-	818
Arthroderma simii(-)	MY03784	-	824
M. canis	331	PN01	794
M. canis	457	PN02	819
M. canis	511	PN03	858
M. canis	551	PN04	817
M. canis	4261	PN05	843
M. canis	4484	PN06	822
M. canis	4487	PN07	823
M. canis	9735	PN08	800 (775)
M. canis	85154	WG09	797
M. canis	90359	WG10	825 (772)
M. canis	729	PN11	840
M. canis	730	PN12	826
M. canis	733	PN13	787 (773)
M. canis	745	PN14	783 (745)
M. canis	761	PN15	786
M. canis	790	PN16	805
M. canis	795	PN17	784 (795)
M. canis	797	PN18	831
M. canis	0767	PN19	801
M. canis	0768	PN20	847
M. canis	blkt2	PN21	782
M. canis	839	PN22	799

APPENDIX B contd.

M. canis	852	PN23	813
M. canis	854	PN24	842
M. canis	855	PN25	844
M. canis	862	PN26	789
M. canis	dog2	PN27	855
M. canis	mb28	PN28	828
M. canis	mb00014	PN29	859
M. canis	397437-leg	WG30A	832
M. canis	397437-face	WG30B	785
M. canis	385195	WG31	816
M. canis	397438	WG32	791
M. canis	no code(x5)	WG33	851
M. canis	no code(x2)	WG34	810
M. canis	no code	WG35	815
M. canis	no code	UK36	811
M. canis	983	PN37	806
M. canis	991	PN38	857
M. canis	993A	PN39A	830
M. canis	993B	PN39B	807 (814)
M. canis	995	PN40	829
M. canis	989	PN41	834
M. canis	1002	PN42	835
M. canis	1001	PN43	790
M. canis	5014	PN44	827
M. canis	295	AK45	852
M. canis	773	AK46	848
M. canis	399svb	AK47	856
M. canis	628	AK48	849
M. canis	760	AK49	850
M. canis	211	AK50	846

APPENDIX B contd.

M. cookei	08	H08	792
M. cookei	30	H30	802
M. cookei	32	A32	837
M. cookei	33	S33	838
M. cookei	39	A39	803
M. cookei	81	A81	845
M. cookei	107	A107	839
M. cookei	125	A125	833
M. cookei	152	A152	841
M. cookei	153	A153	798
M. cookei	154	H154	853
M. cookei	155	A155	812
M. cookei	173	H173	808
M. cookei	192	H192	795
M. cookei	19	H219	772 (793)
M. cookei	23	H223	773 (796)
M. cookei	28	H228	821
M. cookei	551	C551	788
Diheterospora	28	AD28	804
Diheterospora	32	HD32	809

APPENDIX C

The enzyme names, symbols and Enzyme Commission (EC) numbers used are those recommended by the International Union of Biochemistry (IUBNC, 1984).

The isozymes used in this study are 1 to 8.

Enzyme	Symbol	EC Number
1. Catalase	CAT	1.11.1.6
2. Esterase	EST	3.1.1.x
3. Glucose-6-phosphate dehydrogenase	G6P	1.1.1.49
4. Glucose-6-phosphate isomerase	GPI	5.3.1.9
5. Leucine aminopeptidase	LAP	3.4.11.1
6. Malate Dehydrogenase	MDH	1.1.1.37
7. Peptidase (Leu-Ala)	PEP	3.4.11.x.13
8. Peroxidase	PER	1.11.1.7
9. Glutamate dehydrogenase ($NADPH^+$)	GTDHP	1.4.1.4
10. Alkaline phosphatase	ALP	3.1.3.1
11. Acid phosphatase	ACP	3.1.3.2
12. Diaphorase	DA?	1.6.4.3
13. Superoxide dismutase	SOD	1.15.1.1
14. α-glucosidase	α-GLUS	3.2.1.20
15. Lactate dehydrogenase	LDH	1.1.1.27
16. Urease	URE?	3.5.1.5
17. Malate dehydrogenase (NADP+)	MDHP	1.1.1.40
18. Alcohol dehydrogenase	ADH	1.1.1.1
19. (S)-2-Hydroxy-acid oxidase	HAOX	1.1.3.15
20. Carbonic anhydrase	CA	4.2.1.1

APPENDIX C Contd.

21. Aspartate aminotransferase	AAT	2.6.1.1
22. Shikimic acid dehydrogenase	SKDH	1.1.1.25
23. Succinate dehydrogenase	SUDH	1.3.99.1
24 inorganic pyrophosphatase	PP	3.6.1.1
25. Phenol oxidase	PO?	?
26. Thiolsulfate sulfur transferase	TST	2.8.1.1
27. Aryl sulfatase	ARS	3.1.6.1
28. Purine nucleose phosphorylase	PNP	2.4.2.1

isozyme 1-8 gave good enzyme activity staining and were used in this study for indexing allelic variation among the different isolates.

9 showed no variation between species and was therefore not included in the study.

10 could not be successfully resolved with the buffers shown in appendix D.

11 only *Diheterospora* spp. showed activity for this isozyme.

12-13 showed some enzyme activity but the intensity of staining was very poor.

14-28 no enzyme activity was detected on the gels.

APPENDIX D

Buffer systems which were trialled to resolve the alkaline phosphatase enzyme

Enzyme	Electrode buffer	Gel buffer	V/mA
ALP	0.41M citric acid (pH 6.0) adjusted with NaOH	Tris-succinic acid (pH6.0) 0.0025M succinic acid, 0.004M tris	150v
	Tris-citrate (pH 7.0) 0.55M Tris, 0.043M citric acid	Tris-citrate (pH 7.0) electrode buffer diluted 1:15ml	25mA
	Tris-maleic anhydride (pH7.4) 12.1g Tris 11.60g maleic anhydride 3.74g EDTA 2.03g $MgcL_2.6H_2O$ in 1 litre, adjusted with NaOH.	Tris-maleic anhydride diluted 1:9 (pH 7.4)	100v
	Borate (pH 8.0) 0.3M boric acid adjusted with NaOH	Tris-citrate (pH 8.6) 0.076M Tris, 0.007M citric acid,	100v
	Lithium-borate(pH 8.1) 0.06M LiOH 0.03M H_3BO_3	Tris-citrate (pH 8.5) 0.03M Tris 0.005M citric acid	150v

APPENDIX D Contd.

Borate (pH 8.2) 18.55g boric acid in 1 litre	Tris-citrate (pH 9.5) 13.1g Tris 0.525g citric acid in 500ml	150v
Borate (pH 8.5) 18.55g boric acid in 1 litre	Tris-EDTA (pH 8.5) 4.6g Tris 0.09g EDTA in 1 litre	40mA
Tris-citrate (pH 6.3) 12.36g Tris, 11.66g citric acid in 1 litre	Tris-citrate (pH 7.0) 0.73g Tris 0.63g citric acid in 1 litre	250v

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