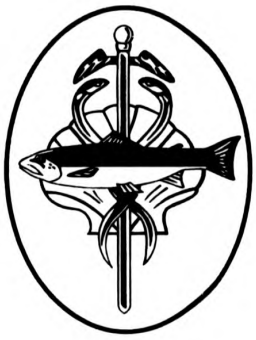

Studies on the Comparative Biology of *Aphanomyces invadans*

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BSc (Hons) Marine Biology, MSc Applied Fish Biology

Thesis submitted to the University of Stirling for the Degree of Doctor of Philosophy

June 1997

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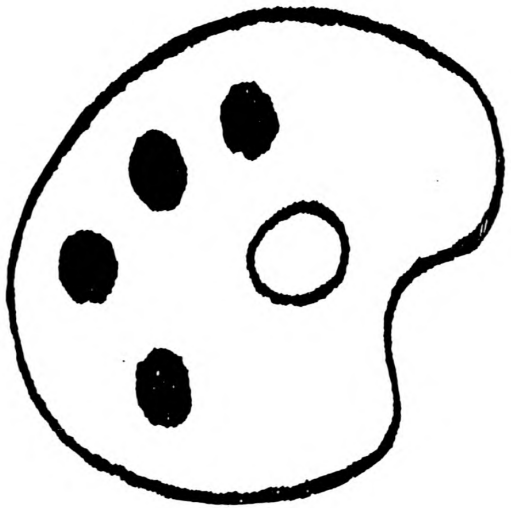

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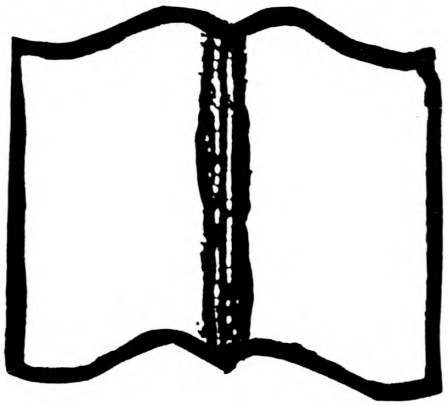
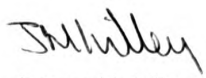


to my parents

Declaration

The work described in this thesis was undertaken by myself. No part of this work has been previously submitted for any other degree or qualification.



James H. Lilley

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ABSTRACT

Aphanomyces invadans Willoughby *et al,* 1995 (as *A. invaderis*) is the recently-named Oomycete fungus that has been shown to be involved in EUS (epizootic ulcerative syndrome), a highly damaging disease of wild and cultured, Asian freshwater and estuarine fishes. The present study shows that *A. invadans* is the only species, out of a number of isolates from EUS-affected areas in Thailand, that is capable of sustained growth in snakehead fish muscle tissue and reproducing EUS lesions, and is therefore pathognomic to the disease. *A. invadans* is characterised, and distinguished from the saprophytic isolates, by means of: growth at various temperatures; growth on different media; level of extracellular enzymes produced; susceptibility to various chemicals; aspects of zoospore and germling behaviour; ultrastructure; immunocytochemistry; protein and carbohydrate electrophoresis banding patterns; lectin and polyclonal antibody binding characteristics by means of Western blot analysis; biochemical fingerprinting using pyrolysis mass spectra (PyMS); and molecular studies involving random amplification of polymorphic DNA (RAPD).

A. invadans is shown to be indistinguishable from pathogenic *Aphanomyces* isolates from two other fish diseases, namely Japanese mycotic granulomatosis (MG) and Australian red spot disease (RSD) using the techniques described above. RAPD analyses, in particular, showed that a wide range of EUS, MG and RSD isolates are not only conspecific, but probably constitute a single genetic clone. This strongly suggests that it is *A. invadans*, and not any other biological aetiology, that has spread across Asia causing ulcerative disease in fish. It is recommended that the name *A. invadans* is used to describe all EUS, MG and RSD pathogenic isolates. This work also shows that *Aphanomyces* isolates obtained from outbreaks of ulcerative mycosis (UM) of American menhaden, are distinct from *A. invadans*, and more similar to the saprophytic fungus *Aphanomyces laevis*. It is conjectured that the invasive UM pathogen has not been studied and that this may show greater similarity to *A. invadans*. In comparison to the other species tested, *A. invadans* is most similar to the crayfish plague fungus,

Aphanomyces astaci, although *A. invadans* is shown to be unable to infect noble crayfish (*Astacus astacus*).

Snakeheads (*Channa striata*) are shown to produce antibodies in response to infection by *A. invadans*, a finding which may have implications for the possible future development of vaccines. *A. invadans* is shown to be culturally and ultrastructurally less robust, and more susceptible to chemical treatment, than other saprolegniacean fungi tested, indicating that strategic water treatments, before fish are infected, should be a relatively effective means of control. It is argued that the culturally-fastidious nature of *A. invadans* could also indicate an inability to compete with natural saprophytes, that may act to restrict it to a pathogenic lifestyle. Possible adaptations of zoospores to pathogenicity include particular chemotactic behaviour; a capability for limited polyplanetism in the presence of a nutrient background, indirect germination, and a form of abbreviated life-cycle. An usually thin zoospore cyst wall, that appears to lack much of the encystment vesicle-derived material apparent on other saprolegniaceans, is believed to have some significance to the ecology of *A. invadans*, although what this may be is undetermined. Despite the obvious ability of *A. invadans* to degenerate muscle tissue in fish, cultures showed relatively low production of extracellular enzymes using agar diffusion techniques, indicating that protease activity may be induced.

A. invadans zoospores and cysts have distinctive lectin-binding characteristics, and of particular interest is their ability to cross-react with monoclonal antibodies raised against *Phytophthora cinnamomi,* a non-saprolegniacean Oomycete. Other features of *A. invadans* that may provide useful species-specific taxonomic markers include temperature-growth characters, a putative K body organelle with a distinctive substructure, specific electrophoretic bands, pyrolysis mass spectra (used here for the first time in Oomycete systematics), and RAPD fingerprints. Polyclonal antibodies (PAbs) proved very non-specific, but peroxidase- and fluorescein- conjugated PAbs provided an effective diagnostic tool for identifying hyphae in infected fish tissue.

ABBREVIATIONS

AAHRI -	Aquatic Animal Health Research Institute, Thailand
ACIAR -	Australian Centre for International Agriculture Research
AHA -	peanut agglutinin (*Arachis hypoaea*)
APW -	autoclaved pond water
ATCC -	American Type Culture Collection
BFAR -	Bureau of Fisheries and Aquatic Resources, Philippines
BSA -	bovine serum albumin
BS-1 -	*Bandeiraea simpicifolia* agglutinin
CDA -	Czapek Dox agar
CMA -	cornmeal agar
Con A -	concanavalin A (*Canavalia ensiformis*) lectin
CVA -	canonical variate analysis
DAB -	3',3-diaminobenzidine tetrahyrochloride
DAPI -	4',6-diamidino-2-phenylindole
DFID -	Department for International Development (new name of ODA)
ECA -	coral tree agglutinin (*Erythrina cristagalli*)
ECP -	extracellular products
EDTA -	ethylenediaminetetraacetic acid
ERA -	EUS-related *Aphanomyces* sp (Lumanlan-Mayo *et al*, 1996)
EUS -	epizootic ulcerative syndrome
FITC -	fluorescein isothiocyanate
FMA -	snakehead fish-meat-extract agar
FME -	fish meat-extract (Hatai *et al*, 1977)
g -	gramme(s)
GMA -	soybean agglutinin (*Glycine max*) = SBA
GP -	glucose-peptone medium
GP-PenOx -	glucose-peptone-penicillin-oxolinic acid medium
GP-PenStrep -	glucose-peptone-penicillin-stretomycin medium
GPY -	glucose-peptone-yeast medium
GY -	glucose yeast medium (Dykstra *et al*, 1986)
H&E -	haemotoxylin and eosin
HCA -	hierarchal cluster analysis
HGA -	horse gram agglutinin (*Dolichos biflorus*)
HRP -	horseradish peroxidase
HSW -	high salt wash buffer
ICBN -	International Code of Botanical Nomenclature
IFAT -	indirect fluorescent antibody technique
IgG -	immunoglobulin G
IHC -	immunohistochemistry
IMI -	International Mycological Institute
IOA -	Institute of Aquaculture, Stirling University
ITS -	internally transcribed spacer
kDa -	kilodalton
LEA -	tomato agglutinin (*Lycopersicon esculentum*)
LSW -	low salt wash buffer
MAb -	monoclonal antibody
MAFF -	Ministry of Agriculture, Fisheries and Food
MEA -	malt extract agar

MG -	mycotic granulomatosis
MIC -	minimum inhibitory concentration
min -	minute(s)
MLT -	minimum lethal time
n/a -	not applicable
NACA -	Network of Aquaculture Centres in Asia and the Pacific, Bangkok
NIFI -	National Inland Fisheries Institute, Bangkok
NSW -	New South Wales, Australia
OD -	optical density
ODA -	Overseas Development Administration (recently renamed DFID)
PAb -	polyclonal antibody
PAS -	periodic acid - Schiffs
PBS -	phosphate buffer saline
PCR -	polymerase chain reaction
PDA -	potato dextrose agar
PG-1 -	peptone-glucose-1 medium
PMSF -	phenylmethylsulphoxylfluoride
PyMS -	pyrolysis mass spectrometry
RAPD -	random amplification of polymorphic DNA
RFLP -	restriction fragment length polymorphism
rRNA -	ribosomal RNA
RSD -	red spot disease
SAPU -	Scottish Antibody Production Unit, Carluke, Scotland
SBA -	soybean agglutinin (*Glycine max*) = GMA
SDA -	Sabouraud dextrose agar
SDS-PAGE -	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAFDEC -	SE Asian Fisheries Development Centre, Iloilo, Philippines
sec -	second(s)
SEM -	scanning electron microscope
TBS -	Tris buffered saline
TEM -	transmission electron microscope
TTBS -	Tween 20-Tris buffered saline
UEA-1 -	gorse seed agglutinin (*Ulex europaeus*)
UM -	ulcerative mycosis (menhaden disease)
WGA -	wheat germ agglutinin (*Triticum vulgaris*)
xg -	times gravity
Ø -	diameter

CONTENTS

LIST OF PUBLICATIONS

Lilley, J.H. and Roberts, R.J. (1997) Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. Journal of Fish Diseases 20, 135-144.

Lilley, J.H. and Inglis, V. (1997) Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi. Aquaculture Research 28(6), 461-469.

Lilley, J.H., Thompson, K.D. and Adams, A. (1997) Characterisation of *Aphanomyces invadans* using electrophoretic and Western blot analysis. Diseases of Aquatic Organisms. (in press)

Thompson, K.D., Lilley, J.H., Chinabut, S. and Adams, A. (1997) The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis*. Fish and Shellfish Immunology (in press)

Lilley, J.H., Beakes, G.W. and Hetherington, C.S. (1997) Characterization of *Aphanomyces invadans* using pyrolysis mass spectrometry. Mycological Research (submitted)

Lilley, J.H., Hart, D., Richards, R.H., Roberts, R.J., Cerenius, L. and Söderhäll, K. (1997) Pan-Asian spread of single fungal clone results in large scale fish-kills. Veterinary Record 140, 11-12.

Lilley, J.H., Cerenius, L. and Söderhäll, K. (1997) RAPD evidence for the origin of crayfish plague outbreaks in Britain. Aquaculture (submitted)

CHAPTER 1. GENERAL INTRODUCTION: BACKGROUND TO EUS AND OTHER SIMILAR DISEASES

1.1 Introduction

Epizootic ulcerative syndrome (EUS) has been the cause of large-scale fish-kills in culture and capture fisheries in Southeast Asia and the subcontinent region for over 15 years (Chinabut, 1995). Outbreaks have been particularly severe in rice-field fish, an important dietary component of many rural communities. The nature of the spread of EUS across Asia has always indicated that an infectious biological agent was the cause, and since the early studies, a number of viruses, bacteria, fungi and parasites have been recovered from affected fish (reviewed by Frerichs, 1995; Boonyaratpalin, 1989; Roberts, Willoughby and Chinabut, 1993; and Tonguthai, 1985). In recent years a slow-growing, thermolabile fungus of the genus *Aphanomyces* has been shown to be capable of migrating through the tissues of susceptible fish and reproducing the characteristic EUS lesions (Roberts *et al*, 1993). At the time of the start of this study, EUS was defined at an ODA-sponsored regional seminar in Bangkok as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response" (Roberts, Campbell and MacRae, 1994a). Soon after, Willoughby, Roberts and Chinabut (1995) named the fungus *Aphanomyces invaderis*. This name will be changed to *A. invadans* in the forthcoming edition of the Index of Fungi [1997, 6(13): 706], and it is this name that is used here.

It was acknowledged at the 1994 meeting that the pathology of two other fish diseases, Japanese mycotic granulomatosis (MG) (Egusa, 1992) and Australian red spot disease (RSD) (Callinan, Paclibare, Bondad-Reantaso, Chin and Gogolewski, 1995a) appeared indistinguishable from EUS. Ulcerative mycosis (UM) (Dykstra, Levine, Noga, Hawkins, Gerdes, Hargis, Grier and Te Strake, 1989) of fish in Western Atlantic estuaries was also recognised as being very similar to the above diseases.

Consequently, EUS research priorities, and the focus of the present study, involved establishing whether *A. invadans* is the only species responsible for the fungus-related pathology of EUS, and if so, to characterise it and compare it with *Aphanomyces* isolates from RSD, MG and UM, and fungi of other aquatic animal mycoses.

1.2 The history of epizootic ulcerative mycoses in the Asia-Pacific region

1.2.1 Mycotic granulomatosis (MG)

The first report of an ulcerative condition similar to EUS came in Summer 1971, in farmed ayu (*Plecoglossus altivelis*) in Oita Prefecture, Japan (Egusa and Masuda, 1971). The characteristic pathology involving a granulomatous response to invasive hyphae was soon recognised and the disease was named mycotic granulomatosis (Miyazaki and Egusa, 1972). It rapidly spread to several parts of the country and affected many species of fish, predominantly cultured ayu and goldfish *(Carassius auratus)*; and wild crucian carp *(Carassius carassius),* bluegill *(Lepomis macrochirus)* and grey mullet (*Mugil cephalus*) (Miyazaki and Egusa, 1972; 1973a; b; c). Significantly, common carp *(Cyprinus carpio)* was not affected. Hatai, Egusa, Takahashi and Ooe (1977a) isolated the invasive fungus from affected fish and subsequently called it *Aphanomyces piscicida* (Hatai, 1980a). Although serious MG epizootics have not been reported in Japan since 1973, outbreaks have continued to occur periodically, and recently Hatai, Nakamura, Rha, Yuasa and Wada (1994) reported a similar disease in imports of ornamental dwarf gourami (*Colisa lalia*) from Singapore.

1.2.2 Red spot disease (RSD)

In 1972, outbreaks of a cutaneous ulcerative condition called red spot disease (RSD) affected estuarine fish, particularly grey mullet (*M. cephalus*), in Queensland, Australia (McKenzie and Hall, 1976). The disease later progressed to coastal rivers in New South Wales (Callinan, Fraser and Virgona, 1989), Northern Territory (Pearce, 1990) and Western Australia (Callinan, 1994a). Seasonal outbreaks continue to occur and the cost to commercial fishermen has been estimated at Aus$ 1 million annually, not

including losses associated with discarded catches and possible long-term declines in fish stocks (Callinan, Sammut and Fraser, 1996a).

An *Aphanomyces* fungus was recovered from diseased fish by Fraser, Callinan and Calder (1992) and was shown to reproduce the disease in fish using bath challenges, but only when the skin of experimental fish was artificially abraded (Callinan, 1994b). Therefore, some other factor was considered to be involved in the disease process. Virgona (1992) showed that RSD outbreaks in estuarine fish in the Clarence river, NSW were associated with lower catchment rainfall and Callinan, Paclibare, Reantaso, Lumanlan-Mayo, Fraser and Sammut (1995b) related this to runoff from acid sulphate soils. Ultrastructural examination of fish gills and skin showed that the low pH and elevated concentrations of monomeric aluminium, representative of estuarine acidification, induces significant lesions in fish (Sammut, Callinan and Cribb, 1996). In aquarium trials, Callinan *et al* (1996a) subsequently induced RSD in fish exposed sublethally to artificially acidified water (at both pH 3 and pH 5) and pathogenic *Aphanomyces* spores, even at low concentrations of monomeric aluminium.

1.2.3 Epizootic ulcerative syndrome (EUS)

Following the outbreaks of MG and RSD, there has been a tangible spread westwards across Asia of a fish condition associated with dermal ulceration and involving large scale mortalities in a number of fish species. The condition was given its present name, epizootic ulcerative syndrome (EUS), in 1986 at the Consultation of Experts on Ulcerative Fish Diseases in Bangkok (FAO, 1986). Outbreaks of EUS have been reported in 18 countries of the Asia-Pacific region, although not all are confirmed EUS according to the Roberts *et al* (1994a) definition. The most recently affected was Pakistan in early 1996 (Figure 1.1).

In 1975-6, an ulcerative disease outbreak, believed to be EUS, occurred in the rivers of Southern Papua New Guinea (Haines, 1983). In 1982-3, there were high mortalities in gudgeon (*Ophieleotris aporos* and *Oxyeleotris heterodon*) from inland areas and

mullet from estuaries in Northern Papua New Guinea (Coates, Nunn and Uwate, 1989). Introduced tilapia (*Oreochromis mossambicus*) are common in these areas, but they proved resistant. Frozen affected fish were later examined by Roberts, Macintosh, Tonguthai, Boonyaratpalin, Tayaputch, Phillips and Millar (1986) and confirmed as pathologically identical to EUS.

In 1980 an epizootic haemorrhagic condition occurred in Java, Indonesia affecting primarily cultured cyprinid and clarid fish, although whether this was EUS is uncertain (Roberts *et al*, 1986). Typically ulcerated snakeheads and catfish have subsequently been reported in the Indonesian states of Sumatra, Sulawesi and Kalimantan (Widagdo, 1990). Invasive hyphae have been identified from sand gobies (*Oxyeleotris marmoratus*) from Eastern Kalimantan (Rukyani, 1994), and D. Bastiawan isolated *A. invadans* from an EUS-affected sand goby from Java in 1993.

Roberts *et al* (1986) discussed unconfirmed accounts of ulcerated walking catfish (*Clarias batrachus*) in Singapore in 1977 and of subsequent occurrences thereafter. Despite Singapore's status as a centre of trade in EUS-susceptible ornamental fishes (Lilley, Phillips and Tonguthai, 1992) there have been no records of high EUS losses to this industry.

An incidence of high fish mortalities occurred in southern peninsular Malaysia in 1979 (Shariff and Law, 1980, cited by Roberts *et al*, 1986), but the first reported typical EUS outbreaks were in December 1980, in rice-field fishes in Northern Malaysia (Jothy, 1981, cited by Roberts *et al*, 1986). These have recurred annually ever since, albeit to a lesser extent (Shariff and Saidin, 1994). Major species affected are snakeskin gourami (*Trichogaster pectoralis*), striped snakehead (*Channa striata*), climbing perch (*Anabas testudineus*) and catfish (*Cl. batrachus*) (Shariff and Saidin, 1994).

Significant, well-documented epizootics have occurred annually in Thailand since 1981 (Ulcerative Fish Disease Committee, 1983; Chulalongkorn University, 1983; 1985; 1987). The second (1982-3) and third (1983-4) outbreaks were particularly devastating as they affected the intensive fish culture systems of central Thailand as well as wild fish in natural waterways. Some of the most severe mortalities were in snakehead (*Ch. striata*) farms and rice-field fish and direct economic losses in 1982-3 alone were estimated at US$ 9 million (Tonguthai, 1985). The original outbreaks started towards the end of the rainy season (September) and persisted throughout the cool season to March. Outbreaks now tend to be restricted to the coolest months of December and January. During the last season (December 1996), EUS was experienced in NE, central and southern provinces (S. Kanchanakhan, pers. comm.). Further discussion of mycological studies carried out in Thailand is presented in Section 1.4.1.

Myanmar, Lao PDR and Cambodia, all bordering Thailand, first reported major outbreaks of EUS in 1983 or 1984 (Lilley *et al*, 1992). Subsequent epizootics were less extensive (eg EUS affected 35 Burmese townships in 1984-85 and 11 townships in 1989-90, Soe, 1990), but given the importance of susceptible fish to rural communities in these countries, the impact continues to be significant.

Several accounts of EUS-affected fish have also come from Vietnam, China and Hong Kong although these are still not validated. The first report of ulcerated snakeheads in Vietnam, and therefore the most likely first occurrence of EUS in that country, came from the Mekong delta in 1983 (Xuan, 1990). Ulcerated *Labeo rohita* were first observed at the Pearl River Fisheries Institute in Guangzhou, South China in 1982 (Lian, 1990). Clarid catfish were affected in the same area in 1987-8 (Lian, 1990) and *Carassius auratus* were reportedly affected over much of Eastern China in 1989 (Guizhen, 1990). Wilson and Lo (1992) reported seasonal mortalities of up to 70% of snakeheads (*Channa maculata*) in late Summer in Hong Kong since 1988.

Laguna de Bay in the Philippines, the largest and most productive lake in Southeast Asia, experienced a serious outbreak of EUS in December 1985. An estimated 5-40% of snakeheads, gobies, gouramies, catfish, crucian carp, *Arius* sp and *Therapon* sp were ulcerated whereas milkfish, bighead carp, and tilapia were unaffected (Llobrera, 1987). The disease continued to spread to at least 11 other provinces affecting wild fish in lakes, rice-fields and swamps and pond cultured fish (Bondad-Reantaso, Paclibare, Lumanlan-Mayo and Catap, 1994). Rejection of ulcerated fish, and reduction in market prices of all fish, significantly reduced the income of fishermen. Mullet, goatfish (*Upeneus bensai*), croaker (*Johnius* sp), *Psettodes* sp and *Scanthophagus argus* in a lagoon in Buguey Province suffered an outbreak in 1990 which was confirmed as EUS by histological examination (Bondad-Reantaso, 1990; S. Chinabut, pers. comm.). These brackishwater and marine species provided an explanation as to how the disease may have spread between the islands. The severity of outbreaks has decreased since 1993. Several *A. invadans* isolates (described as ERA: EUS-related *Aphanomyces* sp by Lumanlan-Mayo, Catap, Paclibare, Callinan and Fraser, 1996) were obtained as described by Paclibare, Catap and Callinan (1994) and provided for the present study. Further studies in the Philippines on bacterial, fungal and environmental involvement in EUS are discussed in other sections.

A major outbreak of EUS in Western Sri Lanka occurred in December 1987, prior to any outbreaks on the subcontinent mainland (Costa and Wijeyaratne, 1989). It is suspected that the disease was imported from Southeast Asia in shipments of infected fish, possibly ornamental angel fish (*Pterophyllum scalare*), which have suffered from high disease-related mortalities (Balasuriya, 1994). Snakeheads with large necrotic ulcers were the most visible sign of the disease, but tilapia, the main commercial species, was not affected. EUS was reportedly still active in Batticaloa lagoon in 1996 (P. Vinobaba and M. Vinobaba, pers. comm.).

In the last few years, EUS has had a serious effect on fisheries throughout the subcontinent, causing losses in important capture fisheries areas and damaging confidence in an aquaculture industry still in the early stages of development. The disease was first reported in the Chandpur district of Bangladesh in February 1988. This first outbreak lasted for 13 months during which time it progressed rapidly throughout the country aided by the flood of September 1988 (Barua, 1994). Ulceration was observed in many wild species, predominantly snakeheads, *Puntius*, *Clarias, Mystus and Mastacembelus.* Cultured Indian major carp were also affected, although mortalities due to the disease were probably restricted to fingerlings (Roberts, Wootten, MacRae, Millar and Struthers, 1989). Direct losses to fisheries during the first outbreak in 1988 were estimated at US$ 3.4 million (Barua, 1990). Extensive local media coverage about the disease at that time fuelled a widespread, but unfounded, fear of possible public health risks from fish consumption, resulting in further losses to fish traders. EUS incidences subsequently declined, but there are reports that, as from 1995, the severity of the disease is again on the increase in Bangladesh (G.U. Ahmed, unpublished report). In January 1993, *A. invadans* was isolated from farmed Indian major carp (*Labeo rohita*) in Northwest Bangladesh and wild fish in the productive flood plain area of Northeast Bangladesh (Plate 1.2).

Incidences of EUS in India have been comprehensively reviewed (Zoological Society of Assam, 1988; Kumar, Dey and Sinha, 1991; Jhingran and Das, 1990; National Workshop on Ulcerative Disease Syndrome in Fish, 1990; ICSF, 1992; Das and Das, 1993; Ninawe, 1993; Mohan and Shankar, 1994; Das, 1994). Discussion of some of the research work presented is given in other sections. The NE Indian states were the first to report losses in May 1988. The disease spread through rivers, reservoirs and paddy fields to most states, affecting some Indian major carp farms as well. EUS had a serious impact on fish in low salinity areas of the rich brackishwater fisheries of Chilka Lake, Orissa in November 1990 (Raman, 1992), and the reservoirs and backwaters of Kerala in June 1991 (Sanjeevaghosh, 1991). Bisht, Bisht, Joshi and Khulbe (1996) also reported serious epizootic mycoses of reservoir fishes in the

Kumaun Himalayas between 1991 and 1994. A number of fungal species were isolated, but no association with EUS was made. To date only Jammu & Kashmir, Punjab and Gujarat have not reported outbreaks of EUS.

Bhutan and the Eastern Terai of Nepal were first affected in 1989 and by 1993, EUS had spread to Himalayan valley regions including Pokhara and Kathmandu where cold water species, including *Tor* spp, were affected (Phillips, 1989; Shrestha, 1994). It is estimated that 20-30% of Nepalese pond fish production (about 3000 mt) is lost every year through EUS (Pantha, unpublished report).

The latest country to be affected by EUS was Pakistan, where EUS was confirmed in snakeheads in Lahore Division, Punjab in April 1996 (Kanchanakhan, 1996a). The blotched snakehead or mud murrel (*Channa punctata*) was the most commonly affected species; *Puntius* spp, *Labeo rohita* and *Cirrhinus reba* were also reportedly affected (N. Akhthar, pers. comm.). An estimated 20% of farms were affected in Sialkot Division, Punjab with the incidence being higher in ponds that were inundated by flooding in 1996 (Callinan, Chinabut, Kanchanakhan, Lilley and Phillips, 1997). Although reported losses have not been high, possibly due to the extensive use of tube-well water for fish farms and elevated salinity in parts of Punjab (Callinan *et al*, 1997), EUS is now established in the upper Indus watershed. As Pakistan has the largest canal system in the world based around the Indus, there are fears of a rapid spread to other areas and potentially serious impacts for the future.

1.3 Species affected

Many species (>100) have been reported to be affected by EUS (Lilley *et al*, 1992), but only relatively few reports have been confirmed by demonstrating the presence of mycotic granulomas in histological section or by isolation of a pathogenic *Aphanomyces* fungus from internal tissues. Table 1.1 lists the fish species in which EUS has been confirmed, including species from MG or RSD outbreaks.

Some authors have commented that the most severely affected species in natural outbreaks are generally bottom dwellers (Llobrera, 1987; Chondar & Rao, 1996) or possess air-breathing organs (Roberts, Frerichs, Tonguthai and Chinabut, 1994b), but examination of Table 1.1 shows that this is by no means always the case.

Cruz-Lacierda and Shariff (1995) found that no particular size group of striped snakeheads appears to be more susceptible, with affected fish ranging from 40g to 900g. A visit to the flood plain fisheries area in NE Bangladesh in 1993, however, revealed a possible variation in susceptibility of either different species or sizes in snakeheads. It was found that striped snakeheads invariably showed severe ulceration, whereas the larger river murrels (*Channa marulia*) only showed petechial haemorrhages or no signs of disease at all (Plate 1.3a). There is also a possibility that size may be significant in other species. For example Indian major carp, and rohu (*Labeo rohita*) in particular, suffer high mortalities as fingerlings (Roberts *et al,* 1989) but larger fish, although appearing ulcerated (Plate 1.4b), have not demonstrated typical mycotic granulomas in muscle tissue, and are not reported as dying in large numbers (Callinan *et al*, 1997).

Reports of MG and EUS *Aphanomyces* isolates capable of experimentally reproducing disease in rainbow trout (*Oncorhynchus mykiss*) at some temperatures (Hatai, 1994; K.D. Thompson, pers. comm.) indicate that EUS may potentially have serious implications for freshwater fisheries and aquaculture activities outside Asia.

Some commercially important species are considered to be particularly resistant to EUS, but few studies have been undertaken to confirm these observations and investigate the mechanism of resistance. Species reported to be unaffected by EUS outbreaks include Chinese major carps, tilapias and milkfish (*Chanos chanos*). Hatai (1994) experimentally injected catfish (*Parasilurus asotus*), loach (*Misgurnus anguillicausatus*) and eel (*Anguilla japonica*) with hyphae of MG-*Aphanomyces* and found them to be refractory to infection. Wada, Rha, Kondoh, Suda, Hatai and Ishii (1996) and Sharifpour (1997) experimentally injected common carp with zoospores of

MG- and EUS-*Aphanomyces* respectively, and demonstrated that fungal growth was suppressed by an intensive inflammatory response. Khan (1997) found that most tilapia (*Oreochromis niloticus*) injected with *A. invadans* zoospores showed very limited pathological signs, but in marked contrast, two moribund tilapias showed extensive fungal growth in muscle tissues and typical associated pathological changes.

Plate 1.1

(a) Publications on EUS from Southeast Asia and South Asia

(b) Fish kill in natural water body in Thailand

a

b

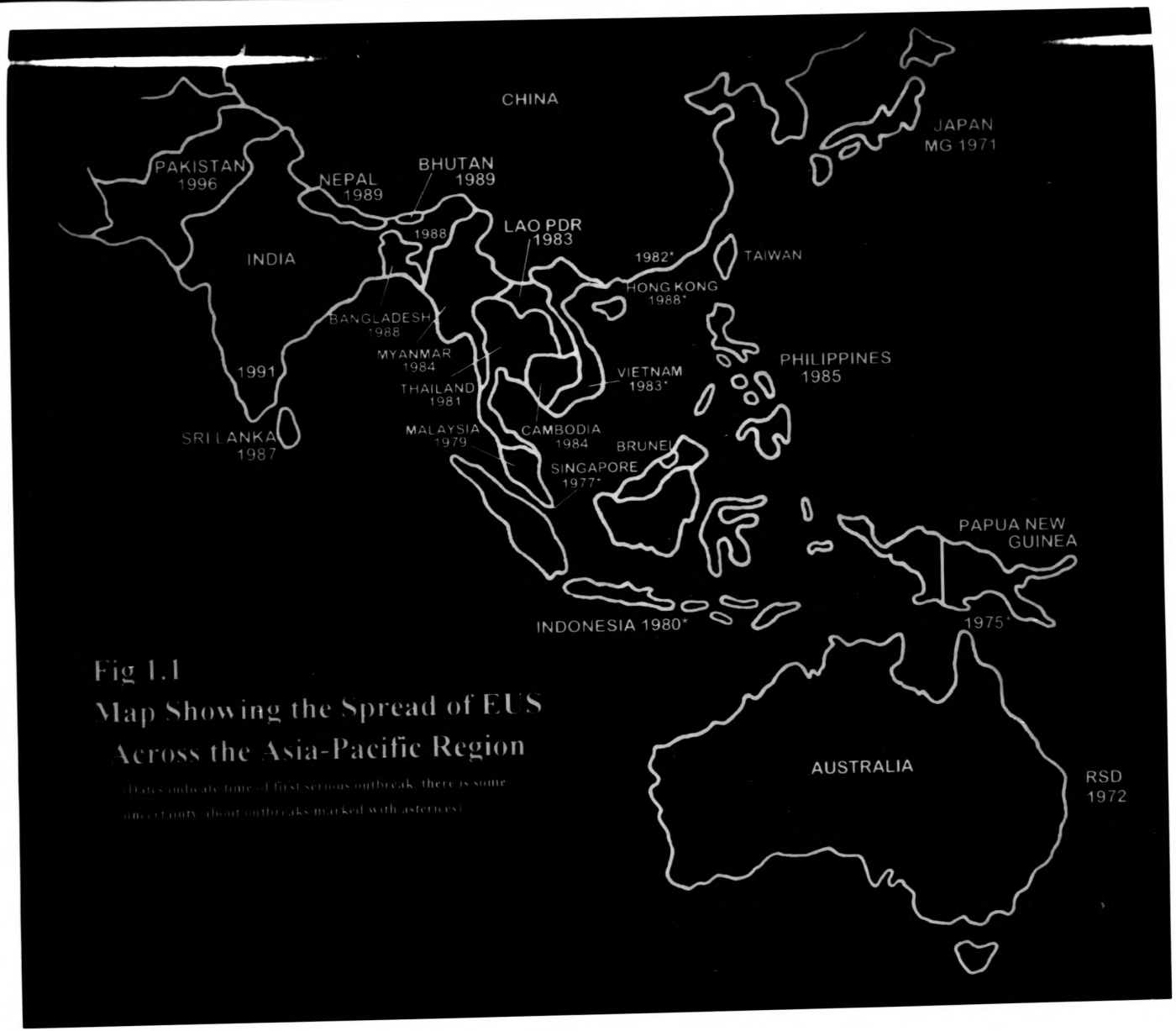


Fig 1.1

Map Showing the Spread of EUS Across the Asia-Pacific Region

(Dates indicate time of first serious outbreak, there is some uncertainty about outbreaks marked with asterisks)

Plate 1.2

(a) EUS-affected fish from an important flood-plain fishery near Srimanagal, Bangladesh

(b) EUS-affected cultured rohu (*Labeo rohita*) juvenile from Bangladesh

a

b

Table 1.1 Species susceptible to EUS (or MG† or RSD‡) as indicated by the presence of typical mycotic granulomas in histological section or isolation of pathogenic *Aphanomyces* from muscle or internal organs (numbers correspond with references given below; *denotes artificial challenge)

***Latin name* (common name)**	Jap†	Aus‡	Ino	Tha	Lao	Mya	Phi	Ban	Ind	Pak	Sco
Acanthopagrus australis (yellowfin bream)		5									
Carassius auratus (crucian carp)	9										
Carassius carassius auratus (gold fish)	9										
*Channa maculata (=Ophicephalus maculatus)*Φ (Formosan snakehead)	9							12			
*Channa marulia (=Ophicephalus marulius)*Φ (river murrel - India)								4			
*Channa micropeltes (=Ophicephalus micropeltes)*Φ (red snakehead)				4							
*Channa punctata (=Ophicephalus punctatus)*Φ (mud murrel - India)								8		7	
*Channa striata (=Ophicephalus striatus)*Φ (striped snakehead)				3*,8	4	4	1		10		
Cirrhina mrigala (mrigal) - fingerlings								12			
Clarias batrachus (walking catfish)							1				
Clarias gariepinus (African catfish)							2*				
Colisa lalia (dwarf gourami)	6										
Fluta alba (swamp eel)				4							
Glossogobius giurus (bar-eyed goby)							1				
Labeo rohita (rohu) - fingerlings								12,8			
Lepomis macrochirus (bluegill)	9										
Liza diadema (mullet)		11									
Mastacembalus armatus (armed spiny eel)								4			
Mastacembalus pancalus (guchi - Bangladeshi)								4			
Mugil cephalus (grey mullet)	9	5					1		10		
Notopterus notopterus (grey featherback)				4							
Oncorhynchus mykiss (rainbow trout) - marginally susceptible	6*										13*
Osphronemus goramy (pla raet - Thai)				4							
Oxyeleotris marmoratus (sand goby)			8								
Oxyeleotris sp (gudgeon)											
Plecoglossus altivelis (ayu)	9										
Puntius gonionotus (silver barb)				4							
Puntius sophore (punti - Bangladeshi)										4	
Puntius sp (puntius)								12	10		
Rhodeus ocellatus (tairiku-baratanago - Japanese)	6*										
Rohtee sp (keti - Bangladeshi)						4					
Scardinius erythrophthalmus (rudd) - marginally susceptible	6*										
Sillago ciliata (sand whiting)		5									
Trichogaster pectoralis (snakeskin gourami)				4							
Trichogaster trichopterus (3-spot gourami)				8							
Tridentiger obscurus obscurus (Japanese trident goby)	9										
Xenentodon cancila (round-tailed garfish)								8			

COUNTRY KEY: Jap = Japan; Aus = Australia; Ino = Indonesia; Tha = Thailand; Lao = Lao PDR; Mya = Myanmar; Phi = Philippines; Ban = Bangladesh; Ind = India; Pak = Pakistan; Sco = Scotland

ΦThe two genera *Channa* and *Ophicephalus* were united as *Channa* by Myers and Shapovalov (1931, cited by Clark, 1991)

REFERENCE KEY:

1 Callinan *et al* (1995a)
2 Callinan (pers. comm.)
3 Chinabut, Roberts, Willoughby and Pearson (1995)
4 Chinabut (pers. comm.)
5 Fraser *et al* (1992)
6 Hatai (1994)
7 Kanchanakhan (1996a)
8 Chapter 2
9 Miyazaki (1994)
10 Mohan and Shankar (1995)
11 Pearce (1990)
12 Roberts *et al* (1989)
13 Thompson (pers. comm.)

1.4 The pathology of EUS, MG, RSD and UM

A brief account of the gross clinical signs and histopathology of EUS is given in Table 1.2 alongside descriptions of MG, RSD and UM, in order to show the similarity between the progression of each of these diseases in fish.

The initial signs of EUS are similar to many other fish disorders, involving reduced appetite, lethargic swimming behaviour, and in the case of snakeheads, swimming with the head out of the water. Petechia and/or small red or grey erosions can rapidly develop and expand into large ulcers, with associated loss of scales, haemorrhaging and oedema. In the case of RSD, Callinan *et al* (1989) commented that erythematous dermatitis lesions and intermediate-type lesions subsequently resolved, whereas necrotising dermatitis lesions consistently developed into dermal ulcers. Healing lesions are scarce among UM-affected fish, however, and Noga, Levine, Dykstra and Hawkins (1988) concluded that that disease was routinely fatal. The most common lesion reported for all of the four diseases described in Table 1.2 is the advanced lesion or dermal ulcer (Plate 1.4a).

Snakeheads are often the most visible sign of an EUS outbreak, as aside from being highly susceptible, they can survive with much more severe, chronic lesions. These may completely destroy the peduncle or erode into the abdominal cavity, sometimes exposing the swim bladder. Head erosion is a common feature of diseased snakeheads and specimens have been found with exposed optic nerves or loosened maxillae and mandibles. A graphic photographic account of naturally and experimentally infected snakeheads is available in Kanchanakhan (1996b).

Callinan *et al* (1989) examined 2560 RSD-affected mullet (*M. cephalus*), each with an average of 1.5 dermal ulcers, and found lesions generally occurred more often in the posterior and dorsal areas of the fish than the anterior and ventral regions. A total of 361 EUS-affected snakeheads (*Ch. striata*) from two separate studies, one in Thailand (Chinabut and Lilley, 1992) (see Plate 1.4a), and one in the Philippines (Cruz-Lacierda and Shariff, 1995) showed that multiple lesions were common and

occurred on all parts of the body, including the head, opercula and fins. The first study showed that dorsal and ventral surfaces were equally affected (80% of fish in both cases); and the second showed a slight preponderance of lesions on the head and towards the posterior sections of the fish. A similar investigation of UM-affected menhaden (*Brevoortia tyrannus*), however, showed that the posteroventral portion of these fish was most frequently affected (67% of all lesions in 424 fish were located here) (Noga *et al,* 1988). It was speculated that this may be due to differences in the immune capability of this part of the fish, or the release of some chemoattractant from the perianal area that stimulates fungal zoospores to attach.

a

b

Plate 1.3

(a) Three striped snakeheads (*Channa striata*) (right) with severe ulceration and three river murrels (*Channa marulia*) only showing petechial haemorrhages

(b) EUS affected striped snakehead with severe secondary mixed fungal infection

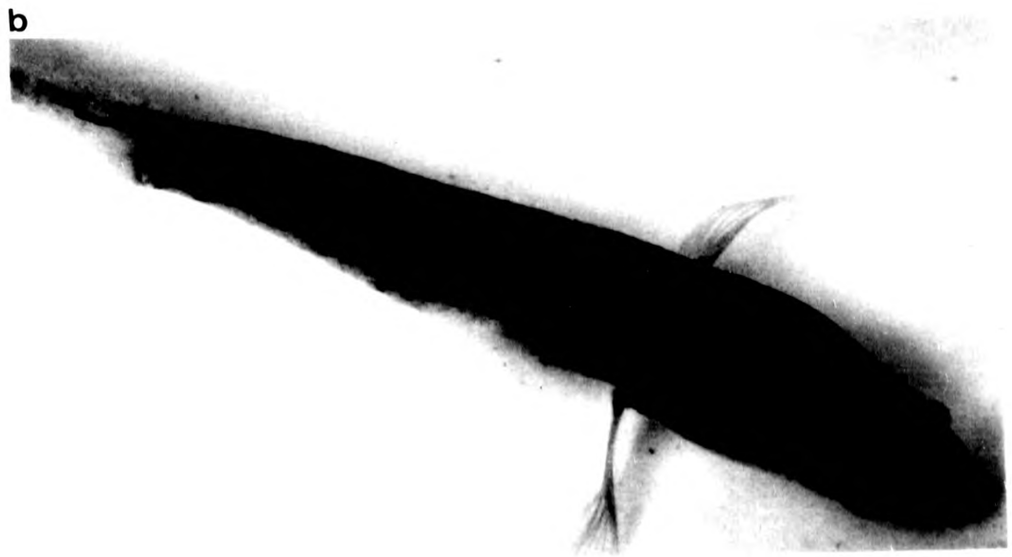


Table 1.2 Classification of lesions in EUS and equivalent descriptions from similar disease conditions

EUS of snakehead (*Channa striata*): Lilley *et al*, 1992	**MG-like disease of dwarf gourami (*Colisa lalia*): Wada, Yuasa, Rha, Nakamura and Hatai, 1994**	**RSD of mullet (*Mugil cephalus*): Callinan *et al*, 1989**	**UM of menhaden (*Brevoortia tyrannus*) Noga *et al*, 1988**
Petechia. Acute dermatitis forming rosacea.		**Erythematous dermatitis**: Yellow skin with irregular reddening. Scales fractured. Usually <10 mm Ø. Epidermis present at margins and irregularly over lesion. Epidermis hyperplastic, oedematous and infiltrated by mononuclear cells. *Stratum spongiosum* mild to severe congestion, oedema and mononuclear cell infiltration. Other tissues normal. No hyphae or granulomas.	
1. Small haemorrhagic surface lesions. Epithelial necrosis with surrounding oedema, haemorrhage of the underlying dermis and some inflammatory cell infiltrate. Few hyphae sometimes seen.		**Intermediate-type dermatitis**: Approx. 10 mm Ø. Epidermis absent over lesion, though sometimes evidence of regeneration, scales usually retained. Mild to moderate chronic active dermatitis with some fungal hyphae and granulomas down to skeletal muscle. Often muscle necrosis.	**Early Type I**: Flat, red or yellow-red area <5 mm Ø. Primarily macrophage response. Aseptate hyphae (7-12 μm Ø) surrounded with small granulomas, appeared to originate near a scale pocket. Fungus invasion associated with necrosis of skeletal muscle and myophagia. Some multinucleated giant cells.
2. Mild epithelial necrosis. Surrounding oedema, haemorrhage of underlying dermis, and inflammatory cell infiltration with severe necrotising myopathy spread over a wide area below the active skin lesion. Hyphae enclosed in epithelioid capsules. No disruption of internal organs.	**Type 1:** Small, discoloured pale areas (3-10 mm Ø) with irregular reddening. Granulomas in *stratum compactum* and muscle, surrounding sparsely- branching aseptate hyphae (8-20 μm Ø). Severe floccular degeneration of skeletal muscle fibres. **=Moderate MG lesion (Miyazaki and Egusa, 1972)**	**Necrotising dermatitis**: yellowish-grey to red, ovoid domed areas (10-40 mm Ø). Epidermis and scales usually absent, dermis swollen and macerated. Few hyphae trailed from lesion (not cotton-wool-like). Moderate to severe, locally extensive, necrotising, granulomatous dermatitis. Large numbers of sparsely-branching, aseptate hyphae (12-18 μm Ø) usually within granulomas down to skeletal muscle. Severe floccular degeneration of muscle.	**Early Type II lesion**: Raised 15-20 mm Ø smooth area with small area of scale loss. Small ulcerated area in centre. Intense inflammation, large coalescing granulomas with necrotic core sometimes identifiable as fungal hyphae.
3. Advanced lesion, with large bacterial ulceration, massive necrotising granulomatous mycosis of underlying muscle involving branching, aseptate Oomycete coated with epithelioid cells. Hyphae may invade abdominal viscera, particularly in the case of major carp (Roberts *et al*, 1989) and spiny eels (Chinabut, 1990) Internal organs other than those infiltrated with hyphae show only mild pathological changes.	**Type 2**: Open ulcers (up to 10 mm Ø) leaving crater-shaped cavity. Large amounts of epidermis and *stratum compactum* sloughed off. Granulomas and hyphae in deeper muscle tissue; commonly throughout the viscera; also within vertebrae and costae; and rarely, hyphae (without cellular response) found in the brain. **=Severe MG lesion (Miyazaki and Egusa, 1972)**	**Dermal ulcer**: About 10-40 mm Ø. Margins sharply defined. Skeletal muscle exposed up to 10 mm below surface. In some cases bone or viscera exposed. Moderate to severe diffuse granulomatous myositis. Hyphae, within granulomas, rarely penetrated internal organs. Some dermal ulcers showed evidence of healing.	**Advanced lesion**: Open ulcers up to 25 mm Ø, containing white friable material consisting of hyphae and necrotic muscle. Hyphae surrounded by intense granulomatous inflammation. Occasional foreign-body-type multinucleated cells. Myophagia and muscle fragmentation often without associated fungus. Often lesions affect viscera or cross to contralateral flank. **End-stage lesion**: Necrotic core sloughed leaving crater-shaped cavity with non-oedematous periphery. Many with large numbers of hyphae, others heavy Gram -ve bacterial infections associated with necrotic cell debris. Some osteoblastic activity. Some mild fibroplasia.
			Healing lesion: <5 mm Ø. Usually overlying peritoneal cavity. Smooth, non-ulcerated areas of tissue loss. Epidermis intact and mildly hyperplastic. Regenerating scales. Mild to moderate granulomatous inflammation. Few hyphae.

KEY: Ø = diameter

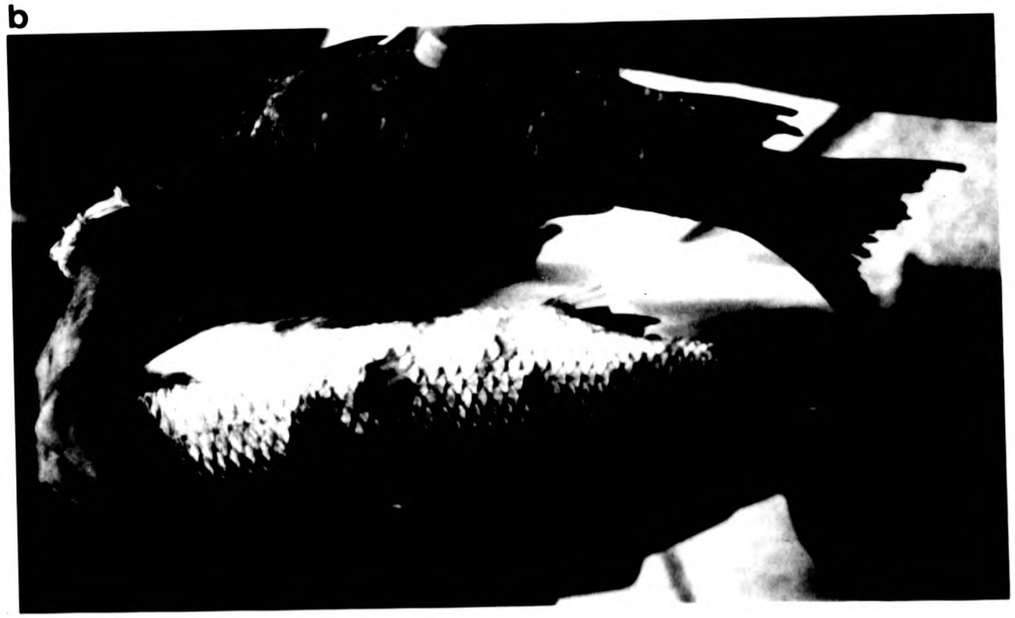
Plate 1.4

(a) EUS-affected snakeheads with multiple dermal ulcers

(b) Adult rohu from Pakistan with dermal ulcers but the fungus does not extend into the muscle

a

b



1.5 Causative agents

Given the nature of the spread of EUS across the region, an infectious biological causative agent has always been suspected. This was confirmed by the successful transmission of EUS to healthy fish by cohabitation with infected fish, feeding of infected material, and exposure to affected water (Balasuriya, Kulathilake and Subasinghe, 1990; Subasinghe and Jayasinghe, 1990; Cruz-Lacierda and Shariff, 1995).

1.5.1 Mycology

Almost since its appearance in Southeast Asia, fungi have been known to be involved in the aetiology of EUS. Limsuwan and Chinabut (1983) described a "severe chronic granulomatous mycosis" in histological sections of lesions from diseased fish in Thailand. At this time *Achlya* and *Saprolegnia* spp were identified from affected fish (Pichyangkura and Bodhalamik, 1983; Limsuwan and Chinabut, 1983) but these were later dismissed as secondary agents (Tonguthai, 1985) (see Plate 1.3b). Roberts *et al* (1993) subsequently isolated the fungus, that was later to be named *Aphanomyces invadans,* from within the muscle of EUS-affected fish in Thailand. It was shown to be slow-growing and thermolabile when compared to other local saprophytic *Aphanomyces,* and when mycelium of isolates RF6 and RF8 were placed below the dermis of striped snakeheads, they were shown to be capable of migrating into the tissues and causing typical EUS lesions (Roberts *et al*, 1993). Chinabut *et al* (1995) further showed that at 26°C and 31°C, challenged snakeheads staged a marked inflammatory response to fungal invasion and by day 14 healing became well established, whereas at 19°C there was only a limited macrophage response and by day 19 all the fish had died. The seasonal nature of EUS has indicated that low temperature is an important factor in the disease process and this study provides an explanation of how it affects the development of lesions.

Nonetheless, as *Saprolegnia* and *Achlya* are commonly observed in squash preparations of EUS lesions (Pichyangkura and Tangtrongpiros, 1985; Ninawe, 1993), and these, along with culturally fast-growing *Aphanomyces* strains, have been

isolated from EUS affected fish (Willoughby and Lilley, 1992; Qureshi, Chouhan, Prasad and Mastan, 1995), Noga (1993a; 1994) called for clinically relevant challenge studies to rule out the possibility that the fungus seen invading tissues is not simply one of any number of environmental opportunists. Such trials were undertaken for this study and are described in Chapter 3.

An account of the characteristics of *Aphanomyces invaderis* (=*A. invadans*) as described by Willoughby *et al* (1995) is given in Section 2.3, along with the published details of the Japanese MG-fungus *(Aphanomyces piscicida)* and the Australian RSD-associated *Aphanomyces*.

Callinan *et al* (1995a) compared RSD-*Aphanomyces* isolates with EUS isolates from the Philippines and found them to be similar in terms of morphological and cultural characteristics and peptide banding profiles. Hatai *et al* (1994) recently isolated an *Aphanomyces* sp from dwarf gourami *(Colisa lalia),* and found it to resemble previous MG isolates, but neither strain has been compared directly with EUS or RSD fungi. Callinan (1994b) and Wada *et al* (1994) have made recommendations for direct comparative studies of the pathogenesis and biological characteristics of the fungi involved in all three diseases. Work described in subsequent sections here attempts to address these recommendations.

1.5.2 Environmental factors associated with EUS

Some EUS-affected areas of Southeast and South Asia have acidic and/or poorly buffered water (eg Udon Thani, NE Thailand and East Pegu, Myanmar: Roberts *et al,* 1986; East Kalimantan, Indonesia: Rukyani, 1994; and North Luzon, Philippines: Callinan *et al,* 1995b). In these cases, the disease process may be similar to that reported for Australian estuaries, with acid-induced dermatitis leading to fungal infection. Statistical studies on EUS outbreaks in Bangladesh have provided further evidence for the importance of pH. These have indicated that routine use of agricultural lime ($CaCO_3$), which is known to stabilise the pH of water, reduces the likelihood of EUS outbreaks (Hossain, Alam and Mazid, 1992; Ahmed and Rab,

1995). However, regional EUS sampling surveys have clearly shown that some outbreaks occurred in areas that did not experience acid water conditions (eg Chiang Mai, N Thailand and Mandalay, Myanmar: Roberts *et al,* 1986; Mymensingh, Bangladesh and Nakorn Ratchasima, NE Thailand: Phillips and Keddie, 1990; and parts of South India: Callinan, Lumanlan-Mayo, Rukyani, and Mohan, 1996b) and in such cases, other pathological agents, as described below, may have a more significant affect.

Relatively low water temperatures, which have been widely associated with EUS outbreaks, are thought to slow the development of the inflammatory response such that fish are unable to prevent extensive fungal invasion (Chinabut *et al*, 1995).

1.5.3 Other pathological agents associated with EUS

Despite overwhelming evidence that the granulomas observed in EUS lesions are formed around fungal hyphae, and that most of the degenerative changes are associated with these hyphae, some workers have attached a causative role in these developments to nocardioform bacteria (Chakrabarty and Dastidar, 1991; Dastidar and Chakrabarty, 1992) or myxosporean parasites (Tika Ram, Chhabra, Batra and Mahipal, 1994; Sanaullah, Hjeltnes and Pittman, 1996). Although there is no reason to doubt the diagnosis of these organisms in some EUS-affected fish, they cannot be considered to be the cause of typical EUS granulomas.

A variety of other bacteria, viruses and parasites have been isolated from EUS-affected fish, some of which may have a role in the progression of EUS, either by facilitating infection by *A. invadans*, or as secondary opportunists.

In areas where acid water does not occur, and therefore the acid-induced dermatitis described by Callinan *et al* (1996a) can not effect EUS, some microbiological, or other environmental agent(s), is probably required to facilitate infection by *A. invadans*. Rhabdoviruses isolated from EUS-affected fish were originally considered candidate

primary pathogens (Frerichs, Millar and Roberts, 1986), but were subsequently found to be incapable of reproducing ulcers in snakeheads (Frerichs, Millar and Chinabut, 1993), and comprised of at least two viral species (Kasornchandra, Engelking, Lannan, Rohovec and Fryer, 1992; Lilley and Frerichs, 1994). However, Kanchanakhan (1996b) showed that rhabdovirus infection resulted in skin damage in juvenile snakeheads, and may be an important precursor to fungal invasion. He reported 100% (17/17) infection of fish injected with a rhabdovirus prior to immersion in *A. invadans* zoospores, whereas only 3/18 fish exposed to spores alone, developed EUS. A birnavirus isolated during the 1983-4 outbreak in Thailand was also shown to be capable of inducing sub-lethal dermatitis in snakeheads (Saitanu, Wongsawang, Sunyasootcharee and Sahaphong, 1986).

Similarly, Subasinghe (1993) showed that EUS transmission to naïve snakehead fry, by feeding with infected material or exposure to affected water, was enhanced by concurrent heavy infestation with *Trichodina*. Skin parasite infestations have been seen to precede EUS outbreaks on several occasions, but the species of parasite varies between localities; *Epistylis* was observed on fish prior to the 1982-3 outbreak in Thailand (Tonguthai, 1985) and significant levels of *Lernea* was reported in the 1996 outbreak in Pakistan (Callinan *et al,* 1997).

Bacteria, and *Aeromonas hydrophila* in particular, have been consistently isolated from EUS ulcers (Llobrera and Gacutan, 1987; Pal and Pradhan, 1990), some strains with particularly virulent (Torres, Shariff and Law, 1990; Suthi, 1991; Karunasagar, Sugumar and Karunasagar, 1995) or cytotoxic (Yadav, Indira and Ansary, 1992) capabilities. Although some bacteria certainly contribute to the development of necrotic ulcers (Lio-Po, Albright and Alapide-Tendencia, 1992), they are generally considered to be secondary opportunists. This conclusion is supported by studies in Bangladesh showing that a large variety of bacteria, including aeromonads, are part of the natural flora in fish farms water (Karim and Chowdhury, 1995) and the mucus and kidneys of healthy, cultured *Labeo rohita* (Baqui and Chowdhury, 1995).

Initial research on RSD was similarly directed towards bacterial pathogens, and the isolation of *Vibrio anguillarum* from diseased fish was originally considered significant (Burke and Rodgers, 1981). A further assessment of the bacteria and parasites involved in RSD dismissed the possibility that they were primary agents (Callinan and Keep, 1989), and subsequent research focused on mycological and environmental aspects of the disease.

1.6 Other similar diseases or mycoses of aquatic animals

Mycotic granulomatosis (MG) and red spot disease (RSD), and the extreme similarity of these diseases to EUS, have already been described above in some detail. Other mycotic fish diseases caused by agents of varying or unknown relationship to *Aphanomyces invadans*, or ulcerative diseases of undetermined aetiology, are reviewed here.

1.6.1 Ulcerative mycosis (UM)

Noga (1994) postulated that ulcerative mycosis (UM) of coastal fish populations of the Western Atlantic may be part of the same syndrome as EUS, given the similarities in clinico-pathological features of both diseases (Table 1.2) and that predominantly *Aphanomyces* fungi are recovered from UM-diseased fish (Dykstra, Noga, Levine, Moye and Hawkins, 1986). However, fish challenged with these *Aphanomyces* isolates have failed to reproduce the disease whereas fish developed UM when lesion material is used as an inoculum, suggesting that some other, unidentified agent is required for infection (Noga, 1993a).

UM was first observed in April 1984, in menhaden (*Brevoortia tyrannus*) in the Pamlico River, North Carolina and in November of that year a massive kill was reported (Noga and Dykstra, 1986). Epidemics of similar diseases were later recognised in estuaries along the eastern seaboard of USA from Connecticut (Noga, 1993a) to Florida (McGarey, Beatty, Alberts, Te Strake and Lim, 1990), although it is

uncertain whether these were first occurrences and represented a spread in the disease. Several fish species were shown to contract UM-like diseases in Pamlico River (Noga, Wright, Levine, Dykstra and Hawkins, 1991) but the incidences of these was markedly lower than in menhaden (Levine, Hawkins, Dykstra, Noga, Moye and Cone, 1990a). In menhaden, a larger proportion of age-0 fish were shown to be affected than age-1 fish (Levine *et al,* 1990b). Levine *et al* (1990b) also provided evidence that specific regions of low salinity within the Tar-Pamlico estuary harboured higher levels of diseased fish, and Noga (1993a) observed that the most damaging outbreaks in the Pamlico River coincided with years of unusually high rainfall and reduced salinity (1984 and 1989). Outbreaks have continued to occur, some of which have resulted in mortalities of millions of fish (Noga, 1993a) and infection rates of menhaden up to 100% (Levine *et al,* 1990b).

Noga, Khoo, Stevens, Fan and Burkholder (1996) showed that sublethal exposure to toxins produced by a recently identified "phantom" dinoflagellate, also responsible for high mortalities in the Pamlico River (Burkholder, Noga, Hobbs and Glasgow, 1992), can result in dermatitis and subsequent ulcerative mycosis infection.

Several species of dead and moribund ulcerated fish, predominantly catfish (*Bagre marinus*) and mullet (*Mugil* spp), have also been observed in and around the estuaries feeding the Baia de Sepetiba in Southeast Brazil (P.C. Scott, pers. comm.) but no post mortem was carried out, and it is unknown if a mycosis was involved.

1.6.2 Other ulcerative diseases in the Asia-Pacific region

There are many accounts of ulcerative disease outbreaks in fish in the region, some of which, although not confirmed as EUS, tend to fit well with the known characteristics of the disease and have been generally associated with EUS. These were discussed in Section 1.2.3. Two other accounts which show gross clinical signs similar to EUS are included here.

Munday (1985) reported the presence of severely ulcerated red cod *(Pseudophycis barbatus)* in the River Tamar near Launceston, Tasmania in November 1980 and 1981. Although a variety of bacteria and parasites were identified from the fish, pollution was considered the main cause of the disease. Munday (pers. comm.) now believes ulcer disease was the same syndrome as EUS although it occurred in higher salinity water. He adds that Launceston's sewerage system has recently been improved and the disease is no longer reported.

Schuurkamp and Hortle (1987) described an ulcerative disease outbreak in freshwater herring (*Nematalosa flyensis*) and 3 other river species in the backwaters of Western Province, Papua New Guinea in 1985 and 1986. Only bacteriological examination was carried out and a number of bacteria were isolated.

163 Other aphanomycoses of fish

The first reported *Aphanomyces* infection of fish, where the fungus was considered to be the primary disease agent, occurred in November 1942 in several aquarium tanks in the University of Illinois (Shanor and Saslow, 1944). Guppies (*Lebistes reticulatus*) and other ornamental species first showed signs of an abnormal dorsal hump, and within a few days hyphae began to protrude from the dorsal musculature. All infected fish died soon after lesions developed. A sexually sterile *Aphanomyces* fungus was the only agent isolated.

An aphanomycosis was reported in Uttar Pradesh, India in 1974, causing mortalities of about 90% in culture ponds of Indian major carp, *Cirrhinus mrigala* (Srivastava, 1979). A sexually sterile *Aphanomyces* species was isolated from hyphae protruding from the body of affected fish, and found to be capable of colonising experimentally injured areas of fish, eventually killing them. Although there was no indication that the fungus was invasive within the muscle, and the infection appears secondary to the physical injury of the fish, the disease fungus is significant as it was given the name

Aphanomyces pisci. However, cultures are now apparently unavailable from either the IMI culture collection where it was lodged, or from the author.

The two accounts above give some information about the growth characteristics of the isolates concerned, and are discussed in comparison to *A. invadans* in Chapter 4.

There are several other reports of superficial infections of fish by a large number of saprolegniacean fungi (see Section 1.6.7). Reported incidences of *Aphanomyces laevis* acting as a wound parasite on fish are given by Vishniac and Nigrelli (1957); Scott and O'Bier (1962); Scott (1964); Srivastava (1980a); Ogbonna and Alabi (1991) and Khulbe, Joshi and Bisht (1995). Similar accounts involving unspeciated *Aphanomyces* are given by Scott and O'Bier (1962); Scott (1964); Willoughby (1970); Pickering and Willoughby (1977) and Srivastava (1980a). Finally there is one report of *Aphanomyces stellatus* affecting eels (Hoshina, Sano and Sunayama, 1960, cited by Neish and Hughes, 1980).

1.6.4 Crayfish plague

Aphanomyces astaci has been the cause of catastrophic mortalities in European crayfish populations since it was first reported in 1860 in Italy (Ninni, 1865, cited by Alderman, 1996). Early workers claimed parasites were the cause (Haldenway; as described by Southgate, 1983), and when *A. astaci* was identified by Schikora (1903; 1906, cited by Alderman, 1996), it was dismissed by other workers as being a saprophyte, probably *Saprolegnia* or *Achlya*. A bacterial aetiology was also described, and it was not until Nybelin's work in 1934 (cited by Alderman, Polglase and Frayling, 1987) that *A. astaci* was accepted as the primary cause. *A. astaci* is now known to be a highly infectious agent that results in 100% mortality of susceptible hosts (Alderman *et al*, 1987); it releases a number of enzymes important in pathogenesis (Söderhäll, 1978); is capable of invading the musculature of infected European crayfish (Southgate, 1983); and has been reported to be similarly slow-growing and fastidious in culture to *A. invadans* (Unestam, 1965; Unestam and Gleason, 1968). Most of this more recent work on crayfish plague has been carried out in Sweden (where it is

known as kräftpesten), which has been transformed from one of the largest exporters of crayfish (Marren, 1986) to the world's largest importer of crayfish (Swahn, 1994) since the introduction of the disease in 1908. The first confirmed occurrence of crayfish plague in the UK was as recent as 1981 (Alderman, Polglase, Frayling and Hogger, 1984). UK isolates of *A. astaci* were included in most of the comparative studies of *A. invadans* described here, and for the first time characterised by comparing polymorphic DNA fragments with those of established *A. astaci* groups from Sweden (Chapter 11).

165 *Aphanomyces* in other aquatic animals

In November 1964, a sterile *Aphanomyces* isolate was obtained from the skin of a freshwater dolphin (*Inia geoffrensis*) that had developed several dermal lesions having been flown from the upper Amazon in Peru to San Francisco (Fowles, 1976). Reported growth characteristics of this isolate are compared to *A. invadans* in Chapter 4.

Valairatana and Willoughby (1994) also reported *Aphanomyces* growing as a wound parasite on the surface of a soft shell turtle (*Trionyx cartilogineus*) in Thailand. This isolate (T1SA) is included among a number of "saprophytic" *Aphanomyces* studied here. Sinmuk, Suda and Hatai (1996) subsequently studied an isolate from a juvenile soft shelled turtle (*Pelodiscus sinensis*) imported to Japan from Singapore, but showed it to have different growth characteristics from MG isolates.

166 Invasive saprolegniasis

The differential ability of particular strains within the *Saprolegnia parasitica-diclina* complex to colonise fish has been reported ever since the taxon *S. parasitica* was established (Coker, 1923; Willoughby, 1978; Hatai, Willoughby and Beakes, 1990; Hatai and Hoshiai, 1993; Beakes, Wood and Burr, 1994). However, "saprolegniasis" is typically an infection of the epidermis or dermis of fish (see next section) and bears little relationship to the fungal involvement in EUS. In some cases, though, hyphae of

Saprolegnia have been reported extending into the underlying musculature and internal organs. Pickering and Richards (1980) classified *Saprolegnia* infections of salmonids, and fungal penetration to the dermis and muscle surface were features of Type 4 and Type 5 lesions respectively, although they commented that muscular (Type 5) lesions were particularly uncommon.

Agersberg (1933, cited by Southgate, 1983); Bootsma (1973); Nolard-Tintigner (1973); Hatai and Egusa (1977); Hatai (1980b); Bruno and Stamps (1987); Hatai and Hoshiai (1992a) and Wada, Hatai and Ishii (1993) all described the penetration of musculature and/or internal organs of small fish due to natural or experimental challenge by *Saprolegnia*. Davis and Lazar (1941, cited by Southgate, 1983) described a similar mycosis of rainbow trout fry and named the causative fungus *Saprolegnia invaderis*. However Seymour (1970) later excluded this taxon as he considered it a representative of *Saprolegnia ferax*. Stuart and Fuller (1968) reported that *Saprolegnia* sp on Atlantic salmon in Ireland has been isolated from the musculature to a depth of 1.5 cm, but the age of the fish concerned was not given.

Neish (1977), demonstrated that *Saprolegnia* hyphae could also penetrate the muscle of adult fish (maturing sockeye salmon, *Oncorhynchus nerka*). Puckeridge, Walker, Langdon, Daley and Beakes (1989) later described a mycotic dermatitis in bony bream (*Nematalosa erebi*) in South Australia whereby *Saprolegnia* (predominantly *S. parasitica*), considered to be the primary infective agent, penetrated deep in the muscle of advanced lesions. Bly, Lawson, Dale, Szalai, Durborow and Clem (1992) reported a similar condition in channel catfish (*Ictalurus punctatus*). In contrast to EUS lesions however, there was no extensive degeneration of muscle tissue or any significant inflammatory response by the fish in all these cases. Whereas the sockeye salmon incidents were considered to be related to increased levels of plasma corticosteroids in the maturing fish, the latter two cases clearly followed rapid reductions in water temperature during cold winter periods, and in this respect, show similarities to EUS. Bly *et al* (1992) described experimental bath challenges with

zoospores of *Saprolegnia* sp from local outbreaks in which a rapid decrease in temperature from 22°C to 10°C sufficiently immunosuppressed juvenile catfish to cause 92% infection and 67% mortality within 21 days. They later showed that similar conditions occurred during natural outbreaks (Bly, Lawson, Szalai and Clem, 1993).

One mechanism that may be important in enabling particular strains to penetrate deeper into the tissues of the host may involve specific enzyme activity, as investigated in *Saprolegnia* spp by Peduzzi, Nolard-Tintigner and Bizzozero (1976) and Rand and Munden (1992). Given the extent of muscle tissue degeneration in EUS-affected fish as a result fungal penetration, specific extracellular enzyme activity may be a particular feature of the fungus or fungi involved (see Chapter 4).

1.6.7 Dermatomycoses

Superficial infections of fish by saprolegniacean fungi are commonly observed and have been reported for almost 250 years. William Arderon's 1748 description of saprolegniasis of roach (*Rutilus rutilus*) may well be the first record of a mycotic disease of any vertebrate (Hughes, 1994).

A great number of Oomycete species have been found to be capable of naturally or experimentally infecting fish. These have been reviewed by Scott (1964); Wolke (1975); Alderman (1976; 1982a); Neish and Hughes (1980); Hatai (1989) and Noga (1993b). Srivastava (1980a) listed 40 speciated and unspeciated fungi that have been reported on over 100 different species of fish. Intensive investigations of Oomycetes colonising fish have been carried out in Japan (Hoshina *et al*, 1960; Hatai *et al*, 1977b; c); India (Bhargava, Swaup and Singh, 1971; Srivastava and Srivastava, 1977a; b; 1978; Srivastava, 1980b; Khulbe, 1980; 1983; 1989; Sati, Mer and Khulbe, 1982; Singhal, Jeet and Davies, 1987; Khulbe *et al,* 1995; Bisht *et al*, 1996); Egypt (El-Sharouny and Badran, 1995); Nigeria (Ogbonna and Alabi, 1991); the UK (Willoughby, 1970; Willoughby, 1997a) and the USA (Tiffney, 1939a; b, cited by Hughes, 1994; Scott and Warren, 1964; Scott and O'Bier, 1962).

In their study of fungi growing on perch (*Perca fluviatilis*) in Lake Windermere, Pickering and Willoughby (1977) identified 4 different genera of fungal colonists on a single lesion and described a apparent succession in species according to the type of lesion. Despite this intricate fungal involvement, they did not attribute the primary cause of the lesion to any of them. Ulcerative dermal necrosis (UDN) is a disease of maturing wild salmonids in which fish can develop heavy *Saprolegnia* infections, but even in these lesions, fungal hyphae generally do not penetrate further than the dermis (Roberts, Shearer, Munro and Elson, 1970).

These latter cases are clearly in contrast to the mycotic involvement in EUS. That so many Oomycete fungi have the ability to colonise fish, but relatively few species have been reported penetrating the internal tissues of the host, indicates that very specific adaptations of the fungus and/or specific conditions within the fish are required for fungi to achieve this. The fungus or fungi involved in MG and RSD are considered the only biological causative agent(s) required for infection, and therefore warrant intensive investigation to understand the processes involved in the pathogenesis of these highly damaging diseases. Whether other aetiologies are involved in EUS and UM or not, the fungi involved again show extraordinary characteristics and are clearly central to the virulence of the disease.

1.7 Objectives of this work

The stated objective of the ODA project, under which this study was funded, was to provide an understanding of the general biology of the fungal pathogen of EUS and resolve questions over its relationship with strains from other similar diseases. Specifically this involved:

(i) Comparing the pathogenicity and invasive capabilities of *Aphanomyces invadans* isolates with other *Saprolegnia, Achlya* and *Aphanomyces* species from EUS affected

fish, to determine whether a single species, or multiple fungal pathogens are responsible for the characteristic mycotic lesions in EUS-affected fish.

(ii) Characterising *Aphanomyces invadans* using a variety of techniques. It was envisaged that in addition to providing a means of identifing the species, further knowledge of the biology of *A. invadans* would be relevant to an understanding of the disease process.

(iii) Comparing the pathogenicity and biological characteristics of *Aphanomyces invadans* with isolates from RSD, MG and other aquatic animal mycoses in order to assess the relationship of EUS to these diseases.

CHAPTER 2. GENERAL MATERIALS AND METHODS: BACKGROUND TO MYCOLOGICAL TECHNIQUES

2.1 Introduction

2.1.1 The taxonomical position of *Aphanomyces*

The genus *Aphanomyces* was established by deBary in 1860, its name meaning "imperceptible fungus", apparently due to the delicate appearance of the hyphae on the body of a decaying insect in the water. In Scott's (1961) "Monograph of the Genus *Aphanomyces*", 26 species were listed as well as 2 isolates of doubtful affinities, comprising Shanor and Saslow's (1944) *Aphanomyces* sp isolated from guppies and Matthew's (1935) *Aphanomyces* sp growing on frog's eggs. *Aphanomyces* species can be categorised into those with smooth or roughened oogonial walls (Cutter, 1941). Scott (1961) erected three subgenera on this basis: *Aphanomyces* (with smooth oogonial walls, including *A. laevis*), *Axyromyces* (with roughened walls and no ornamentation) and *Asperomyces* (with roughened and ornamented walls, including *A. astaci*). Dick (1973) later questioned the validity of this use of subgenera. However, as oogonia have not been demonstrated in any of the EUS, MG and RSD pathogenic *Aphanomyces* isolates, no attempt can be made to allocate subgenera anyway. Early descriptions of oogonia in *A. astaci* are now also considered questionable (Alderman and Polglase, 1988).

Aphanomyces is contained within the family Saprolegniaceae: aseptate, normally eucarpic fungi typically demonstrating two zoospore forms (Dick, 1973). The Saprolegniaceae are the largest family within the order Saprolegniales (class Oomycetes), which is normally characterised by the production, from within a zoosporangium, of zoospores with two flagellae, differing in length and type (heterokont). The Saprolegniales also contain cellulose and ß-1,3-1,6-glucans and lack chitin within their cell walls, although chitin has been demonstrated in some other members of the Oomycetes (Bartnicki-Garcia, 1987). Accumulated evidence indicates that the Saprolegniales are the most primitive order of Oomycetes, and that

Aphanomyces, along with *Achlya*, is one of the most primitive genera of the class (Barr, 1983a); however, particular plant- or animal- specific parasites have developed among the *Aphanomyces*, a feature that requires a high degree of specialisation (Heath, 1987).

2.1.2 The life-cycle of *Aphanomyces*

A generalised life-cycle of *Aphanomyces* is shown in Figure 2.2.

Saprolegniacean genera are distinguished primarily by asexual characters, particularly zoosporangial formation and zoospore release. The zoosporangia of *Aphanomyces* spp are typically no wider than the hyphae. A single row of primary zoospores are formed within a zoosporangium and released from an apical tip, or from lateral evacuation tubes, at which they immediately encyst and form achlyoid clusters (Figure 2.2B). The main free-swimming stage of *Aphanomyces* spp is the secondary zoospore (C) which is discharged from the encysted primary zoospores (=primary cystospore). The secondary zoospore remains motile for a period depending on environmental conditions and location of a host or substratum. Typically the zoospore encysts (=secondary cystospore) and germinates to produce new hyphae (D-E). However, some *Aphanomyces* spp do show a phenomenon known as polyplanetism (Cerenius and Söderhäll, 1984b) whereby several tertiary generations of zoospores may be produced from the secondary cyst (H). This repeated emergence of zoospores was considered to be an adaptation to parasitism among particular *Aphanomyces* species (Cerenius and Söderhäll, 1985). The abbreviated life-cycle (I) and indirect germination (J) illustrated in Figure 2.2 are features associated with fish-parasitic *Saprolegnia* sp (Willoughby, 1977; Willoughby, McGrory and Pickering, 1983), but, as detailed in Chapter 6, are observed in several species of *Aphanomyces* studied here, including *A. invadans*. Chlamydospores (or gemmae) (G) are modified segments of vegetative hyphae containing a dense accumulation of protoplasm. They generally germinate into new mycelium under favourable conditions. Although common among *Saprolegnia* species (Seymour, 1970), Scott (1961) maintained that they did not occur among *Aphanomyces* species. However, Roberts

et al (1993) and Srivastava (1979) observed chlamydospores in *A. invadans* and *A. pisci* respectively.

Sexual reproductive morphology is generally regarded as a conservative characteristic and is therefore important in the taxonomy of saprolegniacean fungi, to the point that it traditionally defines the species status of members of the group. An example of the female oogonia and male antheridia are given in Figure 2.2F. However, it has proved notoriously difficult to demonstrate sexual structures in many saprolegniacean strains. This phenomenon appears to be more prevalent among the more pathogenic species or strains (Alderman and Polglase, 1988). The reports of fish aphanomycoses listed in Section 1.6, for example, invariably describe sterile isolates. Early descriptions of oogonia in the crayfish plague fungus *Aphanomyces astaci* (Rennerfelt, 1936, as cited by Scott, 1961) have not been adequately substantiated (Unestam, 1969a; Rodgers, 1988) and are believed by some workers to be mistaken (Alderman and Polglase, 1988). The common fish parasite, *Saprolegnia parasitica* was originally described as sexually sterile (Coker, 1923) and although oogonia have now been identified from this species (Kanouse, 1932), they remain more rarely observed than in other *Saprolegnia* spp (Wood, 1988). The EUS, MG and RSD pathogenic isolates now provide further examples of important saprolegniacean fungi that cannot be speciated by traditional means due to their sterile nature. The ATCC has recently expanded its computer coding system for the identification of microbial strains to include saprolegniacean fungi in an attempt to address the problem (Jong, Davis, McManus and Krichevsky, 1991).

2.1.3 Descriptions of *A. invadans*, *A. piscicida* and RSD-associated *Aphanomyces*

As mentioned above, oogonia have not been observed in any of the three *Aphanomyces* strains associated with EUS, MG and RSD, which can not therefore be speciated according to traditional mycological characteristics. However, Willoughby *et al* (1995) and Hatai (1980a) have published descriptions of the EUS and MG fungi, naming them *A. invaderis* (=*A. invadans*) and *A. piscicida* respectively. A summary of

these descriptions, and that of the RSD-associated *Aphanomyces* by Fraser *et al* (1992), is given in Table 2.1. It should be noted that Hatai's (1980a) description of *A. piscicida* was, however, not validly published according to the International Code of Botanical Nomenclature, for a number of reasons, but principally because no Latin diagnosis was given (Article 36, ICBN Tokyo Code).

2.1.4 Terminology

It should be noted that *Aphanomyces,* and Oomycetes in general, are no longer phylogenetically regarded as true fungi, but rather fungal-like protists. Zoospore flagellar morphology (Barr, 1981; 1983a), organelle substructure (Powell, Lehnen and Bortnick, 1985; Beakes, 1987) and molecular data (Gunderson, Elwood, Ingold, Kindle and Sogin, 1987) have clearly shown that Oomycetes have a closer relationship with algae than with true fungi. They are therefore usually classed alongside diatoms, brown algae and xanthophytes within the phylum Heterokonta as part of the third botanical kingdom, the Chromista. They are sometimes called pseudofungi, either as a general term or a formal taxon (Cavalier-Smith, 1987). Despite these fundamental changes in the taxonomy of this group, the Oomycetes are still most commonly referred to as fungi, and Dick (1997) argues that physiologically they are fungi, and should be called as such. Therefore, for the purpose of this thesis, the organisms studied here shall remain "fungi".

It is clear from Section 1.6 that the saprolegniaceans have varying abilities to parasitise fish. *Saprolegnia* sp, for example, may or may not be a primary pathogen and may or may not penetrate internal fish tissues (compare Puckeridge *et al*, 1989 with Roberts *et al,* 1974). In this study, a clear distinction made is between pathogens and saprophytes with regards to EUS. In Chapter 3, pathogenicity is determined by the ability of zoospores to germinate and grow invasively in snakehead muscle tissue, eventually producing EUS-like lesions. As the zoospores are introduced directly into the muscle in these challenge studies, even "pathogens" are not assumed to be primary pathogens; although this possibility is discussed, with particular reference to

A. astaci, a highly adapted, primary (possibly obligate) pathogen of crayfish. Saprophytic isolates have been obtained from water samples or from the surface of EUS-affected fish, or other diseased aquatic animals, and cannot grow invasively in snakehead muscle. These isolates probably derive nutrients from dead plant and animal material (saprotrophs) and live facultatively on dead tissue in living fish (perthotrophs). Their ability to derive nutrients parasitically and kill living fish tissue (necrotrophs) is undetermined. However, any involvement these isolates have in EUS can be considered opportunistic, and secondary to the development of a lesion.

Aphanomyces invadans (at present, more commonly known as "*A. invaderis*"), is at this time still only associated with EUS outbreaks. Therefore in the sections describing experimental work it is more often referred to as the EUS pathogen, in order to distinguish it from the MG pathogen ("*A. piscicida*") and the RSD pathogen (*Aphanomyces* sp). A discussion of the species *A. invadans,* and which isolates should be included in the taxon is given in the final section.

The various fungal isolations compared here are referred to as isolates, as this is considered to be the least ambiguous term. Brasier and Rayner (1987) considered isolates that have been asexually propagated in culture to be strains, whereas Tibayrenc (1996) identified microbial lines maintained in laboratories as stocks, and categorically not strains. It is felt that these terms may imply some genetic or physiological variation which is not necessarily the case among all the isolates compared here.

2.2 Materials and methods

2.2.1 Fungal isolates

Tables 2.2 and 2.3 list the origins of the fungal isolates obtained for the purposes of this study, and currently being maintained at the Mycology lab, Institute of Aquaculture, University of Stirling. Included here are: EUS, RSD or MG *Aphanomyces* pathogens; *Aphanomyces* species associated with other diseases of aquatic animals

(principally UM and crayfish plague); *Aphanomyces, Achlya* and *Saprolegnia* isolates obtained from the surface of EUS-affected fish or from EUS-affected water bodies; and the peronosporalean fungus *Phytophthora cinnamomi* obtained for further comparative purposes. Appendix 1 lists the particular fungal isolates used in each of the experiments described. Figure 2.1 maps the origin of the EUS, RSD and MG isolates.

2.2.2 Isolation of fungi

Fungi were isolated using a variety of techniques. The isolation procedures used to obtain *A. invadans* isolates TA1, RF6 and RF8 are detailed in Roberts *et al* (1993). BH, BR and BS isolations are described in Willoughby and Roberts (1994a).

Isolations of *A. invadans* by the present author were made as follows. Where multiple samples were available, a medium-sized dermal ulcer overlying muscle tissue at least 1 cm thick was selected. Small fish were cut it in two using a sterile scalpel, slicing through the fish at the edge of the lesion. A hot scalpel was used to sterilise the exposed surface of the muscle. A small-bladed sterile scalpel was used to cut out a circular block of muscle from beneath the lesion. Muscle samples were about 0.5 cm^3 and not visibly discoloured by the overlying ulcer. With larger fish, the whole lesion was removed, the surface of the remaining tissue was sterilised, and a block of muscle was cut from the exposed area. The muscle sample therefore contained the invasive pathogenic fungus, but not any saprophytic fungi associated with the surface of the fish, and minimal bacterial contamination. The muscle block was then placed in a sterile Petri dish of GP-PenOx broth (see Appendix 2a). The Petri dish was left until fungi could be seen growing out from the muscle block (about 10 hours at 25°C). These hyphal strands were cut out using aseptic technique and transferred to a Petri dish of GP-PenStrep agar (Appendix 2a). In this way, bacterial growth was inhibited and the fungus was able to grow free of bacterial contamination. An agar block containing fungal hyphae from the edge of the colony was then placed upside down on a Petri dish of GP agar and repeatedly subcultured until an axenic stock was

obtained. The only information on these isolates, published by other workers, is by Nakamura, Yuasa, Sinmuk, Hatai and Hara (1995) involving S1PA and G2PA .

Aphanomyces isolates from Australia, Indonesia and the Philippines were all isolated from ulcerated fish on Czapek Dox agar (CDA) with penicillin G (100 units/ml) and oxolinic acid (100 μg/ml). Emerging hyphal tips were repeatedly transferred to fresh plates of CDA or glucose-yeast (GY) agar (5g/l glucose, 2.5g/l yeast, 15g/l agar) with the same antibiotics until bacterial-free cultures were obtained (Fraser *et al*, 1992; R.B. Callinan, pers. comm.). MG-isolate NJM9030 was probably obtained using FME agar (10% fish-meat extract, 1.5% agar) as described by Hatai *et al* (1977a) and NJM9201 was isolated on GY agar (but with 10g/l glucose) with penicillin (500 units/ml) and streptomycin (500 μg/ml) (Hatai *et al,* 1994).

Isolations from UM-affected fish were reportedly made either on cornmeal agar containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (Noga and Dykstra, 1986) or on the same media supplemented with 1% yeast extract and 2% D-glucose (Dykstra *et al,* 1986; Dykstra *et al,* 1989). UK *Aphanomyces astaci* isolates were obtained on RGY agar (1g yeast extract, 5g D-glucose 12g agar in 1l river water) with oxolinic acid (10 μg/ml) and penicillin G (6 μg/ml) as described by Alderman and Polglase (1986). Swedish *A. astaci* isolate J1 was obtained by the method of Unestam (1965) using glucose-blood agar (0.5% glucose, 5% crayfish blood or 20% horse serum, 1.5% agar). All other *A. astaci* were isolated as detailed by Cerenius, Söderhäll, Persson and Ajaxon (1988) using PG-1 agar (Appendix 2a).

The saprophytic isolates in Table 2.2 were easily obtained by techniques described in Roberts *et al* (1993) and Roberts (1989). Previous published information has been given on TF5, TF33, TF41 (Roberts *et al*, 1993), TF54 (Willoughby and Roberts, 1994a), T1SA (=SH1: Valairatana and Willoughby, 1994), W2BAC (Willoughby, 1997b), S. aust (=795: Wood, 1988; Molina, Jong and Ma, 1995), E3 (Wood, 1988; Burr, 1991; Molina *et al,* 1995), and TP41 (Willoughby *et al*, 1983; Wood, 1988; Cross

and Willoughby, 1989; Burr, 1991; Bly *et al,* 1992; Beakes *et al,* 1994; Smith, Chohan, Howitt and Armstrong, 1994; Nakamura *et al*, 1995; Molina *et al,* 1995; Bullis, Noga and Levy, 1996).

2.2.3 Maintenance of fungi

Significant difficulties have been encountered in the maintenance of the slow-growing EUS, RSD and MG isolates due to the rapid staling of the growth media by these cultures (Willoughby and Chinabut, 1996). Stocks were initially maintained in flasks containing 200 ml GP broth at 10°C for 6 weeks before subculturing. Given the large amount of incubator space required to maintain all the isolates, cultures were later kept at room temperature on GPY slopes filled with sterile light paraffin oil (Merck) according to Smith and Onions (1994). Given that cultures of the reputedly fastidious *A. astaci* (Unestam, 1965) can now be maintained for longer periods of time on the buffered medium PG-1 (Söderhäll and Cerenius, 1987), a short comparison of these media was undertaken (see Chapter 4). As a result, all stocks are now maintained on PG-1 slopes under oil at room temperature. Using this technique, cultures of *A. invadans* have been kept for up to a year before subculturing.

Experimental cultures were maintained at 10°C on GPY agar Petri dishes for a maximum of 7 days. Inocula were taken using a 4 mm cork borer.

2.2.4 Water quality analysis of water used for preparing APW

Autoclaved, filtered pond water (APW) is used to induce sporulation of saprolegniacean fungi as described in Section 2.2.5 below. Preparation of APW is described in Appendix 2a.

Water used for APW should first be checked for its ability to support saprolegniacean fungi. Studies undertaken in Stirling or Newcastle used water from Airthrey Loch, a small eutrophic loch on Stirling University campus. This was tested in July 1993 for fungal spores using a GP-PenStrep assay technique (Valairatana and Willoughby, 1993) and a hemp seed baiting technique (Willoughby, 1994). Very high levels of

Saprolegnia spp and *Achlya* spp were recorded at that time and the water was considered suitable. Subsequent water quality analysis of Airthrey Loch throughout 1994 by Kelly and Smith (1996) recorded high pH levels (up to pH 9.54) over the Spring and Summer (see Appendix 2b). This was probably in response to macrophyte and algal respiration, and was shown to result in high autochthonous production of phosphorus. These fluctuations in water quality conditions (and pH in particular) may have affected the suitability of the water for *Aphanomyces* sporulation and could have resulted in low yields of zoospores for experiments conducted at that time. Marked seasonal variations in fungal yields associated with pH changes have been widely reported from natural water bodies in both temperate (Waterhouse, 1942; Roberts, 1963) and tropical (Alabi, 1971; Elhissy, Moharram, Elzayat and Massoud, 1996) localities.

Water used for APW in studies in Bangkok was taken from the fish pond at the National Inland Fisheries Institute (NIFI). Spore counts conducted over an 8 month period in 1992 using the GP-PenStrep assay and snakehead fish scale baits (Willoughby and Lilley, 1992) revealed high levels of *Achlya* spp and *Aphanomyces* spp, particularly over the winter period, when all experiments were carried out (Lilley, 1992). Some water quality data taken in March 1997 are presented in Appendix 2b; this is slightly after the period when experiments were carried out in previous years.

Zoospore reemergence studies undertaken in Uppsala used stocks of Hälsjön Lake water kept since the 1970's, when water quality measurements were taken (Appendix 2b). This water was shown to be a good sporulating medium in experiments at that time (Unestam, 1969a; b; Svensson and Unestam, 1975), and kept in order to standardise procedures.

2.2.5 Zoospore production

The production of suspensions of secondary zoospores was required for several of the experiments described in this thesis. However, it proved very difficult to produce consistent yields of zoospores between isolates, and even different cultures of the

same isolate. As mentioned above, this may be partly explained by varying water quality conditions of the APW used. However, some batches of APW that had initially induced sporulation, subsequently failed to do so in some cultures. It is commonly observed that repeated subculture of laboratory stocks of fungi can result in reduced sporulation (Unestam and Svensson, 1971) and this may have been the case with some of the cultures here. Therefore techniques for producing zoospores were adapted over the course of the study in an attempt to maximise yields.

(a) Initial experiments used a technique based on the description of Willoughby and Roberts (1994b) to prepare suspensions of secondary zoospores. Briefly, 4 mm agar plugs of mycelium were placed in Petri dishes containing GPY broth (Appendix 2a) and cultured for 4 days at 20°C. The resulting mycelium mats were brought through 5 Petri dishes containing sterile distilled water to remove the nutrient media, and finally left overnight at 20°C in APW. Willoughby and Roberts (1994b) used APW during the whole washing and incubation process, but it was found in this study that sterile distilled water could substitute the APW for the washing process without any apparent effect on sporulation. Cerenius and Söderhäll (1980) did likewise for *A. astaci*, but warned that use of distilled water for the incubation process as well resulted in unattached primary cysts and also a reluctance of these cysts to produce motile zoospores.

(b) More effective washing of mycelium mats was later achieved by sequential tranfer through two 500 ml bottles of sterile distilled water, before leaving the fungus overnight in APW (Beakes and Gay, 1980).

(c) Towards the end of the project, an isolate of *Phytophthora cinnamomi* was obtained for comparative studies with *A. invadans*. Sporulation of this fungus is achieved by initial growth in V8 broth (Zheng and Ko, 1996). Although *A. invadans* did not grow well in this medium, it was found to produce generally higher yields of zoospores. This media was therefore adopted using 5 day old mycelium mats in combination with the technique described in (b).

(d) *In vitro* studies undertaken at Uppsala University employed the drop culture technique used in that laboratory (Söderhäll, Svensson and Unestam, 1978). Mycelium squares, approximately 1 mm^2, were used to inoculate drops of 0.25 ml PG-1 broth in sterile Petri dishes. After 3 days growth at 22°C, the medium was replaced with a similar drop of APW, which was then changed for new APW every 30 min for 3 hours. The cultures were then incubated overnight at 22°C.

(e) Large volumes of zoospores were produced according to Cerenius *et al* (1988) for use in the crayfish immersion challenges described in Chapter 3. This technique involved incubating agar plugs of fungus in PG-1 broth for 3 days at 20°C; homogenising the resulting mats in a sterilised blender; and transferring the fragments to a larger volume of PG-1 broth for a further 3 days at 20°C. The mycelia were then filtered and left in approximately 200 ml APW on a shaker for 1 hour. The mycelia were again filtered and left in APW three more times for periods of 1 hour, before being left in a larger volume of APW overnight, at 20°C for *A. invadans,* or 13°C for *A. astaci*.

Willoughby and Roberts (1994c) showed that incorporating 100 mg/l calcium gluconate in the APW improved motile zoospore production, and this should be evaluated as a dehiscence solution in future studies.

2.2.6 Light microscopy and photography

Routine light microscopy was performed using an Olympus BX40F microscope. Petri dishes containing zoospore suspensions were generally observed directly under the microscope, with the mechanical stage removed, at x10 with phase contrast optics. Photographs were taken using An Olympus SC35 Type 12 camera.

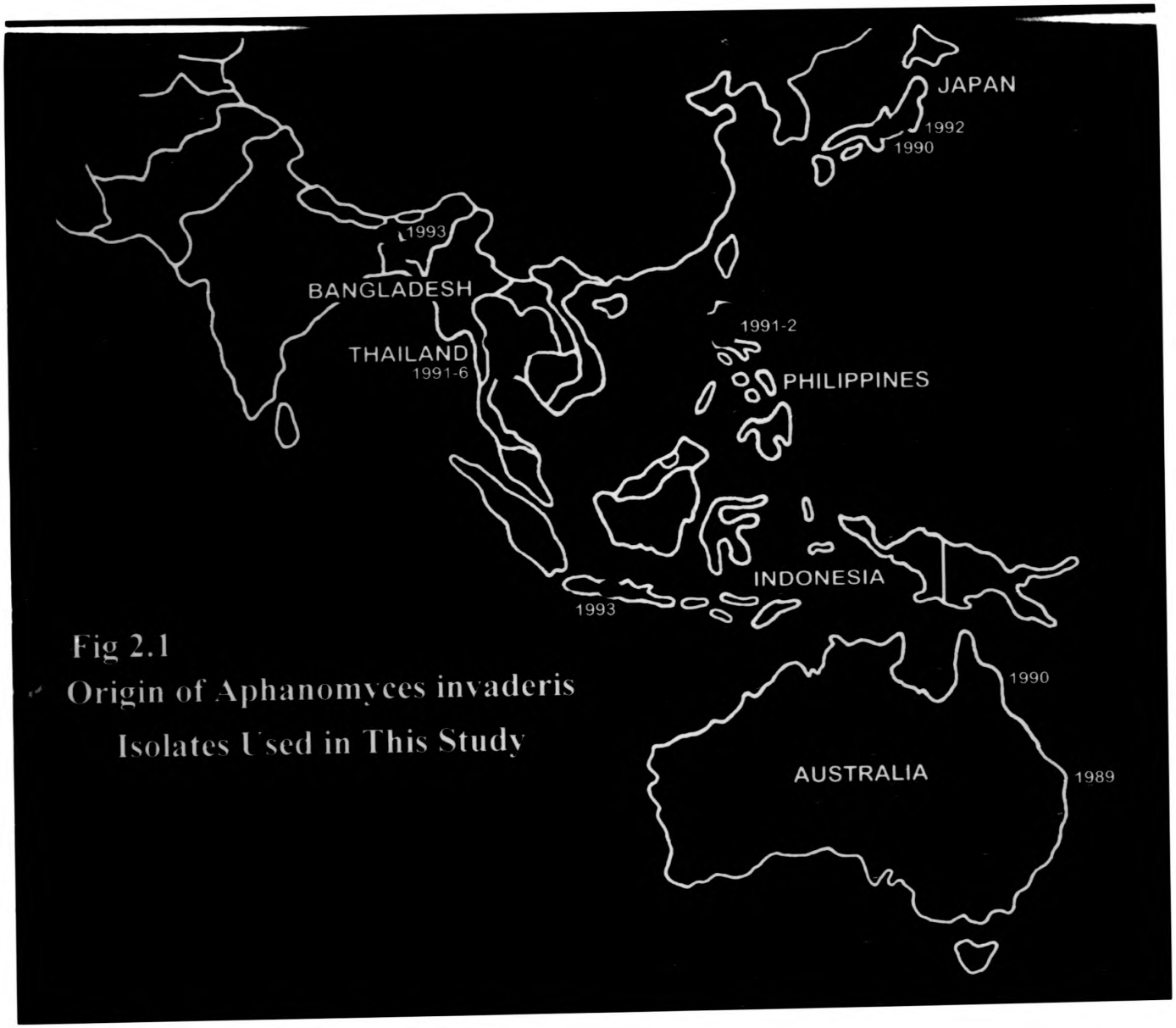


Fig 2.1
Origin of Aphanomyces invaderis
Isolates Used in This Study

Figure 2.2 **Generalised life-cycle of *Aphanomyces* spp**
(features H - J are discussed in Chapter 6)

A - Vegetative hyphae
B - Primary zoosores forming within a hyphal-sized zoosporangium and encysting at the tip to form an achlyoid cluster
C - Motile, laterally biflagellated, hererokont secondary zoospores
D - Encysted secondary zoospore
E - Germling
F - Sexual structures: female oogonium and male antheridium
G - Chlamydospore
H - Repeated zoospore emergence (polyplanetism)
I - Cyst germinates and forms a mini-sporangium capable of releasing a single zoospore (abbreviated life-cycle)
J - Cytoplasmic contents of cyst is transferred via a fine, septate germ tube to the viable hypha (indirect germination)

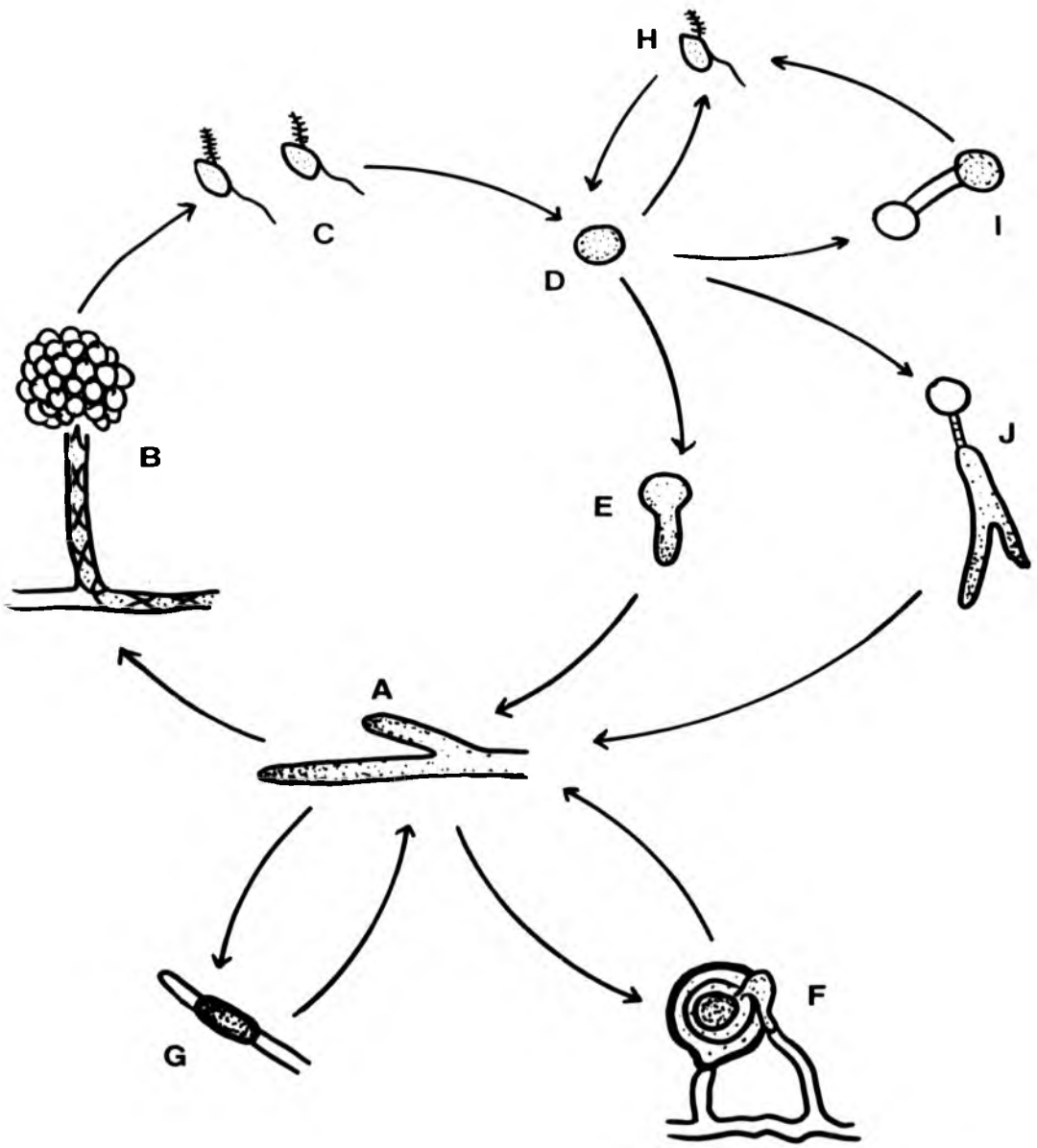


Table 2.1 **Summary of published descriptions of EUS-, MG- and RSD-associated *Aphanomyces***

	***Aphanomyces invaderis* (=*A. invadans*)**	***Aphanomyces piscicida***	**RSD-*Aphanomyces***
Reference	**Willoughby *et al* (1995)**	**Hatai (1980a)**	**Fraser *et al* (1992)**
Hyphal Ø	In fish tissue: 11.7 - 16.7 μm On GP agar: 8.3 μm	On isolation from fish: 12-36 μm In FME broth: 5-20 μm	Growing from muscle: broad, thick-walled
Mycelium appearance	Wavy as dies in culture or diseased fish		Colonies on CDA: flat, opaque, no aerial hyphae
Radial growth rates, and temperature limits for growth	On GP agar (per 24h): 4 mm @ 24°C 0 mm @ 37°C	From Hatai & Egusa (1978): 0 mm on FME agar @ 5°C 0 mm on FME agar @ 37°C 0 mm on FME agar & 10ppt NaCl	(per 24h): 6.4 mm on GY agar @ 22°C 3.9 mm on CDA @ 22°C 3.2 mm on FME agar @ 22°C 0 mm on GY agar @ 37°C 0 mm on GY agar & 12ppt NaCl
Oogonia	Not observed	Not observed	Not observed
Chlamydospores	"Problematic"	-	-
Zoosporangial width	Equal to mycelium, about 10 μm Ø	equal to mycelium	-
Zoosporangial type	Terminal on mycelia, often complex. Complex sporangia have 4 lateral evacuation tubes (630-930 μm long), 3 tubes (430-540 μm long) or 2 or 1 tube (330-470 μm long)	No basal septum. Simple, but on rare occasions complex with lateral evacuation tubes. Isodiametric or tapering. 15-250 μm long, usually 20-40 μm	Separated by thin septa. Terminal or intercalary. Terminal sporangia may or may not have lateral evacuation tubes. Intercalary sporangia only have lateral evacuation tubes
Zoosporangial renewal	Sympodial branching below empty sporangium	-	-
1° zoospore comments	Spores that fail to escape from sporangium fuse and germinate readily as a large unit, possible organs of dissemination	Spherical or elliptical. Form in a line	Single row connected by thin strand of cytoplasm
1° zoospore cyst clusters	Achlyoid	Achlyoid. Usually >10 cysts, rarely >20. Occasionally germinates directly	Achlyoid. Usually 30-50 cysts
1° zoospore cyst Ø	6.7 - 10 μm	5-23 μm, usually 8-9 μm	-
2° zoospore comments	Motile, biflagellate	Motile, biflagellate. Flagella of equal length	Motile, subsperical, biflagellate. Released within 12 hours of sporangial development @ 22°C. Sporulation poor at 2ppt NaCl
2° zoospore cyst Ø	-	-	-

KEY: Ø = diameter

Table 2.2

Details of EUS, RSD and MG *Aphanomyces* isolates used in this study

Species	Isolate	Isolated from	Disease	Date	Location	Worker
Aphanomyces invadans	TA1	striped snakehead	EUS	Feb 91	Thailand	L.G. Willoughby
A. invadans	RF6, RF8	striped snakehead	EUS	Jan 92	Suphanburi, Thailand	L.G. Willoughby
A. invadans	S1PA, S3PA	striped snakehead	EUS	Jan 94	Suphanburi, Thailand	J.H. Lilley
A. invadans	G2PA	three spot gourami	EUS	Jan 94	Bang Na, Bangkok, Thailand	J.H. Lilley
A. invadans	PA1, PA3, PA4, PA5, PA7, PA8, PA10	striped snakehead	EUS	Jan 95	Nonthaburi, Thailand	J.H. Lilley
A. invadans	96PA	snakehead	EUS	Jan 96	Pichit, Thailand	J.H. Lilley
A. invadans	BH	round-tailed garfish	EUS	Jan 93	Srimanagal, NE Bangladesh	L.G. Willoughby & J.H. Lilley
A. invadans	BR	rohu	EUS	Jan 93	Parbatipur, NW Bangladesh	L.G. Willoughby & J.H. Lilley
A. invadans	BS	mud murrel	EUS	Jan 93	Srimanagal, NE Bangladesh	L.G. Willoughby & J.H. Lilley
A. invadans	36/1P	sand goby	EUS	Jan 93	Bogor, West Java, Indonesia	D. Bastiawan
A. invadans	30P	striped snakehead	EUS	Nov 91	Laguna Lake, Sth Luzon, Philippines	J.O. Paclibare
A. invadans	33P	striped snakehead	EUS	Dec 91	Bautista, Central Luzon, Philippines	J.O. Paclibare
A. invadans	34P	grey mullet	EUS	Dec 91	Buguey, Nth Luzon, Philippines	J.O. Paclibare
A. invadans	10D	"snakehead"	EUS	~1992	Philippines	J.O. Paclibare
Aphanomyces sp	3P	grey mullet	RSD	Jun 89	Richmond R., NSW, Australia	G.C. Fraser
Aphanomyces sp	4P	yellowfin bream	RSD	Jun 89	Clarence R., NSW, Australia	G.C. Fraser
Aphanomyces sp	10P	sand whiting	RSD	Jun 89	Richmond R., NSW, Australia	G.C. Fraser
Aphanomyces sp	24P	grey mullet	RSD	May 90	Saltwater Ck, Nth Queensland, Australia	A. Thomas
Aphanomyces piscicida	NJM9030	ayu	MG	1990	Schizuoka, Japan	K. Hatai
A. piscicida	NJM9201	dwarf gourami	MG	1992	Tokyo, Japan (imported from Singapore)	K. Hatai

SPECIES KEY: three spot gourami, *Trichogaster trichopterus* (Pallas); round-tailed garfish, *Xenentodon cancila* (Hamilton); rohu, *Labeo rohita* (Hamilton); mud murrel, *Channa punctata* (Bloch); sand goby, *Oxyeleotris marmoratus* (Bleeker); grey mullet, *Mugil cephalus* L.; yellowfin bream, *Acanthopagrus australis* (Owen); sand whiting, *Sillago ciliata* Cuvier; dwarf gourami, *Colisa lalia* (Hamilton-Buchanan)

Table 2.3 **Saprophytes and non-Asian pathogens used for comparative purposes in this study**

Species	Isolate	Isolated from	Disease	Date	Location	Worker
Aphanomyces sp	84-1240 (=ATCC 62427), 84-1249, 84-1282	menhaden	UM	May 84	Nth Carolina, USA	M.J. Dykstra
Aphanomyces astaci	FDL457, FDL458	white-clawed crayfish	crayfish plague	NA	R. Arrow, Herefordshire, UK	D.J. Alderman
A. astaci	J1	noble crayfish	crayfish plague	1962	Ämmern, Östergötlands, Sweden	T. Unestam
A. astaci	PL	signal crayfish	crayfish plague	1970	L. Tahoe, USA	K. Söderhäll
A. astaci	KV	signal crayfish	crayfish plague	1978	Sweden (imported from Pitt Lake, Canada)	K. Söderhäll
A. astaci	PC	red swamp crayfish	crayfish plague	~1994	Spain	J. Diéguez-Uribeondo
Aphanomyces sp	TF5	striped snakehead	EUS	Jan 91	Thailand	S. Chinabut
Aphanomyces sp	TF33	swamp eel	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Aphanomyces sp	TF41	striped snakehead	EUS	Jan 92	Thailand	L.G. Willoughby
Aphanomyces sp	TF54	striped snakehead	EUS	Jan 93	Thailand	L.G. Willoughby
Aphanomyces sp	T1SA	soft shell turtle	fungal infection	Dec 93	Kasetsart, Bangkok, Thailand	W. Valairatana
Aphanomyces sp	A2SA	aquarium tank	-	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Aphanomyces sp	F3SA	striped snakehead	fungal infection	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Aphanomyces laevis	ASEAN1, ASEAN3	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	L.G. Willoughby
Aphanomyces sp	SSA	striped snakehead	EUS	Feb 94	Suphanburi, Thailand	W. Valairatana
Aphanomyces sp	WSA	fish pond	-	Sep 94	Suphanburi, Thailand	W. Valairatana
Aphanomyces sp	SA11	striped snakehead	EUS	Jan 95	A. Bangkuai, Nonthaburi, Thailand	J.H. Lilley
Achlya sp	S2AC	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Achlya diffusa	W2BAC	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	L.G. Willoughby
Achlya sp	AC2, AC5, AC10	striped snakehead	EUS	Jan 95	Bangkuai, Nonthaburi, Thailand	J.H. Lilley
Saprolegnia sp	TF20S, TF23, TF25, TF26,	striped snakehead	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Saprolegnia sp	TF24	three spot gourami	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Saprolegnia sp	TF29	swamp eel	EUS	Dec 91	Sakhon Nakhon, Thailand	L.G. Willoughby
S. diclina	TF27	striped snakehead	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
S. diclina	TF31	swamp eel	EUS	Dec 91	Sakhon Nakhon, Thailand	L.G. Willoughby
S. australis	*S. aust* (=795) (=ATCC 42060)	skelly	fungal infection	1977	Ullswater	L.G. Willoughby
S. ferax	P32	lake water	-	1957	Lake Windermere, UK	L.G. Willoughby
S. diclina (?)	E3 (=ATCC 36144)	lake water	-	1960	Lake Windermere, UK	L.G. Willoughby
S. parasitica	TP41 (=ATCC 42062)	brown trout	fungal infection	1970	Hatchery, Windermere, UK	L.G. Willoughby
Phytophthora cinnamomi	IMI 335418	Macadamia	plant pathogen	1989	Australia	P.M. Wood

SPECIES KEY: *Aphanomyces laevis* deBary; *Achlya diffusa* Harvey; *Saprolegnia ferax* (Gruithuisen); menhaden, *Brevoortia tyrannus* (Latrobe); white-clawed crayfish, *Austropotamobius pallipes* Lereboullet; noble crayfish, *Astacus astacus* L.; signal crayfish, *Pacifastacus leniusculus* Dana; red swamp (Louisiana) crayfish, *Procambarus clarkii* Girard; swamp eel, *Fluta alba* (Zview); soft shell turtle, *Trionyx cartilagineus* (Boddaert); skelly, *Coregonus lavaretus* L.; brown trout, *Salmo trutta* L.

(?) possibly incorrectly assigned (see Molina *et al*, 1995)

CHAPTER 3. PATHOGENICITY STUDIES[1]

3.1 Introduction

A. invadans has already been shown to be capable of reproducing EUS lesions when mycelium of isolates RF6 and RF8 (Roberts *et al,* 1993) or zoospores of RF6 (Chinabut *et al*, 1995) are injected into the muscle of snakehead fish (*Channa striata*). However, as described in the background Section 1.5.1, a number of *Saprolegnia*, *Achlya* and *Aphanomyces* species have been associated with EUS affected fish, and given that normally perthotrophic fungi can invade muscle tissue in some circumstances (Section 1.6.6), there was a need to conduct challenge studies to compare the pathogenic capabilities of several *A. invadans* isolates with a number of other fungi isolated from EUS lesions, or EUS affected waters (Noga, 1994).

The manifest similarities of MG, RSD and UM to EUS have led to recommendations by workers for the implementation of direct comparative studies to establish the relationship between the fungal aetiologies of each case (Wada *et al,* 1994; Callinan, 1994b; and Noga, 1993a). Isolates of other fungi involved in invasive mycoses of aquatic animals (ie crayfish plague, Section 1.6.4; and saprolegniasis of fish, Section 1.6.6) were also obtained for comparative studies described here.

A tested challenge model based on that described by Chinabut *et al* (1995) was adopted to address the need for comparative pathogenicity studies as described above. In addition to these studies, preliminary immersion challenges were conducted using artificially injured fish and cold-shocked fish to evaluate the ability of *A. invadans* zoospores to initiate an infection. Finally, bath challenges using plague-susceptible noble crayfish (*Astacus astacus*) are described here.

[1]Some of the work described in Chapters 3 and 4 is published in Lilley, J.H. and Roberts, R.J. (1997) Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. Journal of Fish Diseases 20, 135-144.

3.2 Materials and methods

3.2.1 Fish and tanks

Fish challenge experiments were carried out at the Aquatic Animal Health Research Institute (AAHRI), Bangkok. Snakehead (*Ch. striata*) fingerlings were obtained locally and held at AAHRI for at least one month before experiments began. The average length and size range of the fish over the trial period was 15.5 ± 2.5 cm.

Experimental tanks were kept in a 22°C constant temperature room. The testing of each fungal isolate was treated as a separate experiment. For each experiment, three fish were introduced to a tank containing 12 litres of water two days before challenge to allow for temperature equilibration. Following challenge, fish were fed once daily with commercial snakehead feed pellets. Snakeheads are air-breathers and no aeration or water exchange was required during the seven-day long experiments.

3.2.2 Preparation and assay of inocula for injection challenge studies

Fifty eight fungal isolates as listed in Appendix 1 were selected for study. All isolates were maintained at 10°C on Petri dishes of GPY agar (see Appendix 2a). Suspensions of motile secondary zoospores were prepared as described in Section 2.2.5(a) using 4 mm agar plugs incubated in GPY broth at room temperature (~26°C) for 48 hours. The resulting mycelial mats were washed in APW (Appendix 2a) and left overnight at room temperature, with the exception of *Aphanomyces astaci* and *Saprolegnia australis* strains which required a lower temperature for sporulation. The resulting zoospore suspensions were filtered through two sterile sheets of Whatman 541 filter paper to ensure that only motile secondary zoospores were collected. The suspensions were assayed to estimate zoospore numbers and used for fish challenges. Control samples were passed through a syringe and checked under a microscope to ensure that zoospores remained motile.

Zoospore concentrations were determined by performing five 1 ml in 20 ml serial dilutions of each inocula in Petri dishes containing APW, and then adding 0.66 ml of

concentrated assay broth (Appendix 2a). These were left for four days at room temperature, after which the dish containing fungus, but with the lowest number of colonies, was used to calculate the zoospore concentration in the original inoculum. As it took four days to obtain an accurate zoospore count, no attempt was made to adjust zoospore concentration at the time of the challenge experiments.

3.2.3 Injection challenge experiments

Fish were injected intramuscularly with 0.3 ml of the freshly filtered zoospore inoculum into the flank half-way between the anterior parts of the dorsal and pectoral fins. Negative control fish were injected with APW only. All fish were sacrificed seven days post-injection and the site of inoculation excised and fixed in chilled 10% buffered formalin.

3.2.4 Histopathology

The fixed blocks of tissue from each fish were trimmed, embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H&E) and Grocott's stain (Appendix 3). Selected slides were also stained using the periodic acid-Schiff (PAS) reaction (Appendix 3).

3.2.5 Preliminary immersion challenge experiment (a) scraped fish

All procedures for this experiment, including acclimatisation of fish, were carried out at 22°C. Twenty 4-mm agar plugs of *A. invadans* isolate S1PA were used to produce 1 litre of a filtered zoospore suspension as described above. Six snakeheads, 2 of which had a 0.5 cm^2 section of the skin scraped with a scalpel, were placed in a plastic bag containing the suspension for 5 hours. The fish were then transferred to a tank containing 15 litres of tap water and observed daily. An attempt was made to assay the zoospore suspension at the beginning and end of the 5 hour period, but valid counts were not obtained due to excessive contamination of the assay plates. A few motile zoospores were, however, observed directly under a microscope at the end of the 5 hour challenge.

3.2.6 Preliminary immersion challenge experiment (b) cold shock treatment

Forty 4-mm agar plugs of *A. invadans* isolate PA7 were used to produce sporulating mycelium mats as described above. The mats were placed in a tank containing 14 litres of APW at 20°C. Ten snakeheads, acclimatised to normal outdoor temperatures of 26-27°C, were transferred directly to the tank and monitored daily. Water samples from the top, middle and bottom strata of the tank were taken for zoospore assays, however, reliable counts were not obtained due to contamination of the assay plates.

3.2.7 Crayfish immersion challenge experiment

A. invadans isolate PA7 was also tested for its ability to infect noble crayfish, *As. astacus*, a species highly susceptible to the plague fungus *Aphanomyces astaci*. This experiment was carried out at the Department of Physiological Botany, Uppsala University, Sweden. Large volumes of motile zoospores were produced as described in Section 2.2.5(e). Duplicate tanks, each containing two crayfish immersed in 3 litres of PA7 zoospore suspension (at 40,000 spores/ml) were kept at 21°C for 24 hours before transfer to 13°C. A control tank contained two crayfish in a suspension of zoospores of the *A. astaci* strain, Hö (at 10,000 spores/ml).

3.3 Results

3.3.1 Fish injection challenge experiments

Zoospore concentrations were very variable, but in general lower for the group of EUS, RSD and MG isolates (averaging 4,000 spores/ml) than for the other fungi (all the other isolates in combination averaged 12,500 spores/ml). It is significant to note that the zoospore counts of the 1995 *A. invadans* isolates, obtained only a week before the trials, were on average over four times higher than zoospore counts of the older *A. invadans* isolates.

A small number of fish died during the course of the experiments, and in these cases attempts were made to reisolate fungus from within the muscle (see Section 2.2.2).

This proved possible with nine fish injected with slow-growing *Aphanomyces* at times ranging from 2-14 days post-injection. These reisolates were confirmed as slow-growing Aphanomyces and given the epithet (K) (denoting Koch's postulate isolate) for later comparative studies. Two *Saprolegnia* isolates, TF25 and TP41, were also reisolated at day 6 and day 5 post-injection respectively, but these fish showed no gross lesions and no signs of fungal growth in histological section.

3.3.2 Histopathology

The host tissue response after 7 days post-injection fell into two main types. In all cases there was evidence of some mild traumatic damage such as sarcoplasmic degeneration, haemorrhage and cellular inflammatory infiltration. However there was a very distinct difference between the response to the EUS, RSD and MG *Aphanomyces* isolates and that to the other *Aphanomyces*, *Saprolegnia* and *Achlya* spp.

With the EUS, RSD and MG *Aphanomyces* isolates, in all cases there was a typical invasive lesion similar to that described by Roberts *et al* (1993). Numerous delicate fungal hyphae were observed at levels extending from the site of injection, right down to the spinous processes and paravertebral ganglia. In some areas these were associated with very extensive floccular degeneration of muscle sarcoplasm with very little or no myophagia. In other areas there was extensive myophagia and also extensive epithelial and granulomatous accumulation around the developing mycelium. This was accompanied by only limited fibroblastic proliferation and recapillarisation (Plates 3.1 & 3.2).

None of the other strains showed any evidence of frank pathogenicity. The usual reaction was one of varying degrees of myonecrosis in response to physical damage associated with the inoculum at the site of injection. This was often accompanied by organising haemorrhage. In contradistinction to the usually acellular sarcoplasmic degeneration of the pathogenic strains, the myonecrosis was usually associated with

active myophagia, and the entire area showed signs of macrophage activity and fibroblastic proliferation and organisation (Plate 3.3). There was normally no evidence of fungal hyphae present and it was not possible to demonstrate effete spores. In three cases, one *Achlya* (AC2), one *Aphanomyces* (T1SA) and one *Saprolegnia* (TF29), the reaction was similar to the others except that there was evidence of some limited germination of zoospores with vestigial hyphal growth. In all three cases however, it was found that the inoculum had extremely high zoospore counts with 16,000-64,000 spores/ml, some 2-8 times that of the average for all the isolates.

3.3.3 Preliminary immersion challenge experiment (a) scraped fish

During the period up to day 10, both unscraped fish and one scraped fish became moribund, showing signs of "tail rot" with sloughing of epidermal tissue and scale loss. Histology revealed no EUS-associated internal pathology. On day 12, a fish with scale loss, but also mild necrotising dermatitis at the artificially damaged site was sampled for histology. Sections revealed very few hyphal segments in dermis and muscle tissue underlying the lesion. There was also some associated muscle degeneration. On day 14 a fish with similar clinical signs was sampled for fungus isolation. An *Aphanomyces* fungus was obtained from the muscle tissue and was later diagnosed as *A. invadans* on the basis of growth characteristics. The last fish, which had also shown mild necrotising dermatitis around day 12-16, was sampled on day 26. By this time the lesion had healed substantially and this was confirmed by histological section. No hyphal segments could be identified.

3.3.4 Preliminary immersion challenge experiment (b) cold shock treatment

During the period up to day 15, five fish were sampled for histology showing signs of "tail rot". On day 18 and day 22, an external fungal infection was observed on two fish. Isolation and examination of the fungi revealed only *Achlya* sp in both cases. On day 23, two of the remaining three fish were sampled showing small petechial lesions on the tail. All fish were examined histologically but no signs of invasive hyphae or granulomatous response were observed.

3.3.5 Crayfish bath-challenge experiments

Both control crayfish died within 6 days heavily infected with *A. astaci*. All the PA7-challenged crayfish survived showing no significant pathological signs, and the experiment was terminated after two months.

3.4 Discussion

In these studies, all EUS, MG and RSD isolates, and no other fungi, were capable of extensively penetrating muscle tissue and reproducing early EUS-type lesions. Therefore, the possibility that multiple fungal opportunists are involved in producing the distinctive fungus-related pathology of EUS lesions is convincingly ruled out. The distinction made by Hughes (1994) between perthotrophs, parasites and pathogens is clearly relevant here. He suspected that most water moulds are potentially good perthotrophs, and as EUS can result in the exposure of large amounts of necrotic tissue on fish, these lesions provide a substrate for several different competing saprolegniaceans. These vigorous saprophytes are the fungi that are usually isolated from such lesions, and have meant in the case of EUS, that the real pathogen has gone unnoticed for some time. In other mycotic diseases, where the pathogen does not result in such distinctive diagnostic signs as EUS, it may be even more difficult to know whether the true pathogen has been isolated or not.

The pathology induced in snakeheads by the EUS, MG and RSD isolates was indistinguishable, thereby providing evidence that these isolates all constitute the same, fish-pathogenic species. Further studies comparing these fungi *in vitro* are given in subsequent chapters.

Although this study confirms reports of similarities in the histopathology of EUS, MG and RSD, there was no evidence of the invasive nature of the UM fungus. Noga (1993a) reported that attempts to reproduce UM in menhaden using this isolate had also failed. Dykstra *et al* (1989) stated that their UM *Aphanomyces* isolates could be

divided into three categories according to morphological and growth features. The isolate used here (84-1240) belongs to their first category that grew vigorously and produced sexual stages and could therefore be tentatively diagnosed as *Aphanomyces laevis*. This is consistent with *A. laevis* isolates from Thailand used in the present study which also proved non-pathogenic to snakeheads. The third group described by Dykstra *et al* (1989) however, produced "scant mycelium and few zoospores", and if UM has any relationship to EUS, this category may well represent the invasive UM pathogens. A specific request was made to obtain isolates from this group for the present study, but they are no longer being maintained (M.J. Dykstra, pers. comm.).

The slow-growing, pathogenic and sexually-sterile nature of the crayfish plague fungus, *A. astaci*, has led to authors drawing comparisons between this fungus and the EUS and RSD pathogens (Roberts *et al*, 1993; Callinan *et al*, 1995a). Further, Diéguez-Uribeondo, Huang, Cerenius and Söderhäll (1995) have recently isolated a strain of *A. astaci* that is physiologically adapted to warm temperatures. However, this study clearly shows that *A. astaci* does not have the ability to grow in snakehead fish and *A. invadans* is unable to infect noble crayfish, one of *A. astaci*'s natural hosts.

Saprolegnia parasitica (or *S. diclina* type 1) isolate TP41 (ATCC 42062) also could not be detected growing in snakeheads. Other challenge studies by Bly *et al* (1992) using the same isolate failed to reproduce even a superficial infection in channel catfish. In that case, cold-shocked catfish were shown to succumb to "winter saprolegniasis" when exposed to zoospores of local parasitic *Saprolegnia* isolates, but not to TP41. Wood (1988) and Cross and Willoughby (1989), however, used TP41 to induce typical saprolegniasis in salmonids, although this does not involve penetration of the musculature.

Southgate (1983) compared staining techniques of fish and crayfish tissues infected by *S. parasitica* and *A. astaci* respectively, and showed that whereas a variety of

procedures were applicable for *Saprolegnia*, only Grocott's silver impregnation technique was of use for *Aphanomyces*. Of the three histological staining procedures used in this study, Grocott was the only one that clearly demonstrated *Aphanomyces* hyphae in fish tissue. With careful observation, hyphae could be identified with PAS and H&E although the extent of the infection was far more difficult to ascertain. The use of polyclonal antibody staining techniques is discussed in Chapter 8.

Chinabut *et al* (1995) showed that snakeheads succumbed much more readily to infection by *A. invadans* at 19°C than at 26°C or 31°C. Two snakeheads injected here with PA7 were left 2 weeks post-injection at the experimental temperature of 22°C, before sampling. Although these fish had not died, they developed further severe myonecrosis and external haemorrhagic lesions consistent with fish kept at 19°C (Chinabut *et al*, 1995), indicating that the disease was still progressing and that the fungus is also potentially lethal at this temperature.

Preliminary injection challenges were found to be not consistently infective unless an assay count of over 50 spore/ml (15 spores/0.3 ml inoculum) was achieved; and earlier immersion trials had failed to induce EUS in healthy snakeheads at a number of undetermined zoospore concentrations. Unestam and Weiss (1970) reported a reverse situation involving *A. astaci* challenges against *As. astacus*. They calculated an LD_{50} value of 20,000 spores per 0.05 ml inoculum in injection trials and an LD_{50} value of only only 30 spores/ml of water in immersion trials. Alderman *et al* (1987) considered that mortalities would have been 100% in these immersion trials, at even lower zoospore concentrations, if the disease was given enough time to develop.

In contrast to snakeheads, rainbow trout have been shown to be susceptible to infection by *A. invadans* (PA7) only at temperatures above 15°C (K.D. Thompson, pers. comm.). In intramuscular injection challenges carried out over 28 days at 18°C, as many as 10,000 spores per 0.2 ml inoculum were required for fungus to be observed in histological sections of all 25 fish tested; to induce lesions in 44% of fish;

to result in significant mortalities (60% of fish); and to produce a significant antibody titre in injected fish. Percentage mortalities were 84%, 8% and 0% at 100,000, 1000 and 100 zoospores per 0.2 ml inoculum respectively (K.D. Thompson, pers. comm.).

Despite its clear pathogenic qualities in snakeheads, *A. invadans* can not be confirmed as the primary cause of EUS unless fungal propagules are shown to breach the fishes skin unaided, as in the crayfish challenges with *A. astaci* described above. Preliminary bath challenges described here succeeded in reproducing early stage infections in a few artificially injured snakeheads but not in cold-shocked snakeheads. Other workers have succeeded in inducing ulcers using RSD isolates in bath challenges with abraded fish (Callinan, 1994b), and more significantly, with fish exposed sublethally to acid water (Callinan *et al,* 1996a). This latter study confirms that RSD isolates can act as primary pathogens, when combined with particular environmental conditions. As described in Section 1.5.2, acidified water conditions do not occur in many EUS outbreaks, and therefore further investigation of the disease process is required.

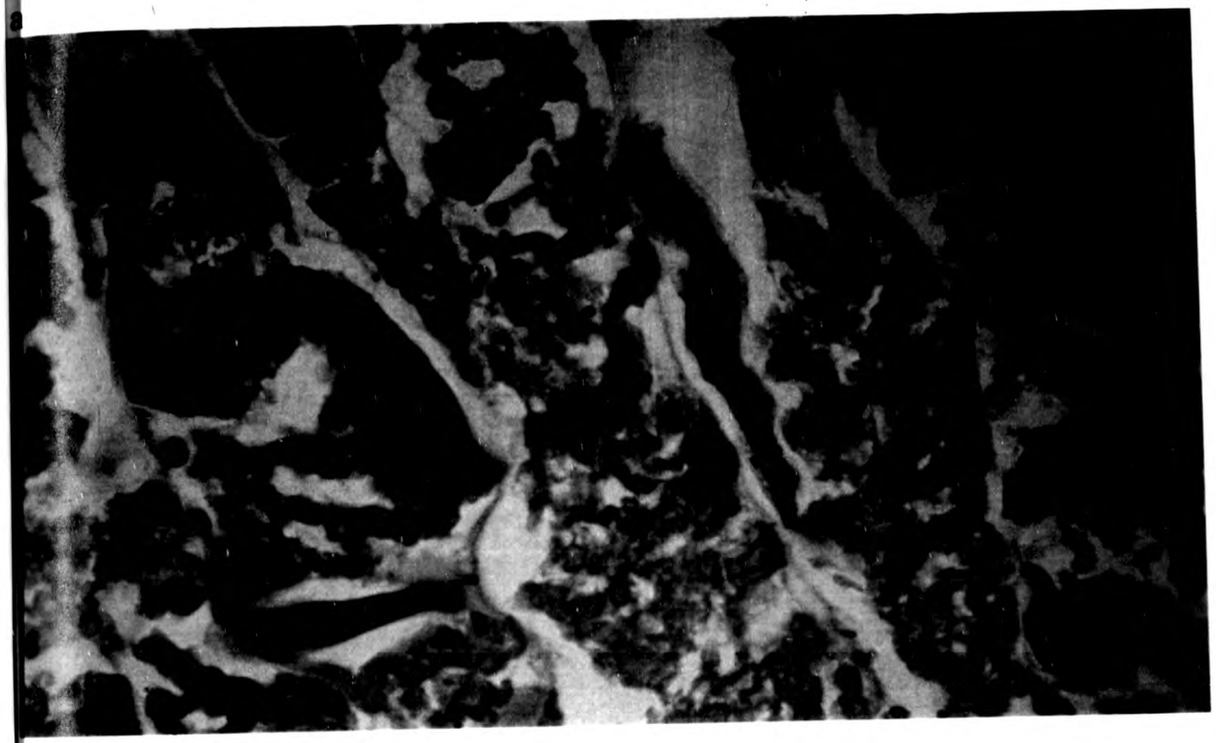
A tempting explanation of the initial EUS outbreaks in an area, which tend to have an explosive impact on fish populations, is that EUS-naïve fish can be susceptible to infection by *A. invadans* zoospores whereas fish in EUS endemic areas have developed an innate resistance. Cruz-Lacierda and Shariff (1995) were consistently successful in transmitting the disease to EUS-naïve snakeheads by simply exposing them to affected fish or water, whereas similar experiments in Thailand, using farmed fish from an area that has suffered repeated annual outbreaks since 1982, were consistently unsuccessful (unpublished). The snakeheads used in those latter studies were from the same source as the fish studied here. Whether any possible innate resistance in these fish is due to selection processes, or the development of a protective immune response as a result of sublethal exposure, is a matter for further speculation. Studies on the immune response of fish to *A. invadans* are presented in Chapter 9.

Another, widely attested theory, is that some other biological agent may be important in the disease process. Recent trials have induced 100% (20/20) EUS infection in snakeheads injected with a rhabdovirus and bathed in *A. invadans* zoospores, whereas vehicle-only injections with zoospore immersion resulted in 35% (7/20) snakeheads infected (Kanchanakhan, 1996b). These experiments were carried out at 20°C, at AAHRI, using fish from the same source as in present trials, and with a rhabdovirus isolated from the same EUS outbreak that G2PA was obtained. Now that isolation techniques have been optimised, Kanchanakhan (1996b) has reported a high recovery rate of rhabdoviruses from EUS outbreaks in Thailand. However, immersion challenges with suspensions of both rhabdovirus and zoospores have yet to be carried out. Parasite-induced infections described by Subasinghe (1993) also require further analysis, as in that case, the burdens of *Trichodina* required were lethal to fish anyway, and EUS affected fish and water, rather than *A. invadans* alone, were used to transmit the disease.

One further consideration for fish challenge experiments is that the virulence of *A. invadans* isolates may become reduced after repeated subculturing. Unestam and Svensson (1971) showed that *A. astaci* isolates lost virulence, zoospore motility and zoospore production capacities after a number of years in culture, but that the first two factors were reversible by selection for these characters. In the present trials, cultures of *A. invadans* isolated the previous week showed considerably more hyphae in histological section than older isolates, but this is thought to be due to the higher zoospore yields obtained and used for inoculation.

Plate 3.1

(a) Floccular degeneration of muscle sarcoplasm showing hyphal segments of Indonesian EUS isolate 36/1P (7 days post-injection, H&E, x700)

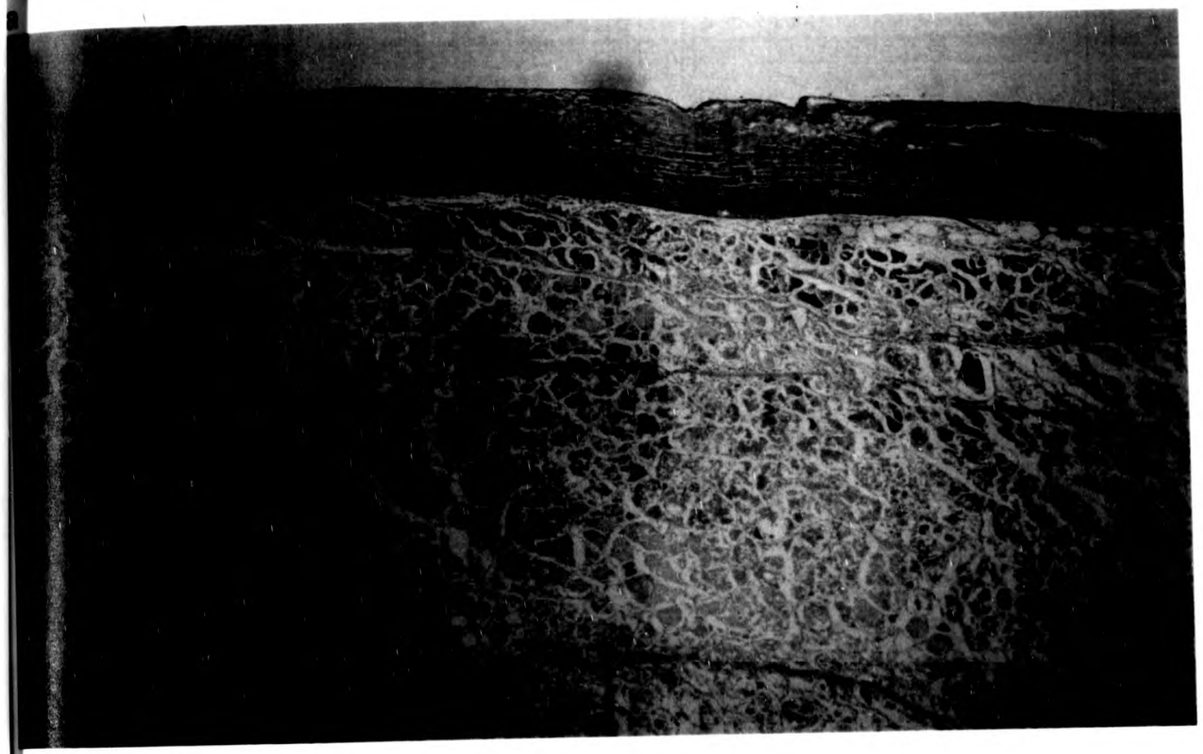


(b) Extensive penetration of Thai EUS isolate PA7 (7 days post-injection, Grocott's stain, x170)

Plate 3.2

Extensive fungal growth and muscle degeneration away from the site of injection, seven days post-injection of MG isolate, NJM9030

(a) Muscle degeneration (H&E x170)



(b) Associated fungal hyphae (Grocott's x170)

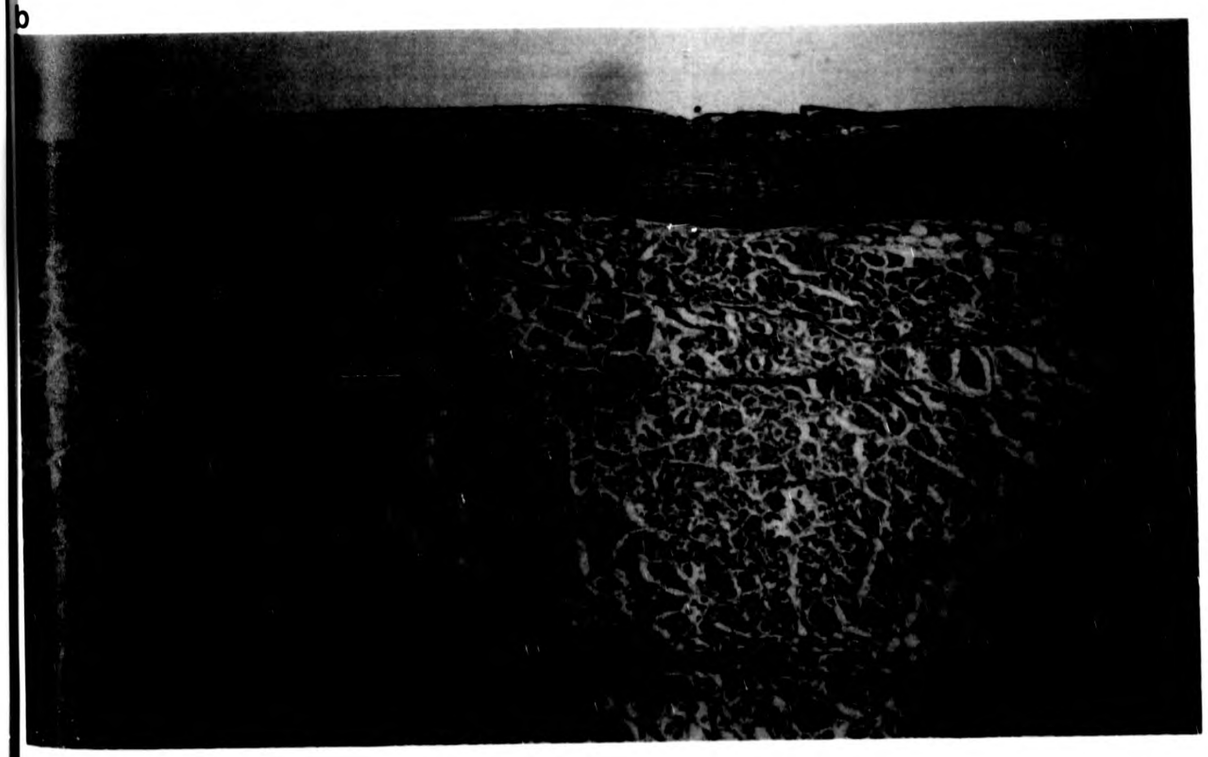
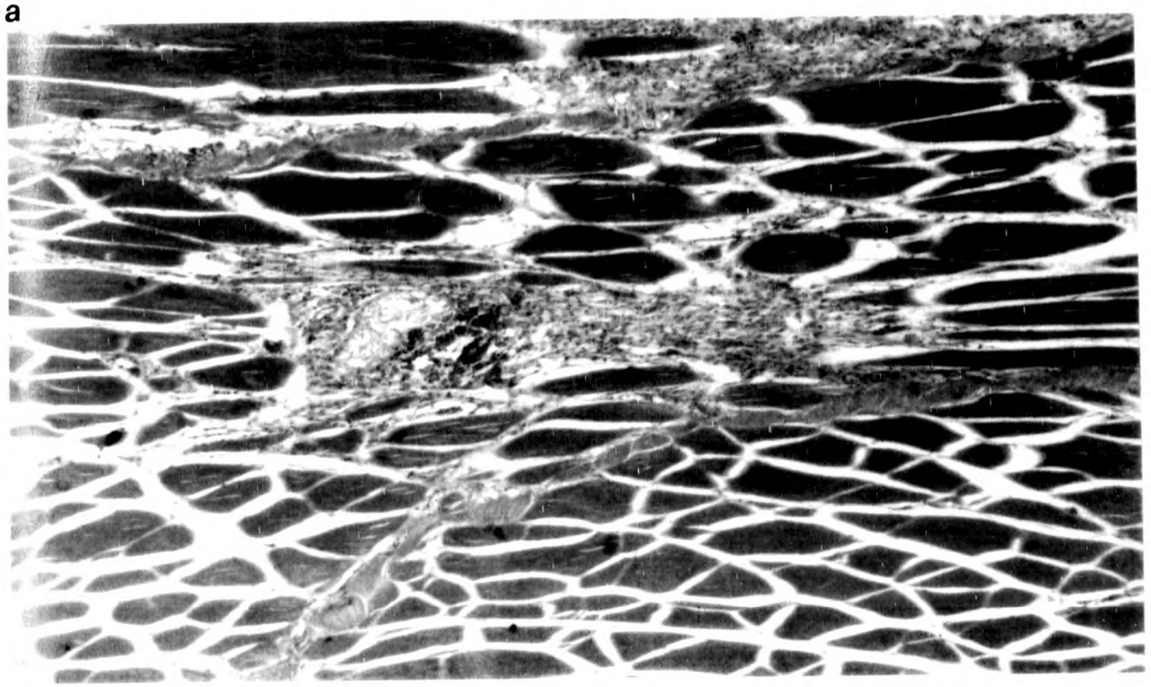


Plate 3.3

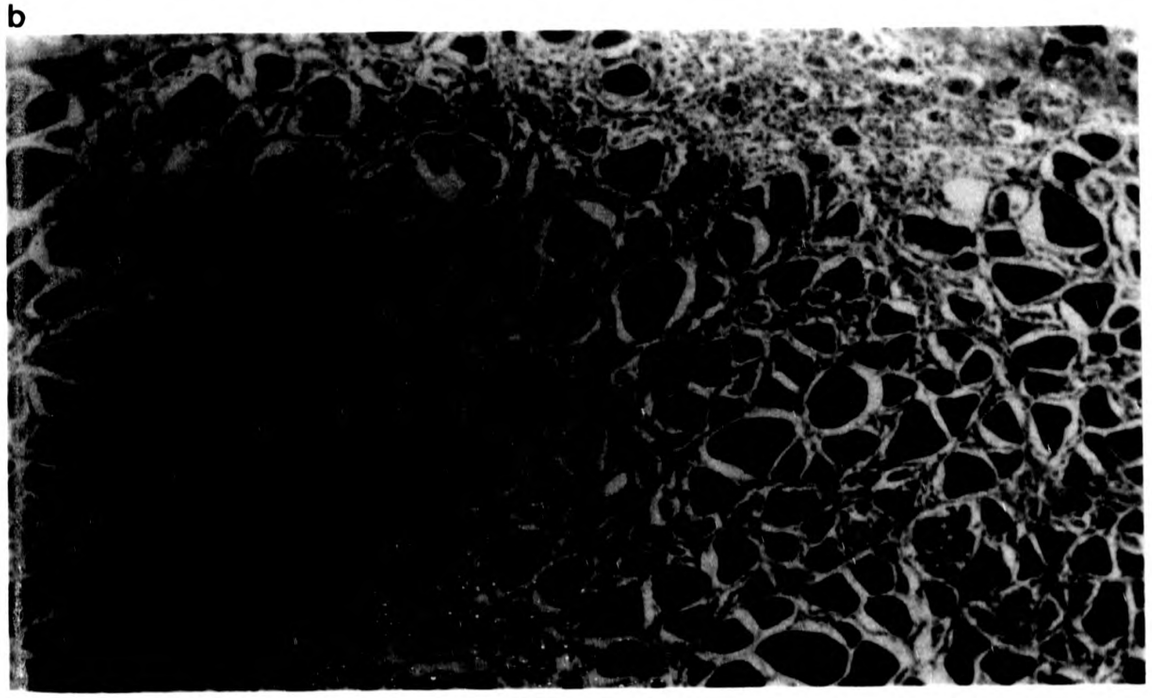
(a) Mild cellular inflammatory infiltration in reaction to injection of saprophytic *Aphanomyces* (TF54) zoospores. No fungus was observed in Grocott-stained sections (7 days post-injection, longitudinal section, H&E x170)

(b) Inflammatory response and active myophagia in reaction to injection of *Saprolegnia* isolate TF25. No fungus was observed in Grocott-stained sections (7 days post-injection, transverse section, H&E x170)

a



b



CHAPTER 4. GROWTH AND CULTURE EXPERIMENTS

4.1 Introduction

Temperature-growth profiles (Willoughby and Copland, 1984; Hatai and Hoshiai, 1992b; Beghdadi, Richard and Dostaler, 1992; Barr, Warwick and Desaulniers, 1996; Diéguez-Uribeondo, Cerenius and Söderhäll, 1996), growth on different media (Hatai *et al,* 1994), growth at different pH (Kitancharoen, Yuasa and Hatai, 1996), colonial morphology (Hansen, Brasier, Shaw and Hamm, 1986; Brasier, Hamm and Hansen, 1993), and pigment production (Kennedy and Duncan, 1995) have all provided rapid methods for distinguishing Oomycete species or strains in culture. As shown in Table 2.1, temperature-growth characteristics are presently the most distinctive means of diagnosing EUS, MG and RSD pathogenic *Aphanomyces* isolates in culture. These characteristics are assessed here, to examine whether they provide a consistent method for distinguishing pathogens from saprophytes, and whether any differences can be identified between the EUS, MG and RSD isolates.

Heterothallism (self-sterility) has been demonstrated in some species of *Achlya*, in which mating only occurs when compatible strains are brought together due to the release of particular hormones (Carlile, 1996). The possibility that this may be the reason for the reluctance of EUS, MG and RSD isolates to produce sexual reproductive structures is investigated here by a programme of pairing isolates. Various other attempts were made to induce oogonia production to aid in the speciation of isolates.

The importance of acidity in RSD and some EUS outbreaks (Section 1.5.2), and in the use of APW as a sporulation medium (Section 2.2.4) has been previously discussed. Short experiments on the effect of pH on fungal growth, the change in pH of the culture medium due to long term growth, and an evaluation of a buffered medium (PG-1) and non-buffered medium (GPY) are described here.

Study of the production of extracellular enzymes by saprolegniacean fungi has been shown to be important to the understanding of their trophic capabilities. For example, Unestam (1966) showed that out of a number of Oomycete fungi, only the three plant pathogenic *Aphanomyces* spp had appreciable cellulase and pectinase activity, and that the crayfish plague fungus, *A. astaci* showed much greater, and constitutive, chitinase activity. A preliminary study of enzyme production, using solid media substrates (Hankin and Anagnostakis, 1975), was therefore undertaken. Protease and DNase activity, as analysed here, has been previously studied in other saprolegniacean fungi (Söderhäll and Unestam, 1975; Alberts, Khan, Lim and Te Strake, 1989; Smith *et al,* 1994).

4.2 Materials and methods

4.2.1 Temperature-growth experiments

Standard 4 mm GPY plugs (Appendix 2a) taken from the edge of actively growing colonies were used as inocula for all culture studies. The effect of temperature on growth was tested on GPY at 6, 10, 14, 18, 22, 26, 30, 34, 38 and 42°C. After the plugs were placed on the test media, the linear extension of mycelia was measured on three points at right angles to each other, from the first day that a growing mycelial border was apparent, and daily thereafter until growth began to slow down or the colony had overgrown the Petri dish. Therefore the growth increment was defined as the daily increase of the mean radius of each colony over the period of the exponential growth phase. All experiments were replicated twice and the average is given.

4.2.2 Growth on different media

Growth was also compared at 22°C on the following unsupplemented media: Czapek Dox agar (CDA), cornmeal agar (CMA) (Difco Laboratories), Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and malt extract agar (MEA) (Oxoid), and on fish-meat-extract agar (FMA: filter-sterilised snakehead meat extract, warmed to 47°C before adding, at 10% v/v, to 1.2% w/v Oxoid No. 3 agar, cooled to 47°C).

4.2.3 Cluster analysis

Numerical taxonomic analysis was performed using the *Aphanomyces* growth data only. The data was not normalised as the same parameter was measured for each variable. A phenogram was constructed by the agglomeration of a squared Euclidean dissimilarity matrix using the unweighted group-average method, UPGMA (Sneath and Sokal, 1973).

4.2.4 Effect of medium pH on growth

In a short, preliminary experiment, GPY broth with 0.1 M Tris buffer was adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10. Petri dishes of media were inoculated with duplicate 4 mm GPY agar plugs of EUS (BH, 30P), UM (84-1240) and saprophytic *Aphanomyces* (TF5, ASEAN3) isolates. Growth was measured after 48 hours. The pH of the media was checked at the end of the experiment to ensure no significant change in pH had occurred.

4.2.5 Change in medium pH due to fungal growth

The change in the pH of the growth medium was monitored using a maintenance technique similar to that described by Willoughby and Chinabut (1996). Glass bottles (500 ml) containing 200 ml GP broth were inoculated with an agar plug of each isolate and kept for 2 months at 10°C before being pH-tested. 143 samples comprising 58 different fungal isolates were tested.

4.2.6 Viability of fungi on different maintenance media

GPY agar plugs (4 mm) of six EUS, RSD and MG pathogens (RF8, S1PA, PA3, 30P, 3P, and NJM9030) and six saprophytic isolates of *Aphanomyces* (TF41, A2SA), *Achlya* (S2AC, W2BAC) and *Saprolegnia* (TF26, E3), were inoculated onto 5 Petri dishes containing GPY agar, GPY-Na_2EDTA (0.044g/l) agar, GPY-sodium phosphate (13 mM) buffered agar, GPY-Na_2EDTA-sodium phosphate buffered agar or PG-1

agar. Dishes were kept at 10°C and plugs of fungi were taken after 21, 36 and 56 days and inoculated onto GPY agar to test for viability.

4.2.7 Clearance zones

Extracellular protease and DNase production were assessed by production of clearance zones on 10% skimmed milk (Oxoid) agar and DNase agar (Oxoid) respectively. Four millimetre GPY agar plugs of fungi were inoculated onto sterile 0.2 μm polycarbonate membranes (Nuclepore) placed over the top of agar plates as described by (Chang, Yang and Ko, 1992). The membrane was peeled off after 7 days at room temperature (19-22°C) and the clearance zone measured in relation to the fungal colony radius. Clearance halos were visualised using DNase agar by flooding plates with 1N NaCl. Some experiments were run without membranes to see if this made a significant difference or using culture medium in wells in the agar to examine the extent of enzyme release.

4.2.8 Pigment production

Between 1995 and 1996, five separate sets of GP broth cultures of most isolates listed in Tables 2.2 & 2.3 (including reisolates from artificially infected snakeheads, cultured separately and labelled K) were assessed for pigment production. Each set of cultures were maintained as described in Section 4.2.5, for 2 months at 10°C, after which both mycelial colonies and culture media were visually graded from 0 to 3 depending on level of pigment production.

4.2.9 Attempts to induce production of sexual reproductive structures

Several combinations of isolates were grown together on GPY agar to investigate the possibility of heterothallism in present asexual isolates (Table 4.1). They were examined for oogonia over a period of 3 weeks during which time plugs were taken several times for sporulation as in Section 2.2.5(a), and again examined for oogonia. Petri dishes were kept in the dark as Fowles (1976) reported that light inhibited the formation of oogonia in *Aphanomyces cochloides*.

Table 4.1 Combinations of *Aphanomyces* isolates grown together on GPY agar plates and then sporulated to test for oogonia production

		TA1	RF6	S1PA	G2PA	BR	BH	10D	84-1240	TF5
EUS path -	TA1									
EUS path -	RF6	X								
EUS path	RF6(K)		X							
EUS path -	RF8		X							
EUS path -	S1PA	X	X							
EUS path -	S3PA			X						
EUS path -	G2PA	X	X	X						
EUS path -	BR	X	X	X	X					
EUS path -	BH	X	X	X	X	X				
EUS path -	BS					X	X			
EUS path -	10D	X	X	X	X	X	X			
UM fungus -	84-1240	X	X	X	X	X	X	X		
sap -	TF5	X	X	X	X	X	X	X	X	
sap -	TF41									X
sap -	TF54									X
sap -	T1SA	X	X	X	X	X	X	X	X	X
sap -	A2SA									X
sap -	F3SA									X

KEY: path = pathogen; sap = saprophyte

In addition to this experiment, attempts were made to induce oogonia production using hemp seed, V8 broth (Appendix 2a), and CMA with 1g/l peptone (as in Paternoster and Burns, 1996).

4.3 Results

4.3.1 Temperature-growth experiments and growth on different media

Average growth rates of all the isolates under various temperature and nutrient conditions are given in Table 4.2. There was no significant difference between the growth rates of the EUS, RSD and MG *Aphanomyces*. All showed optimal growth on GPY at 26-30°C; and failed to survive at 38°C. Further tests showed that they also did not grow at 37°C.

They had a lower growth rate under all culture conditions than any of the Thai saprophytic *Aphanomyces* and were unable to grow on SDA, CMA and MEA. *A. astaci* also failed to grow on SDA, CMA and MEA and was thermolabile and slow-growing, but was clearly more adapted to colder conditions than the Asian pathogens.

The UM *Aphanomyces* also had a preference for colder temperatures and was able to grow on CMA, MEA and relatively well on SDA.

The Thai saprophytic *Aphanomyces* strains were able to grow under all culture conditions tested with optimal growth on GPY at 34-38°C. However growth rates were quite variable within the group, particularly at higher temperatures. In all tests the two *A. laevis* isolates showed a slower growth rate than any of the other Thai saprophytic *Aphanomyces* strains. Growth rates and colonial morphologies on SDA were particularly variable (Plate 4.1).

Some *Aphanomyces* isolates produced distinctive colonial morphologies when grown on SDA. The UM fungus (84-1240) and certain Thai saprophytic *Aphanomyces* strains (TF33, ASEAN1, ASEAN3, SA11 and WSA) formed various patterns of radiating lines which appear to be consistent distinguishing features.

4.3.2 Cluster analysis

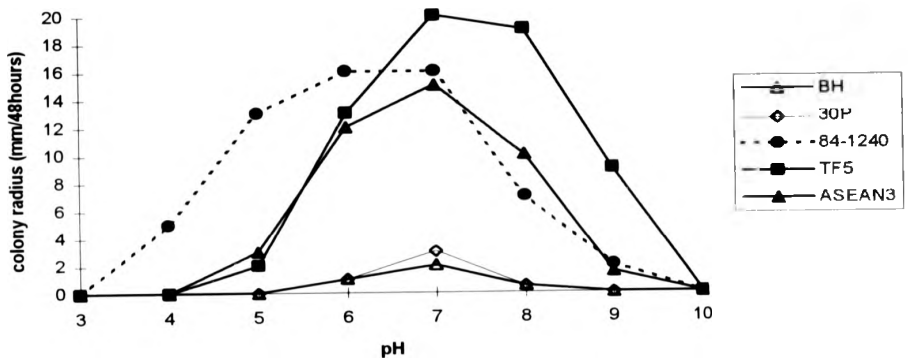
A dendrogram constructed from the growth data is given in Figure 4.1. All EUS, RSD and MG pathogenic isolates clustered together at similarity indices not less than 99.7. Of the other fungi, *A. astaci* was most similar to the pathogens, being separated at an index of 97.1. The Asian saprophytic *Aphanomyces* are shown to be a heterogenous group, separated from the other fungi at a similarity index of 40.3.

4.3.3 Effect of medium pH on growth

The weight of colonies of EUS isolates growing on agar plugs was not significantly greater than the weight of the agar plug alone, and therefore could not be used as an accurate indication of growth. Instead, colony radius was measured 48 hours after inoculation. The mean of duplicate plugs is given in Figure 4.2. Optimal growth for all isolates was at pH 7. The UM isolate tolerated low pH well and the saprophyte TF5 showed better tolerance of high pH. It is probable that the EUS isolates can survive a

much wider pH range than is indicated, but given the small colony radius, growth was not measurable at low and high pH.

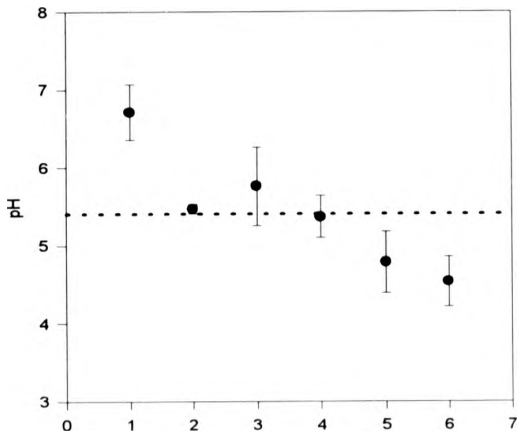
Figure 4.2 **Growth of EUS (BH, 30P), UM (84-1240) and saprophytic *Aphanomyces* (TF5, ASEAN3) isolates at different pH**



4.3.4 Change in medium pH due to fungal growth

The pH of samples from the six major groups of fungi tested, were averaged and are shown in Figure 4.3. The EUS, RSD and MG isolates clearly made the culture medium more alkaline than other isolates, and in contrast, *Achlya* and *Saprolegnia* isolates consistently acidified the medium. Pathogenic isolates were only marginally viable after the 2 month period as some cultures did not grow after subculture onto GPY agar. All other fungi remained viable.

Figure 4.3 **pH (mean ± SD) of GP liquid medium after 2 months growth of isolates at 10°C**



1 - EUS, MG and RSD isolates
(26 isolates, 67 samples)

2 - UM isolate
(1 isolate, 2 samples)

3 - *Aphanomyces astaci*
(2 isolates, 4 samples)

4 - saprophytic *Aphanomyces* spp
(12 isolates, 27 samples)

5 - *Achlya* spp
(5 isolates, 13 samples)

6 - *Saprolegnia* spp
(12 isolates, 30 samples)

dashed line indicates average initial pH of media

4.3.5 Viability of fungi on different growth media

Percentage viability of the six EUS, RSD and MG pathogenic isolates and six various saprophytic isolates, grown on 5 different media, are given in Table 4.3. On the basis of the improved viability of the pathogens after 36 days on PG-1 agar, this medium was adopted for the maintenance of these fungi.

Table 4.3 **Percentage viability of 6 pathogenic *Aphanomyces* and 6 saprophytic fungi grown on Petri dishes with 5 different media at 10°C**

	21 days		36 days		56 days	
media	**pathogens**	**saprophytes**	**pathogens**	**saprophytes**	**pathogens**	**saprophytes**
GPY	100%	100%	17%	100%	0%	100%
GPY-EDTA	100%	100%	33%	100%	0%	100%
GPY-buffer	100%	100%	67%	100%	0%	100%
GPY-EDTA, buffer	100%	100%	83%	100%	0%	100%
PG-1	100%	100%	100%	100%	0%	100%

4.3.6 Clearance zones

The extent and transparency of clearance zones given by a number of isolates are showed in Table 4.4 and Plate 4.2. *A. astaci* and *Achlya* sp showed the greatest protease production compared to growth and the saprophytic *Aphanomyces, Achlya* and *Saprolegnia* spp showed greatest DNase production. Clearance zones shown by EUS, MG and RSD pathogenic *Aphanomyces* were proportionally slightly larger when polycarbonate membranes were not used. Culture media put in wells in the agar leaked out within a few days without showing any discernible clearance halo with any isolate.

Table 4.4 Extent of clearance zones in relation to colony diameter of fungi grown on skimmed milk and DNase agars

	skimmed milk agar		DNase agar	
isolate	**extent**	**transparency**	**extent**	**transparency**
S1PA (EUS pathogen)	x	+	xx	+
34P (EUS pathogen)	x	+	xx	+
10P (RSD pathogen)	x	+	xx	+
FDL457 (*A. astaci*)	xxx	+	x	-
84-1240 (UM fungus)	xx	+	xx	+
TF33 (*Aphanomyces* sp)	xxx	-	xxx	+
ASEAN1 (*A. laevis*)	xx	+	xxx	+
S2AC (*Achlya* sp)	xxx	+	xxx	+
TF27 (*Saprolegnia* sp)	xx	-	xxx	+

x halo less than colony size
xx halo equal to colony size
xxx halo greater than colony size

\+ very transparent
\- not very transparent

4.3.7 Pigment production

Pigment levels, graded from 0 to 3, and averaged from five sets of cultures are given in Figures 4.4 & 4.5. Pigment production was first noticed in Philippine EUS isolate, 10D. Over the period of this study, this isolate retained the ability to colour the hyphae and the culture medium slightly grey-brown. Several other EUS, MG and RSD isolates also spontaneously began to produce pigment in culture, but there was no obvious pattern to this behaviour. Even some reisolated cultures differed from the original

isolate (compare PA4 and PA4(K)). No other growth characteristics appeared to be affected. No comparative analysis of the extracellular products (ECP) was undertaken. Of the non-snakehead pathogenic isolates, only UM isolates (84-1240, 84-1249) and *A. laevis* (ASEAN1, ASEAN3) showed any ability to produce a similar pigment.

Figure 4.4 **Pigment production in EUS, MG and RSD pathogenic isolates**

Lighter section represents mycelial pigment and darker section culture medium pigment
Y axis represents an abitrary pigment scale graded from 0-3 for both mycelial and medium pigments

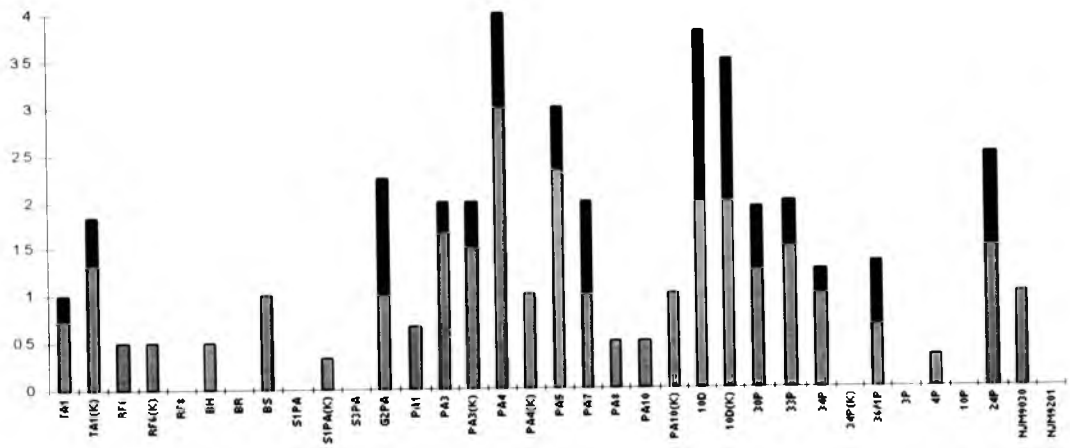
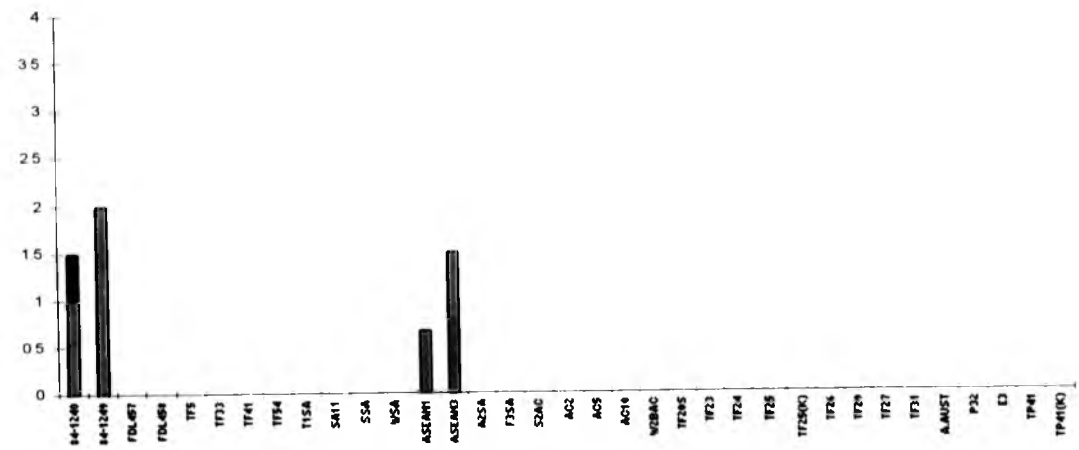


Figure 4.5 **Pigment production in non-snakehead pathogenic fungi**

Lighter section represents mycelial pigment and darker section culture medium pigment
Y axis represents an abitrary pigment scale graded from 0-3 for both mycelial and medium pigments



4.3.8 Attempts to induce production of sexual reproductive structures

No oogonia were observed during any of the experiments described. Growth was poor on hemp seeds, but *A. invadans* could grow on CMA supplemented with peptone.

4.4 Discussion

Growth rate is shown to be relatively consistent among all the EUS, RSD and MG isolates and a good preliminary diagnostic feature of these pathogens. Fowles (1976), in the first direct comparative study of different *Aphanomyces* species, also found temperature-growth profiles to be the most distinctive character studied.

The growth characteristics recorded here agree broadly with those given by other workers. Direct comparison is not possible given that the exact culture conditions vary. The failure of all the EUS, RSD and MG isolates to grow at 37°C on GPY in the present study was a characteristic given by Willoughby *et al* (1995), using glucose-peptone (GP) agar, in their description of *A. invaderis* (=*A. invadans*). The growth data presented by Fraser *et al* (1992) for the isolates 3P, 4P and 10P are similar to that given here, except that in the present study, growth on CDA was very poor in comparison. Growth of RSD isolates on mullet-extract agar (Fraser *et al,* 1992), and of the MG isolate on fish-extract agar (Hatai and Egusa, 1978), are comparable to the growth of all the pathogens on snakehead-extract agar described here. Hatai *et al* (1994) gave very similar results to those presented here for two pathogenic *Aphanomyces* isolates which grew well on glucose-yeast (GY) agar and PDA, but not at all on CDA or malt agar (MA). Hatai and Egusa (1979) also demonstrated the failure of MG isolates to grow on SDA. The fastidious nature of the pathogenic isolates on certain fungal media is consistent with the findings of Yuasa and Hatai (1996a) which showed the inability of MG isolates to utilise any of 25 carbohydrates tested.

Sterile *Aphanomyces* species obtained from guppies, dolphin and Indian major carp (see Section 1.6) are all considered to be distinct from *A. invadans*, given differences in their reported cultural characteristics. In contrast to *A. invadans*, the earlier isolates were shown to grow well on hemp seed (Shanor and Saslow, 1944); up to 45°C and on CMA (Fowles, 1976), and on Sabouraud agar (Srivastava, 1979). These characteristics have more in common with the saprophytic *Aphanomyces* isolates studies here, some of which were also isolated growing parasitically on fish and other aquatic animals.

Given the variability in growth rates and colonial morphology, the *Aphanomyces* saprophytes are considered to be comprised of several species. This has been confirmed by molecular studies undertaken at Glasgow University (see Plate 11.4). Given that most were isolated from fish, several unidentified *Aphanomyces* species are therefore shown to act as perthotrophs and/or wound parasites. The *Achlya* isolates constituted two very different groups, but the Thai *Saprolegnia* isolates appeared fairly homogenous and are possibly all *S. diclina*, although oogonia have been observed in only two isolates. The UK *S. diclina* differed from its Thai equivalents only in terms of its lower temperature preference.

Given the reluctance of *A. invadans* to grow on cornmeal agar, it is possible that if this fungus is involved in UM infections, it may not have been isolated, as Noga and Dykstra (1986) describe the use of CMA in their isolation procedure. Dykstra *et al* (1989) subsequently refer to the use of CMA supplemented with 1% yeast extract and 2% D-glucose, which would more likely support *A. invadans*. Fraser *et al* (1992) showed that the RSD isolates grow on supplemented CMA as described by Dykstra *et al* (1989) and in this study EUS, MG and RSD isolates were capable of growing on CMA with 1g/l peptone.

Other studies have relied on SDA (Bruno and Stamps, 1987) or CMA (Puckeridge *et al*, 1989; Bly *et al*, 1992) to isolate invasive hyphae in fish. In these cases *Saprolegnia*

was obtained, and does appear to be the causative pathogen. Bly *et al* (1992) succeeded in reproducing the disease with their isolates. However, in such cases any involvement of an *A. invadans*-like agent, with an inability to grow on such media, would not have been recognised. In addition, the isolations of Puckeridge *et al* (1989) were made directly from mycelium on the lesion, which would increase the likelihood of obtaining only wound pathogens. Interestingly, Bly *et al* (1992) did not succeed in obtaining fungal cultures from systemic infections using CMA.

Culture medium used for the maintenance of pathogenic isolates was shown here to experience a rise in pH (Figure 4.3), but not to the extent that is lethal for the fungus (Figure 4.2), indicating that the self-staling that occurs in cultures of pathogenic isolates (Section 4.3.4) is due to some other factor than pH. Although buffering the growth media was shown to extend the viability of the pathogenic isolates, the better viability of isolates on PG-1 agar than buffered GPY agar (Table 4.3), also indicated that pH changes are not the only factor involved in the staling of the media. Willoughby and Chinabut (1996) also noted that, in contrast to other fungi that release acids into the growth medium, *A. invadans* culture in GPY broth is accompanied by a pH rise. Those workers maintained their cultures for 2.5 - 4 times as long as in present studies and showed that the medium pH of four pathogenic isolates had continued to rise (pH 7.7 - 8.1), but that the medium of three saprophytic *Aphanomyces* also showed a similarly high pH after this time (pH 7.5 - 8.1). The substance responsible for self-staling that is released in the ECP was considered to be thermally stable, as staled media could not support *A. invadans* growth after autoclaving (Willoughby and Chinabut, 1996).

Hatai and Egusa (1978) demonstrated that MG isolates could grow in fish-meat extract (FME) broth at pH 5 - 10. This is a wider range than demonstrated here for EUS isolates, possibly due to the difficulties experienced in measuring small levels of growth in present samples. It is unlikely, however, that *A. invadans* could have sporulated at pH 9.54, the highest pH recorded for Airthrey Loch water in 1994 at the

time it was being used to prepare APW (Section 2.2.4). Callinan *et al* (1996a) reported that RSD isolates could sporulate at pH 5, but not at values less than 5.

At no time during the course of this current studies work were sexual structures observed in any of the EUS, MG or RSD isolates. Experience with the *A. laevis* isolates (ASEAN1 and ASEAN3) indicates that oogonia are produced more readily soon after isolation, and may lose this ability after repeated subculture. However, even EUS fungi isolated a matter of weeks before these experiments did not produce sexual fruiting bodies. Klebs (1899) expounded the theory that in aquatic fungi, sporangial production can be induced by the dilution of the standard growth medium, and oogonial production can be induced by transferring the fungus from a very rich medium to a very dilute one. The first idea is routinely practised for *A. invadans* in the technique of transferring mycelium to APW. The second was tested by Willoughby (pers. comm.) by growing EUS and MG isolates (RF6 and NJM9030) and saprophytic *Aphanomyces* isolates on x1, x2, x4, x8 or x16 strength GPY agar and then transferring them to APW. However, as in all previous tests, no oogonia resulted. It was noted, however that unlike the saprophytes, the pathogens were unable to grow on x16 GPY agar.

Previous workers have tested a variety of methods designed to produce sexual reproductive structures in other asexual saprolegniacean isolates, but these have invariably proved unsuccessful. Fowles (1976) grew an *Aphanomyces* wound parasite on a variety of substrates including snake skin, cow's horn, porcupine quill, blonde hair, grass and *Drosophila,* but failed to induce oogonia production. Stuart and Fuller (1968) investigated the possibility of heterothallism in sterile saprolegnians by a programme of pairing cultures, as tested here. In one case, sexual structures formed at the interface of two of Stuart and Fuller's (1968) isolates, however other studies using sterols failed to induce sexual stages, and no further evidence of heterothallism in *Saprolegnia* has emerged. Willoughby (1968) experimented on *Saprolegnia* using oatmeal and cholesterol agars of different strengths, and pairing isolates in natural

conditions with sterile soil, but was similarly unsuccessful. However, growth on hemp seeds contaminated with *Penicillium* did produce oogonia in some *Saprolegnia* isolates (Willoughby, 1968). Although *A. invadans* does not grow well on hemp seeds, this latter method will be tested in future studies.

Alderman and Polglase (1988) observed that asexuality appeared to be more common among the more pathogenic saprolegniaceans. The slow-growing nature of *A. invadans* may also be an indication of its pathogenicity. A distinction between obligate and facultative microbial parasites is often made, based on the theory that the former can never be cultured in artificial media (Willoughby, 1993). However, given that several fungi that are only known as parasites can now be cultured, Lewis (1973) felt that culturability should be abandoned as a basis for classification, it is rather the details of ecological and nutritional behaviour that are important. For example, it is likely that the reduced ability of an organism to compete with natural saprophytes could restrict it to a parasitic mode of life. Unestam (1969a) suggested that given *A. astaci*'s fastidious nature in culture, it would have little chance of competing with saprophytes outside its natural crayfish host. This was supported by the finding that once all the crayfish in an area are killed by *A. astaci*, and uninfected but susceptible crayfish are introduced one year later, the disease does not recur for a number of years (Unestam, 1969a). This finding also formed the basis of control strategies against crayfish plague (Söderhäll, Svensson and Unestam, 1977). Despite extensive effort in Thailand, no EUS isolates have been obtained other than from diseased fish; and in Australia, Fraser and Callinan (1996) have identified slow-growing *Aphanomyces* from RSD-affected water, but only during active outbreaks. More sensitive, (molecular-based) assays are required to verify this work, but this evidence, when combined with the present studies showing the delicate nature of *A. invadans* in culture, suggests that *A. invadans* is adapted to an ecological niche as a fish pathogen and would not survive long as a saprophyte.

Peduzzi and Bizzozero (1977) suggested that a high capacity to produce extracellular proteolytic enzymes would increase the power of saprolegniacean fungi to invade

tissues, and Lewis (1973) regarded the copious production of various tissue degrading enzymes to be characteristic of pathogenic, necrotrophic fungi. However, EUS and RSD isolates examined here showed relatively little proteolytic enzyme activity on skimmed milk agar. In contrast, Callinan (pers. comm.) found significant production of clearance zones on skimmed milk agar using RSD-*Aphanomyces*, although it is not known how this activity compared with other fungal strains. The results of Thompson (pers. comm.), using spectrophotometric values of azocasein hydrolysis, were more in agreement with the present work. She showed that protease activity per weight of mycelium was higher in mycelial extract and extracellular products (ECP) of saprophytic *Aphanomyces*, F3SA, than the EUS isolate, PA7. For both fungi, protease activity was generally higher in the ECP than the mycelial extract, and increased with pH (on a pH range from 4.5 to 6.5). Attempts to determine the molecular weight of the proteases involved, by means of clearance bands on polyacrylamide gels, produced only very faint bands. It is therefore apparent that any large scale protease activity by *A. invadans*, either can not be detected by present methods, or needs to be induced by some sort of environmental cue. Such a stimulus may come from the fish host (Rand and Munden, 1992; 1993a), or as a result of low temperature (Bly and Clem, 1992), as has been suggested with regards to *Saprolegnia*. If this is the case for *A. invadans,* it could be considered a less specialised parasite than *A. astaci,* in which constitutive production of chitinase has been regarded as evidence that that species is an obligate pathogen of crayfish (Unestam, 1966). Alternatively, protease activity by *A. invadans* may be highly specific and not required in large quantities to confer invasive growth capabilities on the fungus. In this regard *A. invadans* may be more like the biotrophic fungi described by Lewis (1973), although this is somewhat at odds with the amount of tissue damage resulting from *A. invadans* infection.

Table 4.2 **Influence of temperature and culture medium on mean radial growth (mm/day) of all isolates**

°C	6	10	14	18	22	26	30	34	38	42	22	22	22	22	22	22
MEDIA	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	FMA	PDA	CDA	SDA	CMA	MEA
TA1	0.1	1.1	2.1	2.9	4.3	4.8	4.9	3.6	0.0	0.0	5.0	3.8	0.8	0.0	0.0	0.0
RF6	0.2	0.9	1.6	2.6	3.5	4.3	4.1	2.9	0.0	0.0	2.7	3.4	0.8	0.0	0.0	0.0
RF8	0.1	0.7	1.7	2.8	3.9	4.4	4.4	3.5	0.0	0.0	4.2	3.6	0.9	0.0	0.0	0.0
S1PA	0.1	0.8	1.8	2.4	3.7	5.0	4.8	4.0	0.0	0.0	4.2	3.9	0.9	0.0	0.0	0.0
S3PA	0.2	0.8	2.0	2.6	3.7	4.5	4.9	3.9	0.0	0.0	3.5	3.2	0.8	0.0	0.0	0.0
G2PA	0.1	0.8	2.1	2.7	3.9	4.6	4.8	3.7	0.0	0.0	3.6	3.6	1.0	0.0	0.0	0.0
PA1	0.1	0.8	1.5	2.8	3.9	4.4	5.0	3.6	0.0	0.0	3.6	3.6	0.7	0.0	0.0	0.0
PA3	0.1	0.7	1.7	3.2	3.9	4.4	5.2	4.3	0.0	0.0	3.5	3.5	0.8	0.0	0.0	0.0
PA4	0.1	0.8	2.0	2.8	4.0	4.5	5.1	4.1	0.0	0.0	3.7	3.8	0.7	0.0	0.0	0.0
PA5	0.1	0.7	2.1	2.8	4.0	4.5	4.7	3.6	0.0	0.0	3.5	3.4	0.6	0.0	0.0	0.0
PA7	0.1	0.8	1.8	2.9	4.2	4.6	5.5	4.0	0.0	0.0	3.7	3.9	0.8	0.0	0.0	0.0
PA8	0.1	0.8	1.8	2.9	4.0	4.7	5.0	3.5	0.0	0.0	3.5	2.9	0.7	0.0	0.0	0.0
PA10	0.1	0.7	2.0	2.9	3.9	4.6	4.9	3.7	0.0	0.0	3.6	3.6	0.6	0.0	0.0	0.0
BH	0.2	0.8	1.5	3.4	3.8	4.5	3.8	3.4	0.0	0.0	3.5	3.4	0.8	0.0	0.0	0.0
BR	0.1	1.0	1.7	2.6	3.6	4.3	4.4	3.4	0.0	0.0	4.0	3.2	0.9	0.0	0.0	0.0
BS	0.2	1.1	1.8	2.8	4.1	4.6	4.4	4.2	0.0	0.0	4.2	3.9	0.8	0.0	0.0	0.0
36/1P	0.2	1.0	1.9	2.8	4.1	4.6	4.6	3.7	0.0	0.0	4.1	4.5	1.1	0.0	0.0	0.0
30P	0.1	1.0	2.2	3.0	3.8	4.5	4.0	2.4	0.0	0.0	4.5	3.7	0.6	0.0	0.0	0.0
33P	0.2	1.2	2.3	2.8	4.1	4.5	4.2	2.8	0.0	0.0	4.8	3.9	0.7	0.0	0.0	0.0
34P	0.1	0.5	1.8	2.6	3.2	3.9	3.4	2.0	0.0	0.0	3.4	2.9	1.0	0.0	0.0	0.0
10D	0.1	1.0	2.1	2.4	3.9	4.6	4.6	2.0	0.0	0.0	4.0	2.9	0.6	0.0	0.0	0.0
3P	0.1	0.8	1.6	2.8	3.7	5.0	4.5	3.5	0.0	0.0	3.3	3.5	1.0	0.0	0.0	0.0
4P	0.1	0.5	1.5	2.7	3.7	4.6	4.3	3.5	0.0	0.0	3.2	3.3	0.9	0.0	0.0	0.0
10P	0.1	0.9	2.0	2.8	3.5	4.1	3.7	2.8	0.0	0.0	2.8	3.0	0.9	0.0	0.0	0.0
24P	0.2	0.8	2.1	2.6	3.8	4.7	4.9	3.5	0.0	0.0	3.5	3.4	0.9	0.0	0.0	0.0
NJM9030	0.1	0.5	1.7	3.1	4.0	5.2	5.3	3.8	0.0	0.0	3.6	3.3	1.0	0.0	0.0	0.0
84-1240	1.7	2.6	4.9	6.4	7.8	9.1	7.0	0.0	0.0	0.0	8.6	10.1	8.1	7.2	5.9	6.9
FDL457	1.2	2.3	3.1	3.7	4.3	2.3	0.2	0.0	0.0	0.0	5.6	5.0	0.9	0.0	0.0	0.0
FDL458	1.2	2.4	3.2	3.8	4.7	2.6	0.5	0.0	0.0	0.0	5.4	4.6	0.9	0.0	0.0	0.0
TF5	0.7	2.4	5.3	7.6	10.3	13.8	15.6	17.5	18.7	10.9	9.7	10.2	8.4	4.0	6.5	6.5
TF33	1.2	2.4	5.1	7.3	10.4	13.7	15.5	17.7	15.7	0.9	10.2	11.6	9.7	8.1	8.8	9.4
TF41	0.6	1.9	5.1	6.9	9.4	12.7	15.4	17.2	18.4	9.1	9.0	9.7	7.6	5.3	6.4	6.6
TF54	0.4	1.9	4.5	6.3	9.7	11.9	14.0	15.9	14.9	7.1	7.0	7.2	5.1	1.0	5.6	5.0
T1SA	0.6	2.3	4.4	7.0	9.3	12.7	14.9	17.3	18.8	11.2	10.0	10.9	10.0	5.4	7.9	8.1
A2SA	0.7	2.1	4.9	7.1	9.8	12.8	14.6	16.7	18.6	13.2	10.1	10.6	9.6	4.1	7.0	7.7
F3SA	0.7	2.2	4.6	7.2	10.0	12.8	15.1	16.6	18.3	12.8	10.0	10.8	9.8	4.2	6.9	7.7
ASEAN1	0.2	1.5	3.4	4.0	6.5	9.0	10.3	11.5	11.4	1.2	7.3	6.0	4.9	3.8	4.8	5.0
ASEAN3	0.2	1.4	3.1	5.0	6.5	8.8	10.1	11.4	11.0	1.2	7.4	6.4	5.1	3.1	5.0	5.0
SSA	0.6	2.1	4.3	7.2	10.0	12.5	15.8	18.2	19.5	11.7	10.3	9.7	8.5	4.5	7.8	8.0
WSA	1.4	2.9	5.4	8.3	11.0	14.5	16.8	19.0	18.1	2.8	10.9	13.7	8.6	11.0	9.8	11.1
SA11	2.1	3.6	5.8	8.1	10.3	12.5	14.0	14.8	6.6	0.0	10.1	11.0	9.4	5.6	7.9	8.8
S2AC	0.1	1.6	4.3	5.2	7.8	9.7	12.0	12.8	9.3	0.0	7.6	7.3	2.7	4.1	6.5	6.3
W2BAC	2.0	4.5	8.3	10.6	14.0	17.2	20.2	19.8	11.0	0.0	16.3	19.6	10.2	15.0	13.5	14.6
AC2	0.1	0.9	2.5	5.9	8.0	10.4	11.9	14.5	13.4	0.0	9.0	9.1	4.4	6.5	7.1	7.4
AC5	0.1	0.6	2.4	5.7	7.5	10.3	10.0	13.3	12.0	0.0	9.1	8.4	3.4	5.9	6.7	6.3
AC10	0.1	0.7	2.3	5.6	7.5	10.2	9.8	13.8	11.7	0.0	9.1	8.4	3.5	5.8	6.9	6.5
TF20S	4.5	6.9	11.7	13.4	18.0	22.2	22.5	21.0	5.8	0.0	21.7	21.4	13.8	17.5	19.0	18.8
TF23	4.5	6.9	11.4	14.5	18.3	22.1	24.5	21.1	5.5	0.0	21.5	21.3	14.7	17.1	19.6	18.7
TF24	4.7	6.6	12.4	13.6	18.3	22.2	23.5	21.0	6.0	0.0	22.3	21.3	13.2	17.1	18.5	17.5
TF25	4.7	6.9	11.6	14.4	18.0	21.8	23.0	21.1	5.5	0.0	21.1	21.6	14.7	17.2	19.2	18.5
TF26	4.7	6.9	11.6	14.5	18.0	21.2	23.0	21.2	5.0	0.0	21.1	21.3	13.8	17.7	18.9	18.6
TF29	4.5	6.5	10.3	13.3	17.0	21.1	23.5	21.0	6.8	0.0	20.1	21.9	14.7	17.6	18.5	18.5
TF27	4.3	6.6	10.8	14.0	17.0	21.0	23.0	20.7	6.1	0.0	20.6	22.4	13.4	17.5	18.0	18.1
TF31	4.2	6.5	10.1	13.2	17.1	21.2	23.7	21.2	8.6	0.0	21.0	22.2	15.6	17.2	18.3	19.0
S.AUST	2.5	4.2	6.1	8.3	8.8	10.2	0.8	0.3	0.0	0.0	14.3	16.3	9.9	11.8	10.8	11.2
P32	6.2	8.3	11.7	14.4	17.8	15.9	0.8	0.0	0.0	0.0	20.3	19.8	10.8	13.6	16.0	16.5
E3	6.8	9.1	12.6	15.7	18.5	20.6	5.6	0.8	0.0	0.0	20.6	24.2	13.5	17.9	18.5	18.5
TP41	5.1	7.2	9.6	11.5	13.8	15.8	8.0	0.8	0.0	0.0	18.5	19.2	9.2	13.4	11.9	12.9

Figure 4.1

Dendrogram of *Aphanomyces* isolates, constructed using cluster analysis of the 16 growth parameters in Table 4.2, showing the extreme similarity of the culture characteristics of the fish-pathogenic isolates compared to other *Aphanomyces* spp.

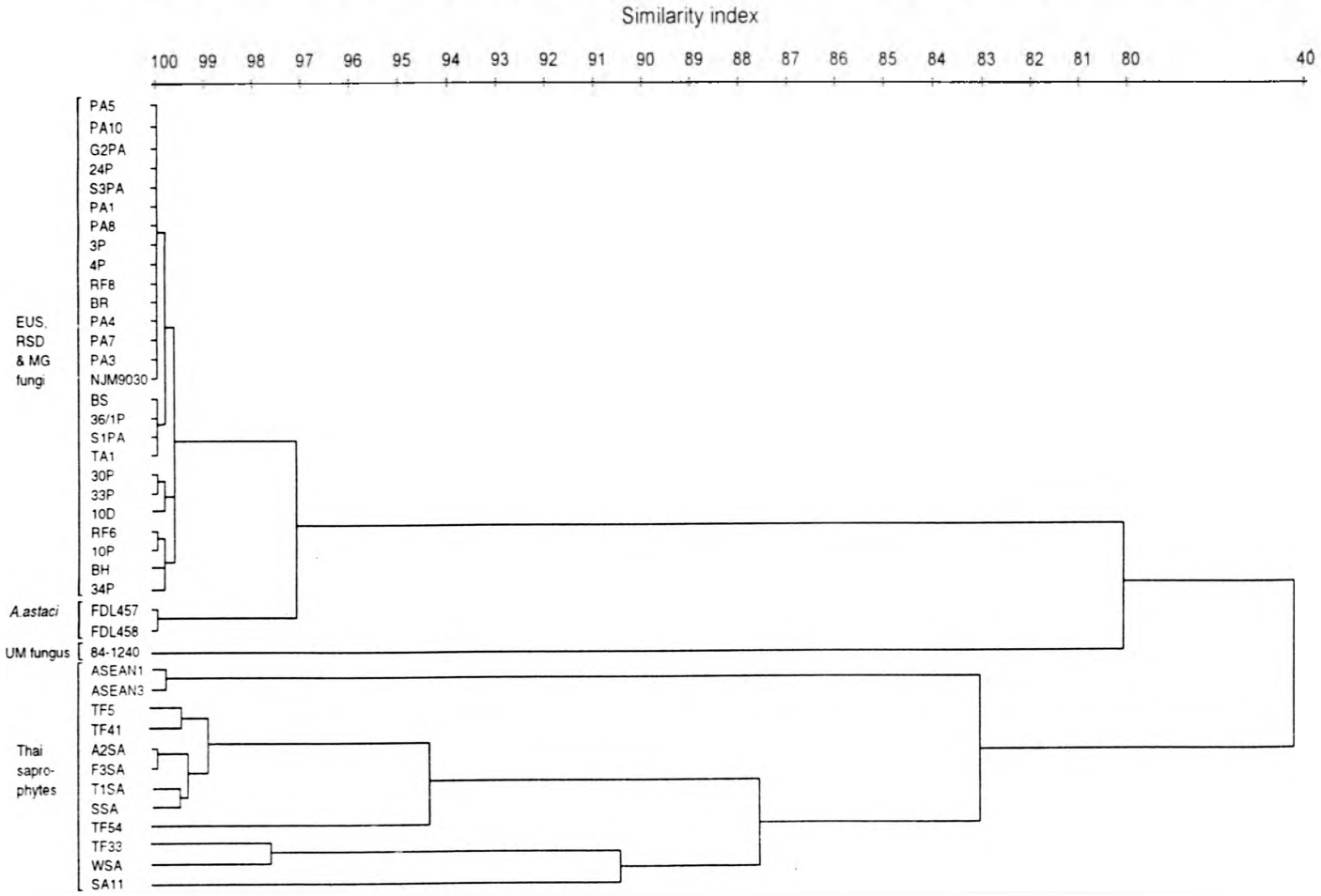


Figure 4.2

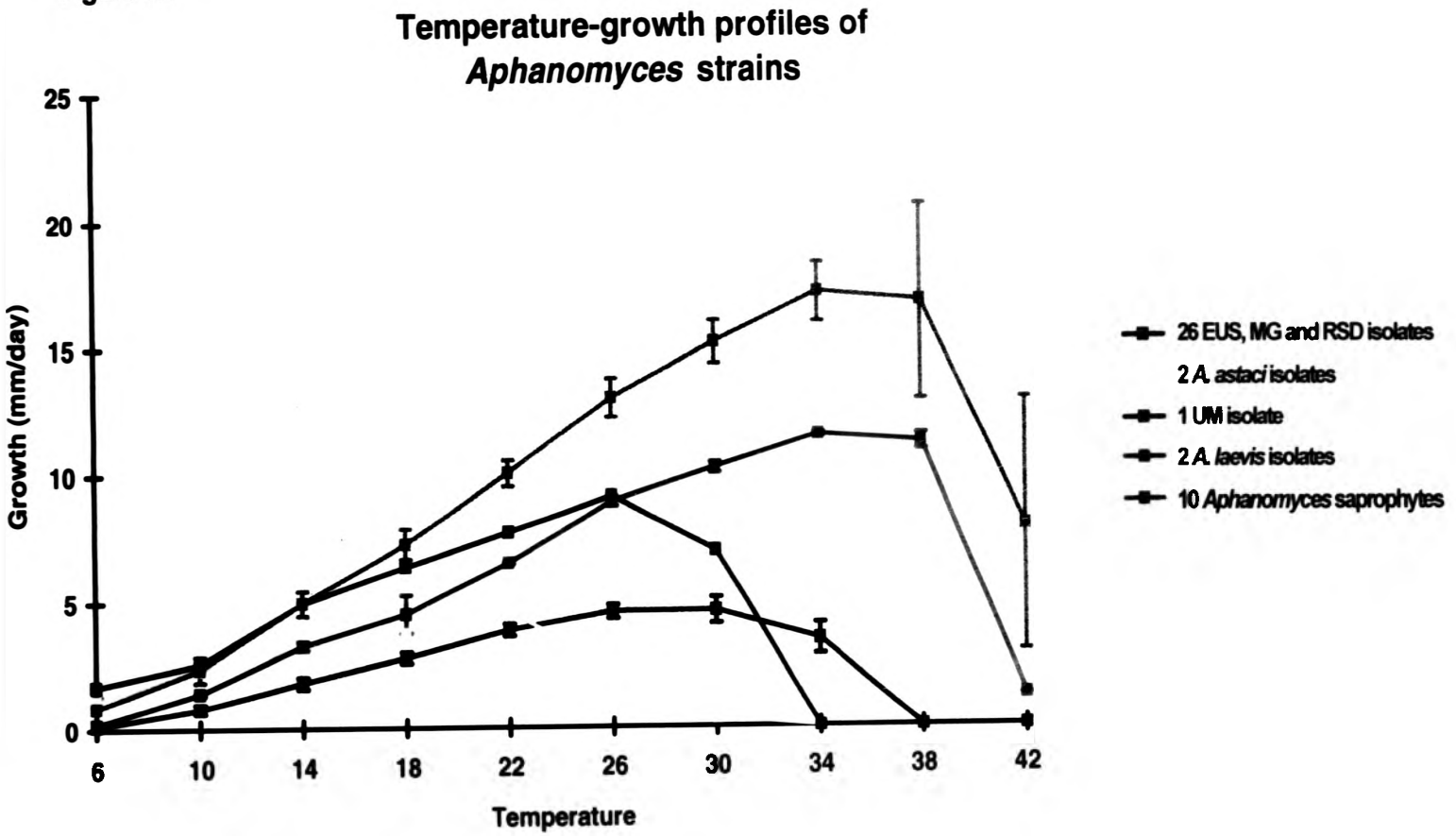

Temperature-growth profiles of
Aphanomyces strains
Growth (mm/day)
25
20
15
10
5
0
6
10
14
18
22
26
30
34
38
42
Temperature
26 EUS, MG and RSD isolates
2 A. astaci isolates
1 UM isolate
2 A. laevis isolates
10 Aphanomyces saprophytes

Plate 4.1

Differing colonial morphologies of 3 saprophytic *Aphanomyces* isolates on SDA. These patterns were consistent on several replicate cultures of the isolates

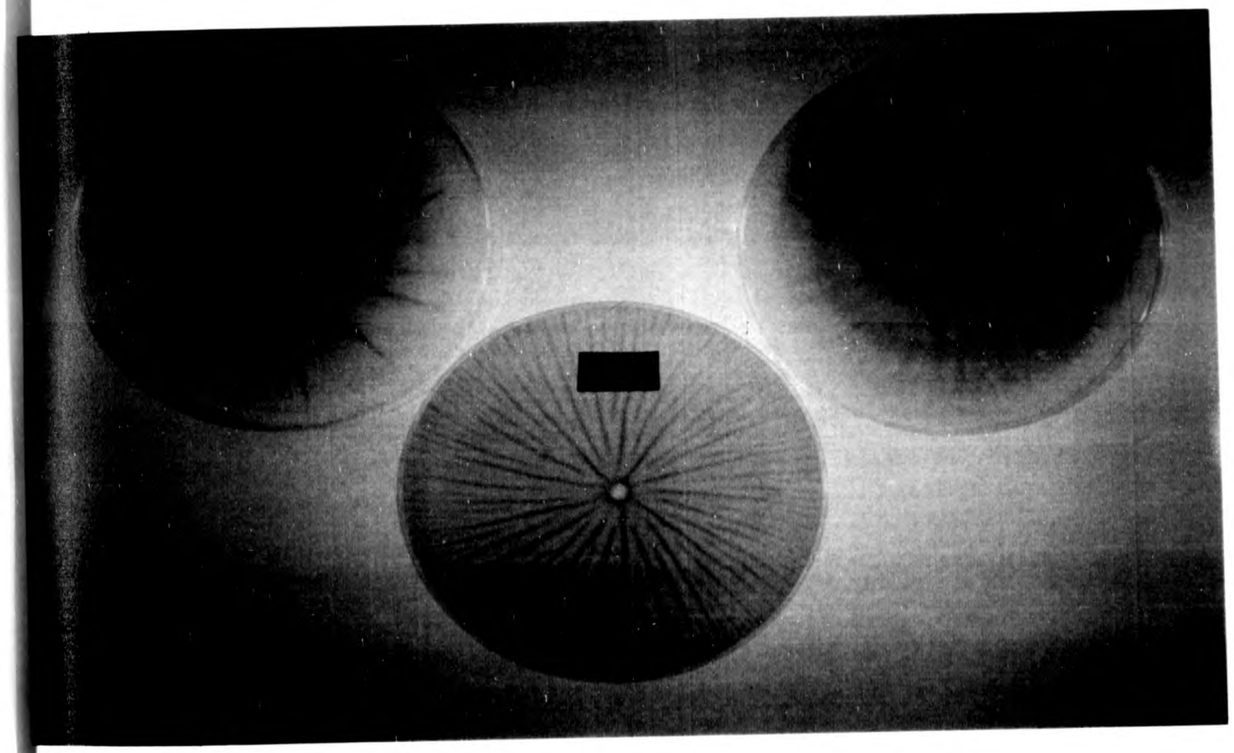


Plate 4.2

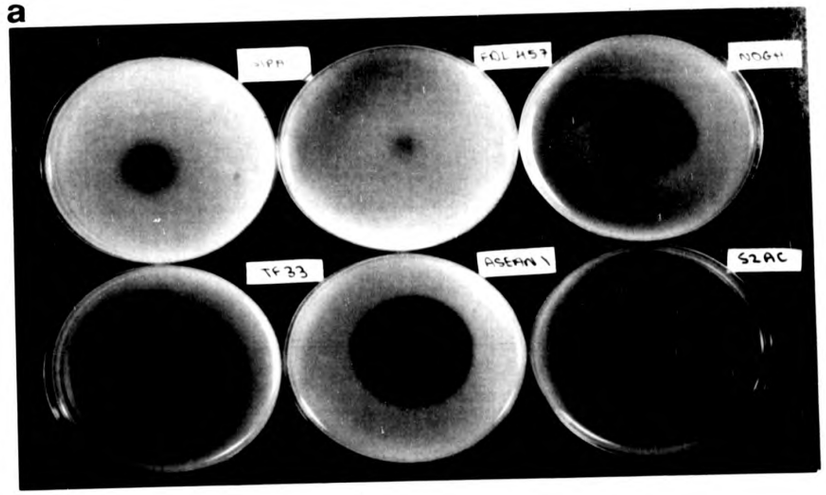
Clearance zones produced by *A. invadans* (S1PA), *A. astaci* (FDL457), UM-*Aphanomyces* ("NOGA" = 84-1240), *Aphanomyces* sp saprophyte (TF33), *A. laevis* (ASEAN1) and *Achlya* sp saprophyte (S2AC)

(a) Using DNase agar

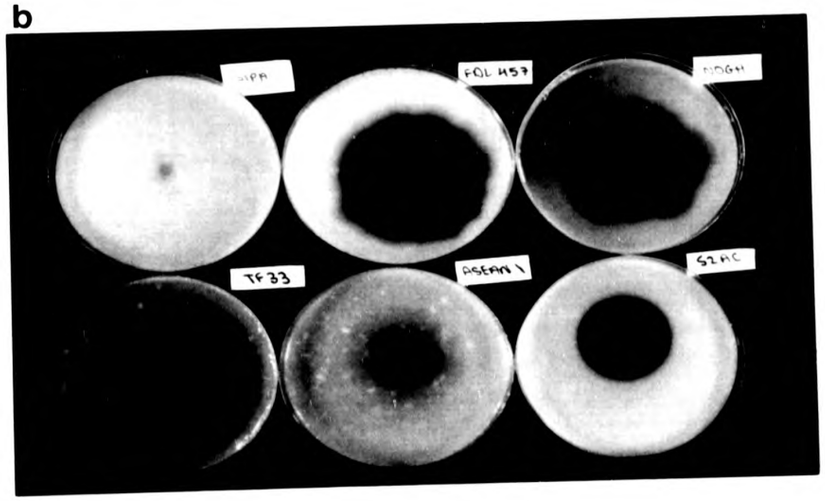
(b) Using skimmed milk agar

(c) Polycarbonate membrane showing extent of *A. invadans* growth (right) and corresponding clearance zone on skimmed milk agar (left)

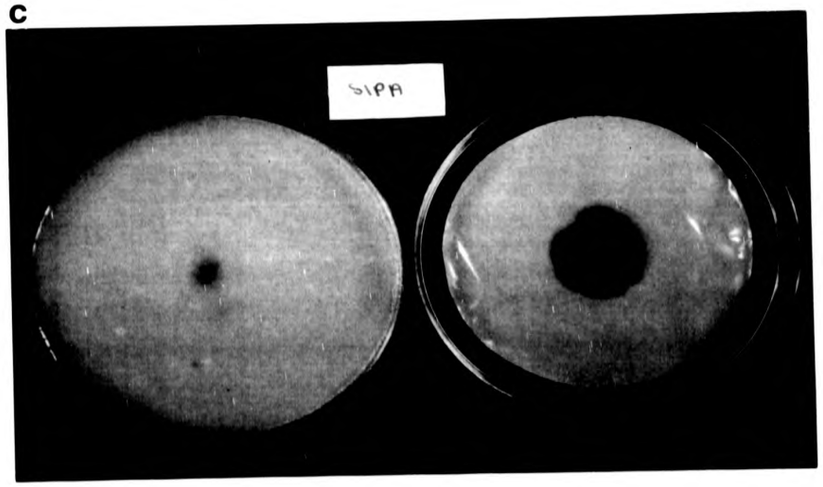
a



b



c



CHAPTER 5. CHEMICAL SUSCEPTIBILITY TESTS[2]

5.1 Introduction

Previous studies on the susceptibility of aquatic fungi to chemical compounds have tended to concentrate on the *Saprolegnia* pathogens of salmonid fish (Willoughby and Roberts, 1992a; Marking, Rach and Schreier, 1994a; b; Yuasa and Hatai, 1996b), and the crayfish plague fungus, *A. astaci* (Hall and Unestam, 1980; Alderman and Polglase, 1985). Yuasa and Hatai (1994a; b; 1995a) have also investigated the drug-susceptibility of the MG pathogenic fungus.

Some articles have been published giving recommended treatments for EUS in ponds (Das and Das, 1993; Areerat, 1990) but no studies have investigated the *in vitro* susceptibility of *A. invadans* to chemical treatment. The present work attempts to characterise isolates of *A. invadans* in comparison with other fungi, in terms of their susceptibility to various chemical agents. This data can then be used to determine optimum concentrations of antibiotics, fungicides and disinfectants for the respective isolation, treatment and disinfection of these fungi.

5.2 Materials and methods

5.2.1 Fungal isolates

The 54 fungal isolates used in this study are listed in Appendix 1. The two fungi S1PA(K) and TF25(K) are reisolations of the isolates S1PA and TF25 after artificial injection into fish. All fungi were grown at 10°C on GPY agar (Appendix 2a). Four millimetre agar plugs taken from the edge of colonies less than seven days old were used in all experiments.

[2]The information presented in this Chapter is published in Lilley, J.H. and Inglis, V. (1997) Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi. Aquaculture Research 28(6), 461-469.

5.2.2 Preliminary tests using antibiotic sensitivity disks

Oxoid antimicrobial sensitivity disks were used as a means of preliminarily testing fungal susceptibilities to different antibiotics. A 4 mm GPY plug of fungus was placed in the centre of a GPY agar Petri dish, with 6 different disks arranged around the plug. Dishes were left at room temperature (16-20°C) until the periphery of the fungal colony had grown about 1 cm passed the disks.

The antibiotics and their concentration on the disks were: trimethoprim (W1.25, 1.25μg), kanamycin (K5, 5μg), erythromycin (E10, 10μg), sulphamethoxazole (RL25, 25μg), oxytetracycline (OT30, 30μg) and furazolidone (FR50, 50μg) (all Oxoid). All are broad spectrum antibiotics except erthromycin which is mainly active against gram positive bacteria. Of these, only furazolidone is considered to have significant anti-fungal activity.

5.2.3 Growth in agar incorporating antibiotics

Three antibiotics were tested: penicillin-K (Sigma: 1575 units penicillin-G base per mg), streptomycin sulphate (Sigma: 763 units streptomycin base per mg) and oxolinic acid (Sigma). Each antibiotic powder was added at 10, 50, 100 and 500 ppm (equivalent to 16, 79, 158 and 788 units/ml penicillin-G; 8, 38, 76 and 382 ug/ml streptomycin and 10, 50, 100 and 500 ug/ml oxolinic acid) to molten GP agar (Appendix 2a) after autoclaving and cooling to about 50°C. Care was taken to continually mix the media when pouring into the Petri dishes. Control plates contained no antibiotics.

Duplicate agar plugs of each fungus were placed upside-down on five agar plates, each incorporating a different concentration of antibiotic. The plates were kept at room temperature (17-20°C). The growth rate of the fungus was measured as the mean increase in radius of the colony after 72 hours. An average of the duplicate colonies was taken and expressed as a percentage of the growth of the controls.

Penicillin is active against gram positive bacteria, whereas oxolinic acid and streptomycin sulphate are both broad spectrum antibiotics.

5.2.4 Fungicide tests

Three antifungal agents were selected for minimum inhibitory concentration (MIC) testing. Malachite green (Sigma) was tested at 0.5, 1, 5 and 10 ppm; hydrogen peroxide (Solvay Interox Ltd) at 50, 100, 500 and 1000 ppm and sodium chloride (Merck) at 5, 10, 20 and 30 ppt. Control treatments contained no antifungal agent.

For each fungal isolate, 5 ml of each concentration of fungicide was pipetted into a different compartment of a multi-compartment "Replidish" (Bibby Sterilin Ltd) (Alderman, 1982b). Triplicate agar plugs of each fungus were placed in each of the five compartments and left at room temperature (19-22°C) for one hour. For the malachite green and sodium chloride experiments, the plugs were then washed three times in separate Replidish compartments containing distilled water over a period of one hour before blotting on sterile filter paper and placing upside-down on GPY agar. In the case of hydrogen peroxide, the plugs were transferred to Replidish compartments containing neutralising solution (0.25g/l catalase) for one hour before blotting and placing on GPY agar as with the other fungicide test treatments.

Growth was monitored daily and recorded as "no effect" if growth was apparent at the same time as controls; "inhibition" if growth started after controls; or "control" if no growth took place.

5.2.5 Disinfectant tests

Three disinfectants, the iodophore FAM 30 (Evans Vanodine Ltd), sodium hypochlorite (Merck) and Proxitane 0510 containing 5% peracetic acid in hydrogen peroxide (Solvay Interox Ltd) were assessed in terms of their minimum lethal time (MLT) to each fungal isolate.

Three replicate plugs of each fungus were immersed in a single concentration of the test disinfectant for 10 sec, 1 min, 5 min, 20 min or 1 hour and then removed and immersed in a neutralising solution for 20 min before being blotted on sterile filter paper and inoculated onto GPY agar. Triplicate control plugs were placed directly into neutralising solution without treatment and then inoculated onto GPY agar.

FAM 30 was used at a test concentration of 100 ppm available iodine, sodium hypochlorite at 100 ppm available chlorine and Proxitane 0510 at 100 ppm peracetic acid. The neutralising solution for FAM 30 and sodium hypochlorite contained 0.5g/l sodium thiosulphate pentahydrate, 15g/l Tween 80 and 3g/l lecithin; and for peracetic acid it comprised 50g/l sodium thiosulphate pentahydrate and 0.25g/l catalase.

As in the fungicide experiments, growth was checked daily and the treatment was recorded as "no effect", "inhibition" or "control" compared to negative controls. Plates were maintained at room temperature (19-22°C) throughout the experiments.

5.3 Results

5.3.1 Preliminary tests using antibiotic sensitivity disks

The interpretation of the inhibitory effect of the antibiotic test rings on fungal growth was problematical as clearance areas varied according to the growth rate of the fungus past the disks. For example, the fast growing *Achlya* and *Saprolegnia* fungi probably showed inhibition behind some of the disks because of the colonial morphology that occurs as the fungus grows around the physical obstruction, rather than any effect of the antibiotic itself. Nevertheless, some significant inhibition of *A. invadans* by kanamycin and furazolidone was recorded, as shown in Table 5.1.

Table 5.1 Inhibition of fungal growth around antibiotic disks judged after the main colony had grown passed the disk

	growth time (days)*	W1.25	K5	E10	RL25	OT30	FR50
TA1 (EUS pathogen)	**17**	0	+++++	0	0	0	++++
RF6 (EUS pathogen)	**17**	0	+++++	0	0	0	++++
BH (EUS pathogen)	**17**	0	+++++	0	0	0	++++
84-1240 (UM fungus)	**10**	0	0	0	+	0	+++
TF54 (*Aphanomyces* sp)	**10**	0	+++	0	0	0	+++
S2AC (*Achlya* sp)	**10**	+	0	0	+	0	++++
W2BAC (*Achlya* sp)	**6**	++	+	0	+	++	++++
TP41 (*Saprolegnia parasitica*)	**6**	+++	0	0	++	+	++++

0 no effect
\+ reduction in fungal growth, no thinning of mycelium
++ thinning of mycelium behind antibiotic disk
+++ thinning of mycelium all around disk
++++ no fungal growth behind disk
+++++ no fungal growth all around disk

*The number of days taken for the fungus to grow about 1 cm passed the plug

5.3.2 Growth in agar incorporating antibiotics

The effects of antibiotics on fungal growth are shown in Figure 5.1. Oxolinic acid caused slight inhibition of most isolates at concentrations as low as 10 ppm, however virtually all fungi continued to grow at 60% of controls or faster, even at the highest concentration of 500 ppm. Streptomycin sulphate had the most severe effect, particularly on the EUS, RSD and MG *Aphanomyces* with most of these isolates growing at 0-30% of controls when 500 ppm was incorporated in the media. Penicillin-K had no significant effect on most fungi even at concentrations of 500 ppm.

The effects of the antibiotics on different fungi were quite variable, even between different isolates of the same species (Figure 5.1). All the most recent isolates of *A. invadans* (PA1, PA3, PA4, PA5, PA7 and PA10) showed lower than average resistance to penicillin. The two *A. astaci* isolates (FDL457 and FDL458) were significantly more susceptible to penicillin and streptomycin than most other fungi. The *Saprolegnia australis* isolate was among the most resistant of the fungi to all three antibiotics.

5.3.3 Fungicide tests

The only treatment that succeeded in killing all isolates was exposure to 10 ppm malachite green for one hour. However, over three quarters of the isolates failed to survive treatment with 5 ppm malachite green, 500 ppm hydrogen peroxide or 30 ppt sodium chloride (Figure 5.2).

A clear difference in susceptibility to malachite green and sodium chloride between the EUS, RSD and MG *Aphanomyces* isolates and the saprophytic *Aphanomyces* spp was apparent. All the former were killed by exposure to 0.5 ppm malachite green or 20 ppt sodium chloride for one hour, whereas all the latter survived these treatments.

5.3.4 Disinfectant tests

The MLT for all the fungi was 20 min with sodium hypochlorite or Proxitane 0510 and 1 hour with FAM 30 at the specified concentrations (Figure 5.3).

All the EUS, RSD and MG *Aphanomyces* isolates were sublethally affected by sodium hypochlorite after 5 min whereas none of the saprophytic fungi were affected by this treatment. The effect of FAM 30 was even more variable between strains: all the fish-pathogenic *Aphanomyces* isolates failed to survive after 1 min, the saprophytic *Aphanomyces* and the *Saprolegnia* spp were killed by 5 min treatments but the *Achlya* spp were all killed only after 1 hour treatments.

5.4 Discussion

A feature of the results of this work is the variability in susceptibility of different fungal species to the compounds tested. Previous workers have used growth in inhibitory compounds as an aid to separating and comparing individual strains and species (Alderman and Polglase, 1984; Coffey and Bower, 1984; Paterson and Bridge, 1994; Yuasa and Hatai, 1994a; b; 1995a; b). This study shows that *Aphanomyces invadans* can be clearly distinguished from the saprophytic *Aphanomyces* spp tested by its greater susceptibility to malachite green, sodium chloride and sodium hypochlorite. In

addition, the RSD and MG isolates were indistinguishable from EUS isolates in all of the tests. The UM *Aphanomyces* (84-1240), differed from *A. invadans* particularly in terms of resistance to sodium chloride. The unusually high salt tolerance of this strain has been previously reported by Hearth and Padgett (1990).

The effect of 1 hour NaCl treatments on the subsequent growth of RSD and MG isolates shown here is very similar to the effect on these isolates when NaCl is incorporated into GY agar, as reported elsewhere. Fraser *et al* (1992) showed significant inhibition of growth of RSD isolates 3P, 4P and 10P at 8 ppt NaCl, and failure to grow at ≥12 ppt at 22°C. Hatai *et al* (1994) reported significant inhibition of an MG isolate at 10 ppt NaCl and failure to grow at 15 ppt at 25°C.

The identification of antimicrobial compounds to which *A. invadans* is differentially more resistant would help to improve the selectivity of isolation media. However, *A. invadans* was found to be generally more sensitive to most antibiotics tested. Antimicrobial sensitivity disks have been used elsewhere to test the effect of antibiotics on saprolengiacean fungi (Oláh and Farkas, 1978; Bly, Quiniou, Lawson and Clem, 1996), but as in the study of Alderman and Polglase (1986), they were found here to be of limited use with filamentous fungi. They did, however, reveal significant growth inhibition of all fungi by furazolidone, and of *A. invadans* by kanamycin. This is of interest as kanamycin has been used an alternative to the antibiotics listed in Appendix 2a for the isolation of *A. invadans*, but is shown here to be unsuitable for this purpose. The results also suggest that trimethoprim, erythromycin, sulphamethoxazole, and oxytetracycline could be used for the isolation of *A. invadans* at the concentrations tested. An agar diffusion method that can also measure MIC's of antimicrobial agents has also been developed (Brown and Brown, 1991), and adapted for use with yeasts (Anon, 1994a), but its use with filamentous fungi may be similarly problematical as for the test disks used here.

The three antibiotics tested by incorporation in the growth medium, have previously been shown to have relatively little effect on some saprolegniacean fungi (Oláh and Farkas, 1978; Alderman and Polglase, 1986) and are therefore considered suitable for use in fungal isolation media to exclude bacteria. However, Beakes and Gay (1980) noted some inhibitory effects of streptomycin against *Saprolegnia*; and at the 100 ppm level of streptomycin sulphate and oxolinic acid advised by Willoughby and Roberts (1994a), significant growth inhibition of EUS, RSD, MG and crayfish plague fungi can occur (Figure 5.1). As it is common to use two or more antibiotics in combination when isolating fungi, it is likely that growth may be significantly slowed. Further, Griffin and Coley-Smith (1971) showed that streptomycin toxicity increases with temperature, and in the case of the *A. invadans* it may be necessary to attempt isolation in temperatures of 30°C or above, resulting in further inhibition in growth of the fungus. A reduced level of 10 ppm of streptomycin sulphate and oxolinic acid is therefore advised for the isolation of *A. invadans* (Appendix 2a).

It is possible that some of the variation in susceptibility to the antibiotics between similar isolates may be the result of different antibiotic levels used in their isolation. For example, the *A. invadans* isolates PA1-PA10 were isolated using a low penicillin-K concentration of 10 ppm, and these are shown to be generally more susceptible to penicillin than other EUS isolates. Likewise, both *A. astaci* isolates are consistently among the most susceptible of the fungi, and these were isolated as described by Alderman and Polglase (1986) using penicillin-G and oxolinic acid at 10 ppm.

Bailey (1983a; b) showed that the agar plug method of testing fungicide activity *in vitro* correlated well with *in vivo* treatments of surface infections of fish. The results of the present fungicide tests should therefore adequately project the activity of these agents against *Saprolegnia parasitica* and the fungi involved in saprophytic infections of EUS-affected fish. The *Achlya* strains examined here have been found to be the most common fungal saprophytes growing on the surface of fish lesions in Thailand (unpublished results), and these, along with the *Saprolegnia* spp, were usually the

most resistant species to the compounds tested. As *Saprolegnia* spp are probably most commonly used in studies evaluating new compounds, it is possible that some of the agents dismissed by previous studies may be useful for treating *A. invadans*.

Given the possible carcinogenic and teratogenic properties of malachite green (Alderman, 1992), an extensive search is underway to find an alternative agent for the treatment and control of fungal diseases of fish (Bailey, 1984; Marking *et al*, 1994a; b; Fitzpatrick, Schreck and Chitwood, 1995; Li, Wise and Robinson, 1996). Some substances, including magnesium chloride (Rantamäki, Cerenius and Söderhäll, 1992), chitosan (Min, Hatai and Bai, 1994), amphotericin B and diquat (Bly *et al*, 1996) and cyclohexamide (Yuasa and Hatai, 1996b) have been identified as candidate fungicides. Two compounds currently being considered for use in aquaculture, hydrogen peroxide and Proxitane 0510, are shown here to have some potential for fungicidal treatments and disinfection respectively.

The treatment of several mycoses of fish is difficult because the fungi involved penetrate their hosts and are, to a large extent, protected from exposure to the chemicals. In these cases, a more strategic use of fungicides in culture systems is required, in which the infective stages of the fungus are treated in the incoming water. Willoughby and Roberts (1992a) showed that only very low concentrations (0.25 ppm) of malachite green are required to kill *S. parasitica* zoospores and cysts and preliminary work during the present study showed that cysts of *A. invadans* isolates failed to germinate after exposure for 1 hour at 20°C to malachite green concentrations as low as 0.08 ppm.

Fraser *et al* (1992) has shown that RSD isolates are unable to sporulate in water above 2 ppt NaCl. This would correlate with reports on EUS outbreaks that water of increased salinity (20 ppt) acted as a barrier to the spread of the disease in Sri Lanka (Costa and Wijeyaratne, 1989). Similarly, Raman (1992) reported that EUS did not occur in highly saline zones (8 - 12 ppt) of Chilka Lake in India; and that in affected

zones, the incidence of EUS subsided as the salinity increased from 1.0 - 5.2 ppt in December to 2.8 - 5.8 ppt in February.

EUS has been reported only in tropical and sub-tropical areas and it should be remembered that the antifungal activity of chemicals and their effect on fish varies with temperature. Malachite green, for example, is very toxic to fish at higher temperatures (Alderman, 1985). Chinabut (1993) noted that malachite green is generally more toxic to scaleless fish than scaled fish, but advised that 0.1-0.15 ppm indefinite bath treatments of malachite green should be safe for most tropical fish, preferably in the early morning when temperatures are lowest. Willoughby and Roberts (1992a) warned however that given the rapid infection rate by fungi in tropical conditions, one water treatment daily is probably not sufficient to prevent infection.

Further special considerations have to be made with regard to the control and treatment of EUS given that affected cultured fish are often in ponds of very high organic content when the efficacy of many fungicides is reduced. In these cases farm management techniques such as reducing stress to cultured fish, excluding wild fishes and sterilisation of equipment are advised (Lilley *et al,* 1992). The high relative susceptibility of *A. invadans* to the disinfectants studied here shows that sterilisation can be easily achieved and is a useful management technique which can help prevent outbreaks of EUS.

Pond trials carried out at the Bureau of Fisheries and Aquatic Resources (BFAR) in the Philippines have indicated that 5 ppm Coptrol (a copper chelated compound) can prevent the development of lesions in *A. invadans*-challenged, abraded catfish (*Clarias gariepinus*), but concluded that this compound is too expensive for use in aquaculture (R.B. Callinan, pers. comm.). In the same trials, 0.1 ppm malachite green was partially effective, and agricultural lime (2 kg/100 m^2) failed to prevent the development of EUS lesions. The main affect of agricultural lime ($CaCO_3$), however, is only to stabilise the pH of the water, whereas slaked lime ($Ca(OH)_2$) is known to have

an additional biocidal effect, and Lilley *et al* (1992) suggested that at prescribed concentrations, this may be a more efficacious treatment for diseased fish.

The spread of EUS to islands and other isolated areas in Asia is probably the result of the uncontrolled movement of fish within the region (Costa & Wijeyaratne, 1989). To minimise the risk of further spread of EUS, one of the effective fungicidal treatments identified in this study should be applied to all fish, including ornamental species, imported to areas free from EUS. This should be combined with a period of quarantine for species susceptible to EUS.

Figure 5.1

Radial growth of test fungi on GP agar incorporating different antibiotic concentrations, given as a percentage of controls. Antibiotics tested were:

(a) Penicillin-K

(b) Streptomycin sulphate

(c) Oxolinic acid

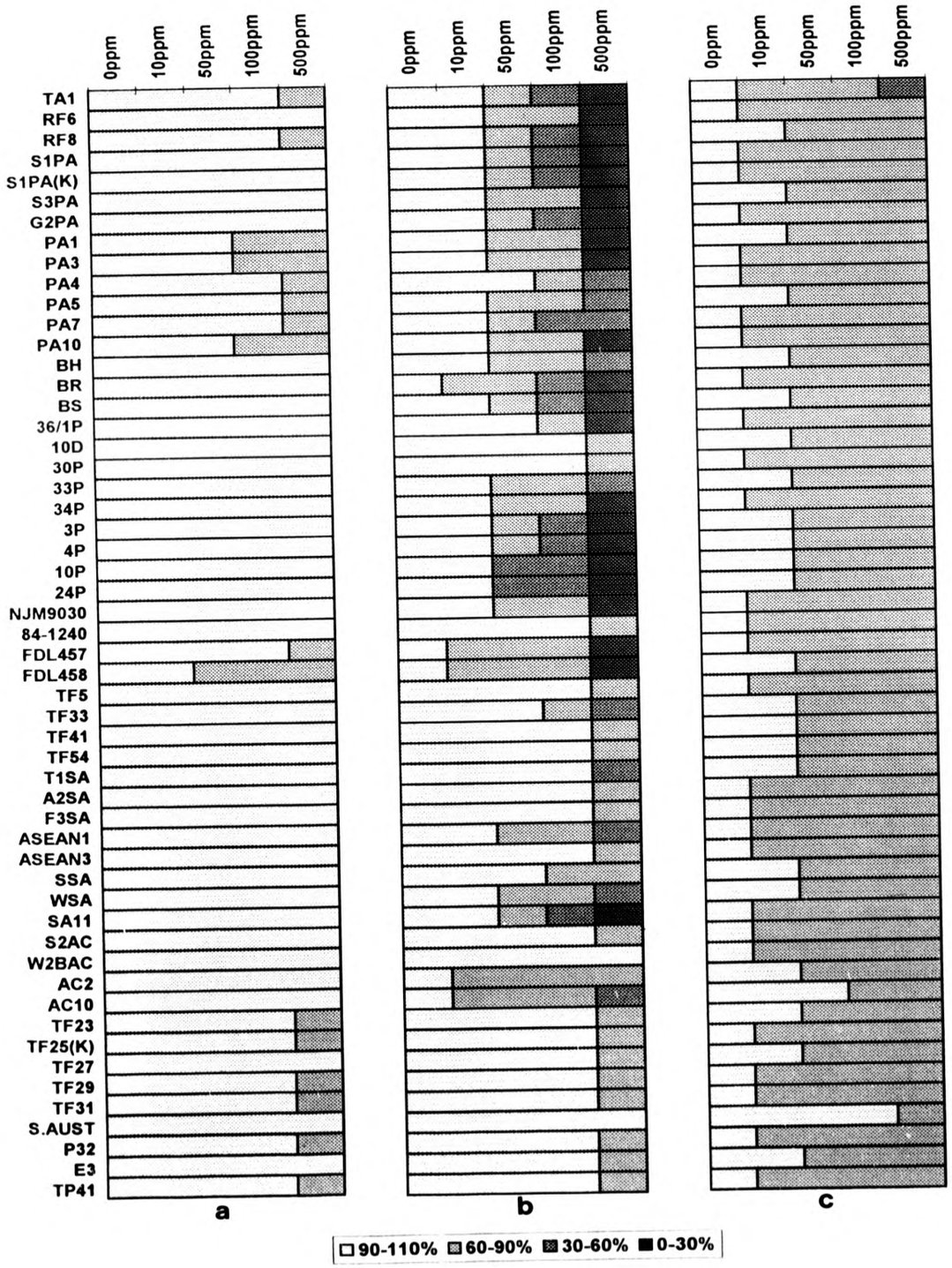


Figure 5.2

Minimum inhibitory concentration (MIC) of three fungicides against test fungi. This was assessed by radial growth of test fungi on GPY agar compared to controls, following one hour fungicide treatments of fungal agar plugs. Fungicides tested were:

(a) Malachite green

(b) Hydrogen peroxide

(c) Sodium chloride

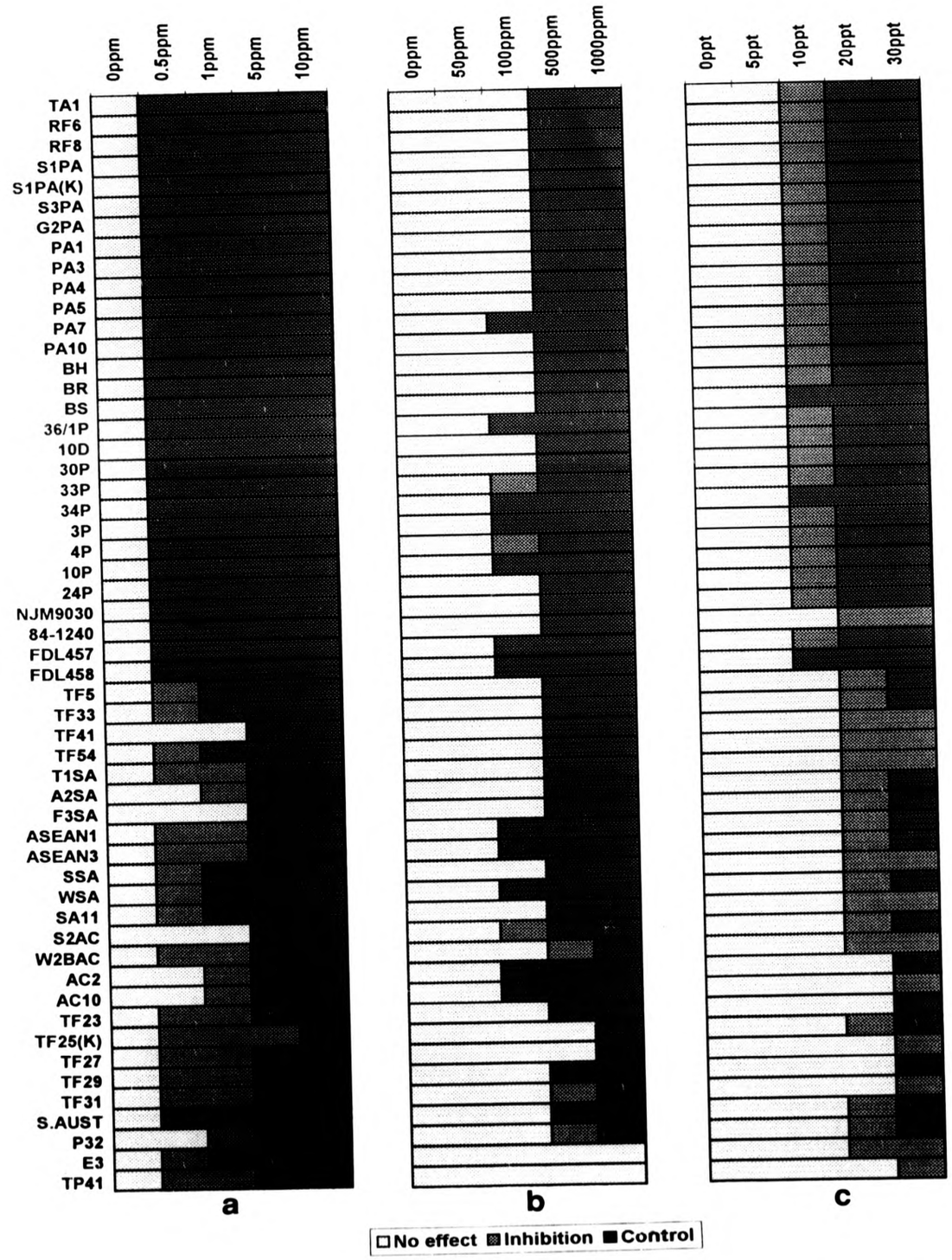


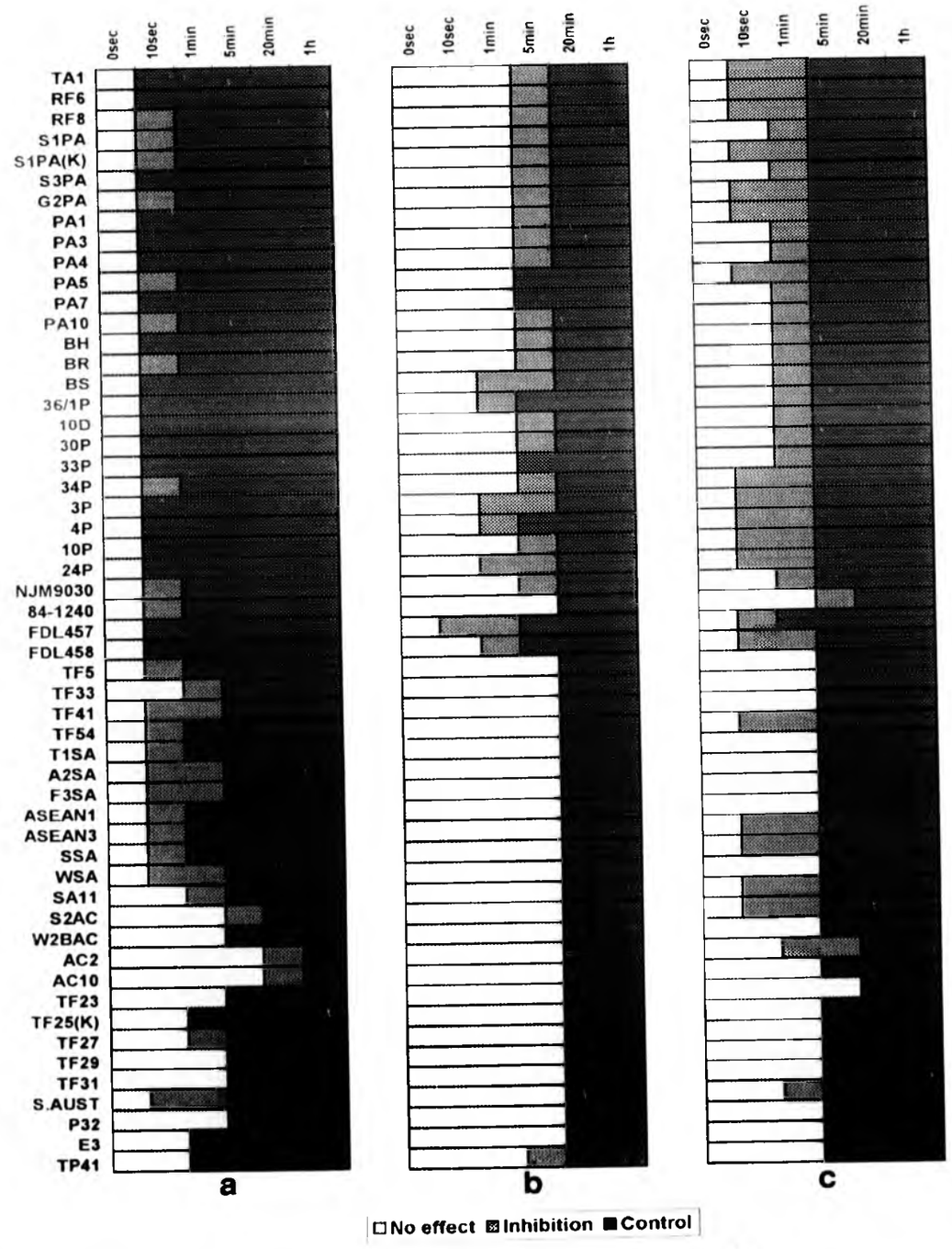
Figure 5.3

Minimum lethal time (MLT) of three disinfectants to test fungi. This was assessed by radial growth of test fungi on GPY agar compared to controls, following disinfectant treatments of fungal agar plugs at various exposure times. Disinfectants tested were:

(a) FAM 30 (at 100 ppm available iodine)

(b) Sodium hypochlorite (at 100 ppm available chlorine)

(c) Proxitane 0510 (at 100 ppm peracetic acid)



CHAPTER 6. ZOOSPORE BEHAVIOUR

6.1 Introduction

Motile zoospores provide Oomycete fungi with a capacity for dispersal (Lange and Olson, 1983) and host/substrate location (Deacon and Donaldson, 1993). Therefore the study of adaptations in the structure, physiology and behaviour of the secondary zoospores of *Aphanomyces invadans* are likely to provide valuable information on the processes by which the fungus locates and infects hosts. Chapter 7 deals with the comparative structure of *A. invadans* propagules, and selected aspects of zoospore behaviour in relation to different environmental conditions are investigated here.

Chemotaxis to certain host-derived substances (Cerenius and Söderhäll, 1984a; Rand and Munden, 1993b), encystment induced by certain substances (Burr, 1991), indirect germination (Willoughby *et al*, 1983) and polyplanetism (Cerenius and Söderhäll, 1985) have all been studied in saprolegniacean fungi as possible adaptations to parasitism. These aspects were studied in relation to *A. invadans*, and compared with other related fungi.

As no measurements of secondary zoospores or cysts have been described for EUS, MG and RSD isolates (Table 2.1), this chapter also includes an assessment of size-distribution counts of present fungi using a Coulter counter. Schoulties and Yang (1971) showed that reliable counts of *Aphanomyces* zoospores could be obtained using an older Coulter instrument.

6.2 Materials and methods

6.2.1 Size-distribution of zoospores

A selection of EUS (TA1, PA7, 36/1P), MG (NJM9201), RSD (24P), UM (84-1240), saprophytic *Aphanomyces* (TF33, WSA), *Achlya* (W2BAC, AC2), and *Saprolegnia* (TF27) isolates were analysed. At least two spore suspensions of each isolate were used. These were prepared using (b) or (c) technique described in Section 2.2.5 and then filtered through 2 sheets of Whatman 541 filter paper. An initial aliquot was taken

and added gently to Isoton II electrolyte solution (Coulter Corporation) at 1 part zoospore suspension to 5 parts Isoton II. Size distribution readings were taken of the secondary zoospores using a Coulter Multisizer (Coulter Electronics Ltd). The original sample was then shaken for 1 min in a universal tube, and left for at least 5 min, before another aliquot added to Isoton II (1:5) and used to obtain counts of encysted zoospores. Work described in Section 6.2.5 had shown that zoospores rapidly encyst following agitation. Cerenius and Söderhäll (1984b) and Burr (1991) reported that zoospores of *A. astaci* detach their flagellae within 2 min after 20 sec vortexing, and *S. parasitica* encyst within 5 min after 30 sec vortexing. At least three counts were taken to allow the sample to stabilise, before readings were downloaded into a computer and plotted using Coulter Multisizer AccuComp software Version 1.19.

6.2.2 Chemotaxis

Chemotaxis in *A. invadans* was investigated using a technique based on that described by Bimpong and Clerk (1970). Test substances were incorporated into 1.2% technical agar (Oxoid no.3). Spore suspensions were prepared as in Section 2.2.5(a). APW (Appendix 2a) was drawn up into a sterilised haematocrit tube, which was then inserted into the test agar. The test agar was pushed further into the tube a further few millimetres using a needle, and the tube was left in a Petri dish containing a test suspension of motile zoospores. After 1 hour zoospore attraction was examined under a microscope and compared to agar-only control plugs.

6.2.3 Geotaxis

From observations of sporulating EUS, MG and RSD cultures in Petri dishes, it has appeared that motile zoospores of these isolates do not tend to accumulate at the surface of the APW to the extent seen in other isolates. In order to investigate this further, two 5 ml glass pipettes were stuck together using silicon sealant giving a total height of 40 cm. Suspensions of zoospores were drawn up the pipettes, which were then left vertical for 30 min to 4 hours with the bottom end plugged with plasticine. After a set time, aliquots of zoospore suspension were taken from the pipettes and

counts were made of motile zoospores per field of view at x10 magnification under the microscope.

6.2.4 Chemically induced encystment of motile zoospores

This work was undertaken at the University of Newcastle upon Tyne, using a method adapted from Burr (1991). Encystment of secondary zoospores of an EUS isolate (36/1P), a saprophytic *Aphanomyces* sp (WSA) and *Saprolegnia diclina* (E3) was tested in the following solutions: Con A (5 µl/ml), $CaCl_2$ (50 mM), pectin (50 µl/ml), GPY (1/10). The first three solutions have been shown to accelerate encystment in some Oomycetes at present concentrations by Hardham and Suzaki (1986); Grant, Griffith and Irving (1986) and Grant, Irving and Radda (1985) respectively. Glucose, the main ingredient of GPY has also been used at 50 mM to induce encystment in *Phytophthora cinnamomi* (Byrt, Irving and Grant, 1982a). Distilled water was added to control suspensions. The drop culture technique (Section 2.2.5d), with GPY broth as a growth medium, was used to produce suspensions of motile zoospores. Triplicate 90 µl samples were pipetted into separate sterile plastic Petri dishes for testing each fungal zoospore suspension against each test solution. Ten microlitres of test solutions were added so as to give the end concentrations indicated above. After a specific time, the suspensions were fixed in 1% glutaraldehyde. A large cover slip was placed over the drop and sealed with clear nail varnish. The proportion of zoospores encysted was calculated from counts of 200-300 cells under a microscope. These experiments were carried out allowing 1 min or 1 hour for encystment. In a further experiment, GPY (1/10 concentration) was added to the suspensions, following the addition of the test substances, and samples were left for 15 hours before fixation to check that viable hyphae resulted from germination.

6.2.5 Germination following mechanically induced encystment

This study assessed the relative tendency of encysted zoospores to germinate, or produce a further zoospore generation (polyplanetism), given a general nutrient background (GPY). This work was carried out at the University of Uppsala, Sweden, and as well as three *A. invadans* isolates (96PA, 36/1P, 33P), and three

Aphanomyces saprophytes (F3SA, WSA, ASEAN3), two fungi previously tested in that laboratory were also included: *A. laevis* (107-52) and *A. astaci* (Hö). The technique of Diéguez-Uribeondo, Cerenius and Söderhäll (1994) was adopted, whereby motile zoospore suspensions were obtained as in Section 2.2.5(d) and encysted by vortexing for 45 sec. An equal volume of GPY broth was added immediately. After a further 2 hours, the percentage of cysts that had germinated, released zoospores (indicated by empty "ghost" cyst), or were undifferentiated (neither germinated nor empty), were determined by examining 200-300 cells under a microscope.

In other experiments, encysted zoospores were left in APW with no added nutrients, and features of the resulting starved germlings were observed.

6.2.6 Polyplanetism following mechanically induced encystment

This work was carried out at the University of Uppsala with the isolates listed above, and an additional control isolate, *Saprolegnia parasitica* (SPT). Zoospore suspensions were produced and vortexed as described above to induce encystment. After a period of time, which varied between isolates, the next generation of laterally biflagellated zoospores was vortexed and so on until no subsequent zoospore generations were released. At each stage the proportion of germinated, empty and undifferentiated cysts were counted.

6.3 Results

6.3.1 Size-distribution of zoospores

Coulter counts of all suspensions showed a peak at about 2 μm, which probably constituted particulate matter from the pond water or paper debris derived from the paper-filtering the APW. This peak was excluded from analyses. The zoospore size distributions clearly distinguished the smaller *Aphanomyces* propagules from those of *Achlya* and *Saprolegnia* (Table 6.1, Figure 6.1). EUS, MG and RSD isolates were indistinguishable, and often quite variable. However, spore counts of these isolates

were generally lower than the other fungi tested, which reduces the accuracy of the machine. The size of saprophytic *Aphanomyces* propagules varied between isolates; WSA as shown in Figure 6.1, is clearly intermediate between *A. invadans* and the *Achlya-Saprolegnia* group. The encysted zoospores of several isolates were measured at up to 0.7 μm larger than motile zoospores.

Table 6.1 **Average (mean ± SD) diameter of fungal zoospores determined using a Coulter counter**

	motile secondary zoospore (μm)	encysted secondary zoospore (μm)
EUS, MG and RSD isolates	6.0 ± 0.3	6.4 ± 0.4
84-1240 (UM isolate)	6.8 ± 0.1	6.8 ± 0.2
TF33 (*Aphanomyces* sp)	7.5 ± 0.2	7.6 ± 0.2
WSA (*Aphanomyces* sp)	6.9 ± 0.1	7.3 ± 0.1
AC2 (*Achlya* sp)	8.3 ± 0.1	9.0 ± 0.3
W2BAC (*Achlya* sp)	8.9 ± 0.2	9.3 ± 0.5
TF27 (*Saprolegnia diclina*)	8.5 ± 0.2	9.1 ± 0.3

6.3.2 Chemotaxis

Attraction of zoospores to a few test substances are shown in Table 6.2.

Table 6.2 **Chemotaxis of motile secondary zoospores after 1 hour**

	GPY	CDA	1% casein	MEM + 1% FBS	trout blood	salmon blood	tilapia blood
RF6 (EUS pathogen)	++	-	-	+	+	+	++
NJM9030 (MG pathogen)	++	-	-	++	+	+	++
FDL457 (*A. astaci*)	-	-	++	++	+	+	+
84-1240 (UM isolate)	++	-	+	-	+	+	+
TF5 (*Aphanomyces* sp)	++	-	+	+	+	+	+
ASEAN1 (*A. laevis*)	+	-	+	+	+	+	+

++ strong chemotaxis
\+ some chemotaxis
\- no discernible chemotaxis

MEM minimum essential medium Eagle (ICN)
FBS foetal bovine serum

6.3.3 Geotaxis

Using the present technique, counts giving the distribution of zoospores along the vertical pipettes were not consistent. Zoospores of several EUS, MG and RSD isolates tested appeared to be more randomly distributed than zoospores of *Aphanomyces* saprophyte (TF33) or *Achlya* sp (S2AC), which showed an accumulation at the top of the tube, but given the variation between experiments, no significant differences could be recorded. The effect of positive or negative chemotaxis to the plasticine was not tested.

6.2.4 Chemically induced encystment of motile zoopores

Various difficulties associated with obtaining cyst-free suspensions for experiments, maintenance of suspensions in contained drop cultures, and the drying of samples under coverslips were experienced with this study. For these reasons, the results given in Table 6.3 were the only data obtained by averaging triplicated experiments.

Table 6.3 Encystment and germination of zoospores in response to various substances

	distilled water			Con A (5 µl/ml)			$CaCl_2$ (50 mM)			pectin (50 µl/ml)			GPY (1/10)		
	%Z	%C	%G	%Z	%C	%G	%Z	%C	%G	%Z	%C	%G	%Z	%C	%G
36/1P (*A. invadans*)															
fixed after 1 min	78	22	0	57	43	0	55	45	0	52	48	0	57	43	0
fixed after 1 hour	8	77	15	3	79	18	14	65	21	7	73	20	6	79	15
WSA (*Aphanomyces* sp)															
fixed after 1 min	96	4	0	97	3	0	96	4	0	98	2	0	98	2	0
E3 (*Saprolegnia diclina*)															
fixed after 1 min	81	19	0	80	20	0	66	34	0		nd		61	39	0

%Z = % zoospores remaining
%C = % cysts
%G = % germinated cysts
nd = not done

Table 6.4 Encystment, germination and polyplanetism of zoospores kept in 1/10 GPY for 15 hours after exposure to various substances

	distilled water			Con A (5 µl/ml)			$CaCl_2$ (50 mM)			pectin (50 µl/ml)			GPY (1/10)		
	%C	%G	%Z	%C	%G	%Z	%C	%G	%Z	%C	%G	%Z	%C	%G	%Z
***A. invadans* (36/1P)**	35	20*	45	0	100	0	0	100	0	3	86	11		nd	
saprophytic *Aphanomyces* (WSA)	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0

%C = % undifferentiated cysts
%G = % germinated cysts (including abbreviated life cycle - see Section 6.3.5 - *highest level of occurrence 40% of %G)
%Z = % zoospores released (ie number of empty cysts)
nd = not done

The proportion of cysts in test suspensions after 1 min was not much higher than in controls, indicating that this was not sufficient time to allow for encystment. Numbers of encysted 36/1P zoospores were high in controls after 1 hour, making it difficult to demonstrate any induced encystment by test substances. However, the fact that some zoospores remained in test samples showed that none of the substances could induce 100% encystment in *A. invadans* at present concentrations (Table 6.3). All of the 36/1P propagules kept in 1/10 GPY for 15 hours germinated only when exposed to Con A or $CaCl_2$, indicating that these substances do encourage germination in *A. invadans* (Table 6.4). Pectin appears to have a marginal effect on germination. In contrast to *A. invadans*, all the propagules of the saprophytic *Aphanomyces* WSA had germinated after 15 hours, even in controls (Table 6.4), indicating that 1/10 GPY is enough to induce germination in this isolate. The lack of undifferentiated cysts in 36/1P samples exposed to Con A and $CaCl_2$ shows that all *A. invadans* propagules counted were capable of germination, and therefore the undifferentiated cysts in controls here (and in Table 6.5 below) are attempting to release zoospores.

6.3.5 Germination following mechanically induced encystment

The relative importance of germination and polyplanetism to the various isolates is indicated by the fate of encysted zoospores after the addition of media as shown in Table 6.5.

Table 6.5 **Effect of adding equal volume GPY to zoospore suspensions immediately after vortexing, as observed after 2 hours**

	% zoospore release (ie empty cysts)	% germination	% undifferentiated cysts
96PA (*A. invadans*)	24	71	5
36/1P (*A. invadans*)	16	60	24
33P (*A. invadans*)	44	56	0
Hö (*A. astaci*)	56	0	44
F3SA (*Aphanomyces* sp)	0	100	0
WSA (*Aphanomyces* sp)	0	92	8
ASEAN3 (*A. laevis*)	0	99	1
107-52 (*A. laevis*)	0	98	2

In other experiments at IOA, Stirling University, involving more isolates, no media was added after encystment was induced. In this case, several isolates showed evidence of the production of "starved germlings". Examination of these showed indirect germination commonly occurred in several EUS, MG and RSD isolates (Plate 6.1a); a saprophytic *Aphanomyces* sp (WSA) and *Saprolegnia* isolates (TF20S, TF26, TP41). This phenomenon is where a fine, septate germ tube connects an empty cyst to the viable cytoplasmic hypha (Willoughby, 1977). Less obvious, and more rarely observed evidence of indirect germination was shown by a UK *A. astaci* isolate (FDL458), a UM isolate (84-1240) and saprophytic *Aphanomyces* sp (TF33, TF54). Indirect germination was not observed in a Swedish *A. astaci* isolate (Hö), *A. laevis* (ASEAN1), a saprophytic *Aphanomyces* sp (T1SA) and *Achlya* sp (S2AC). RSD isolate, 24P, was used to show percentage indirect germination out of all germlings in distilled water (59%), 1/10,000 GPY (53%), 1/1000 GPY (43%) and 1/100 GPY (43%), although these percentages were variable among the pathogenic isolates.

EUS, MG and RSD isolates, and also the saprophyte, WSA, all demonstrated structures consistent with an abbreviated life-cycle (Willoughby, 1977). In these cases, a cyst germinates but does not grow far before producing another cyst-like structure which is thought to be capable of releasing a zoospore. Although partial dehiscence of the cyst-like structures was observed, under present conditions they

appeared incapable of fully releasing zoospores, as in the "undifferentiated cysts" recorded in Table 6.4. The abbreviated life-cycle appeared as illustrated in Figure 2.2(I).

6.3.6 Polyplanetism following mechanically induced encystment

A. invadans isolates (96PA, 36/1P, 33P) were shown to be capable of producing one generation of motile zoospores from artificially encysted secondary zoospores, but no further generations could be induced (Table 6.6). There was however, a fairly high proportion of undifferentiated cysts (neither releasing zoospores, nor germinating). The same was the case for an *A. astaci* isolate (Hö). This contrasts with work by Cerenius and Söderhäll (1984b), who succeeded in inducing three generations in a different isolate of *A. astaci*. Up to six successive generations of *S. parasitica* (SPT) zoospores could be induced, as previously reported by Diéguez-Uribeondo *et al* (1994) and Heinäaho (1996) for the same isolate. A higher proportion of *A. laevis* (ASEAN3, 107-52) secondary cysts produced zoospores than reported by Cerenius and Söderhäll (1985), but percentage germination was high in the second polyplanetic generation. Other saprophytic isolates (F3SA, WSA), however, showed a high level of polyplanetism, each producing up to four successive generations of zoospores.

Table 6.6 **Maximum number of repeated zoospore generations induced in each isolate**

	generation number	hours between vortexing	% zoospore release (ie empty cysts)	% germination	% undifferentiated cysts
96PA (*A. invadans*)	1	-	82	9	9
		12	0	48	52
33P (*A. invadans*)	1	-	65	6	29
		12	0	35	65
36/1P (*A. invadans*)	1	-	58	3	39
		12	0	47	53
Hö (*A. astaci*)	1	-	57	1	42
		17 at 13°C	0	54	46
F3SA (*Aphanomyces* sp)	1	-	82	18	0
	2	4½	80	20	0
	3	5½	63	35	2
	4	3¼	10	88	2
WSA (*Aphanomyces* sp)	1	-	58	31	11
	2	4½	85	4	11
	3	5½	92	5	3
	4	3¼	3	92	5
ASEAN3 (*A. laevis*)	1	-	67	4	29
	2	3½	20	74	6
107-52 (*A. laevis*)	1	-	30	32	38
	2	4	13	45	42
SPT (*Saprolegnia parasitica*)	1	-	99	0	1
	2	2½	98	0	2
	3	3¾	99	0	1
	4	2¾	96	0	4
	5	2½	84	0	16
	6	10	2	3	95

6.4 Discussion

The diameter of hyphae and propagules of EUS, MG and RSD isolates are highly variable (Table 2.1) compared with other saprolegniacean fungi. However, size of these structures are one of the few morphological features available to distinguish these isolates from other *Aphanomyces* spp under the light microscope. Therefore, Coulter counter analysis was used as a rapid method to see if a particular size-distribution profile could be obtained for zoospores of EUS isolates, and whether this was consistent with MG and RSD isolates as well. This method did indicate that propagules of these isolates were generally smaller and had a higher level of variation compared to other species tested, but this made it more difficult to obtain a consistent, normal distribution, particularly in cyst suspensions.

It would be of interest to evaluate the presence of "giant cysts" (up to 27 μm in diameter) that appeared to be particularly frequent in sporulating samples of some of the present EUS, MG and RSD isolates (Plate 6.1b & c). Scott (1961) considered these to be abnormally large encysted primary zoospores resulting from incomplete cleavage of the protoplasm prior to discharge from the sporangia, which go on to produce two secondary zoospores. However, in present samples, individual cysts were observed fusing to form giant cysts, and these have only been observed germinating, not dehiscing to release any number of zoospores. DAPI stained giant cysts illustrated in Chapter 7 show them to be multinucleate.

Putative clamydospores, as illustrated for EUS isolate, RF6, by Roberts *et al* (1993) were observed here in sporulating cultures of EUS, MG and RSD isolates. Although Scott (1961) maintained that these structures did not occur in *Aphanomyces* species, Srivastava (1979) gave illustrations of distinctively shaped chlamydospores as a major distinguishing feature of *A. pisci*. The structures observed in EUS, MG and RSD isolates are formed by simple segmentation of small sections of hyphae; they do not show any extraordinary morphology and can not be regarded as a significant diagnostic feature.

Observations of *A. invadans* zoosporangial structure during the course of these experiments revealed apical evacuation of primary zoospores from terminal sporangia (Plate 6.2a), and either lateral evacuation tubes from terminal sporangia, or intercalary sporangia (Plate 6.2b & c). The differentiation between the long, complex, terminal sporangia commonly observed in EUS isolates by Willoughby *et al* (1995) and the intercalary sporangia described as common by Fraser *et al* (1992) depends on locating the septa that delimit the sporangia. These were found to be very difficult to trace, so much so that Hatai (1980a) felt that they did not exist in MG isolates (see Table 2.1).

Specific chemotactic responses are a clear indication of adaptation to a parasitic mode of life. Initial trials using eel mucus in an agar diffusion system as described by Cerenius and Söderhäll (1984a) gave some indication of a positive chemotactic response in *A. invadans*. However, problems were encountered with this technique, and the mucus was unavailable for the present haematocrit tube trials. These shown significant differences between the response of EUS/MG pathogens and *A. astaci* to GPY and casein. It is hoped to extend these trials using further substances.

Chemotropism of fungal hyphae growing on GP agar plates was also tested using a well of trout blood serum. Each fungus tested (S1PA, TF33, TP41) showed slightly increased growth in the area where serum had visibly diffused into the agar, but no directional change in hyphal growth could be detected. Chemotropism towards casein hydrolysate (Musgrave, Ero, Scheffer and Oehlers, 1977) and various amino acids (Schreurs, Harold and Harold, 1989) has been previously reported for *Achlya bisexualis*.

Cameron and Carlile (1977) demonstrated negative geotaxis in *Phytophthora* spp by comparing the top and bottom 1 mm of zoospore suspensions in 4 cm tubes. Using the present technique, neither negative nor positive geotaxis could be demonstrated. Callinan (pers. comm.) has observed possible positive geotaxis in RSD isolates, but no quantitative data are available.

The finding of Cerenius and Söderhäll (1985), that the more parasitic *Aphanomyces* isolates showed a higher level of polyplanetism was not sustained in the present studies. The two unspeciated saprophytic *Aphanomyces* (F3SA and WSA) showed a much greater facility for polyplanetism than did either *A. invadans* or *A. astaci*. Although the latter two species showed a high proportion of undifferentiated cysts, which may, under more suitable conditions produce zoospores, the three generations demonstrated by Cerenius and Söderhäll (1984b) for *A. astaci* was still less than that induced in the saprophytes here. It may be the case, however, that F3SA and WSA

have a more parasitic lifestyle than *A. laevis* (isolated from water samples), which showed a low level of polyplanetism. The finding of Willoughby and Roberts (1994b; c) that *A. invadans* zoospores lost and regained motility following physical shock, without an intervening encystment stage, was not observed here. Empty "ghost" cysts left by the tertiary zoospore generation of *A. invadans* were clear evidence of the intervening encystment stage. However, these structures were very much more difficult to observe compared with all the other species, which is consistent with the finding in Chapter 7 that *A. invadans* cyst walls have a very thin amorphous outer layer and are therefore much less obvious under the light microscope.

The differentiation between pathogens and saprophytes was also unclear in the experiments on indirect germination, which Willoughby *et al* (1983) considered to be a possible adaptation to parasitism in saprolegnians. In the present study, indirect germination was shown to be common in *A. invadans* but possibly absent in *A. astaci*. Saprophytic *Aphanomyces* species showed varying abilities to produce septate germ tubes depending on the isolate. The fact that *Saprolegnia* fungi tested here clearly showed indirect germination, and *Achlya* clearly did not, also indicates that this phenomenon varies between taxa, but its relevance to the lifestyle of the fungus is uncertain.

Structures that probably represented an abbreviated life-cycle appeared very similar morphologically for EUS, MG and RSD pathogenic isolates as well as the saprophyte, WSA. They were also morphologically different from those illustrated by Willoughby (1977) for *Saprolegnia* sp. Structures described here invariably formed after only after a short section of the hypha had grown, and appeared to resemble a cyst rather than a saprolegnian sporangium. On dehiscence, present structures also appeared to be attempting to release a single zoospore, whereas Willoughby (1986) described the release of two zoospores from the mini-sporangium. Willoughby (1986) also described the release of a single zoospore from germlings ("micro life-cycle") but this was only from the original secondary zoospore cyst.

A clear difference between pathogens and saprophytes was demonstrated here by the addition of nutrient media to suspensions of encysted zoospores. In this case, none of the four saprophytic *Aphanomyces* isolates produced any zoospores (Table 6.5), whereas some *A. invadans* cysts germinated and some released zoospores. Under these conditions, none of the *A. astaci* cysts germinated. This appears to indicate that *A. invadans* is intermediate between the saprophytes and *A. astaci* in its requirement for a specific stimulus before germination takes place. Diéguez-Uribeondo *et al* (1994) showed that almost 100% of each of 5 generations of zoospores of *S. parasitica* would germinate in a nutrient background (PG-1). In this way, *S. parasitica* is acting similarly to the *Aphanomyces* saprophytes described here.

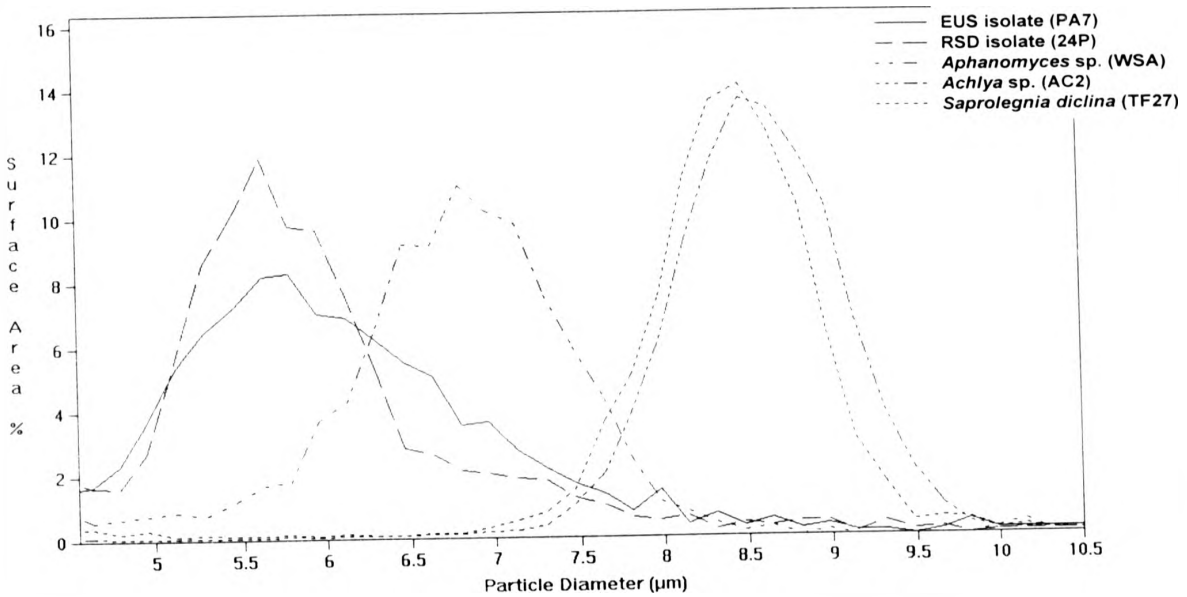
Work by Svensson and Unestam (1975) on *A. astaci,* Byrt *et al* (1982a; b) on *Phytophthora cinnamomi,* Jones, Donaldson and Deacon (1991) on *Pythium aphanidermatum,* and Burr (1991) on *Saprolegnia* spp has shown that often different stimuli are involved in the mechanisms of encystment and germination. Con A and $CaCl_2$, at present concentrations, did not induce 100% encystment in *A. invadans* zoospores (Table 6.3), but did stimulate germination in 100% of *A. invadans* cysts (Table 6.4). Willoughby and Roberts (1994c) reported that $CaCl_2$ inhibited *A. invadans* zoospore motility temporarily at 2.5 - 5 mM Ca^{2+} and permanently at 10 mM Ca^{2+}, but zoospores did not appear to encyst. Immunocytochemical studies described in Chapter 7 show that another lectin, WGA, has stronger binding characteristics to *A. invadans* propagules than Con A, and may also act to stimulate encystment and/or germination in this fungus. Burr and Beakes (1994) have previously reported variation in the response of different species to WGA. They noted that WGA bound to the ventral groove region, and induced encystment, of zoospores of a saprolegnian saprophyte (E3), but not of the fish parasite TP41.

In studying the natural infection cycle of the fungus, it is preferable to examine the specific stimuli that induce the zoospores to encyst, and distinguish these from

physical and chemical shock treatments that result in encystment, and at higher levels, can cause cell lysis (Singh and Bartnicki-García, 1975; Burr, 1991). An examination of the latter may, however, lead to the development of control strategies against pathogenic fungi. Work on *A. astaci* has shown that $MgCl_2$ lyses 80% zoospores at 50 mM Mg^{2+} (Persson and Söderhäll, 1986), and also prevents sporulation at 20 mM and growth at 200 mM, and has therefore been recommended for use in the control of crayfish plague (Rantamäki *et al*, 1992). Although the undifferentiated cysts counted in several experiments described here sometimes showed evidence of lysis, this also occurred in control samples in APW and was not necessarily a result of the test substance.

Figure 6.1 **Coulter counter generated size distribution of (a) motile secondary zoospores and (b) encysted secondary zoospores**

(a) motile zoospores



(b) zoospore cysts

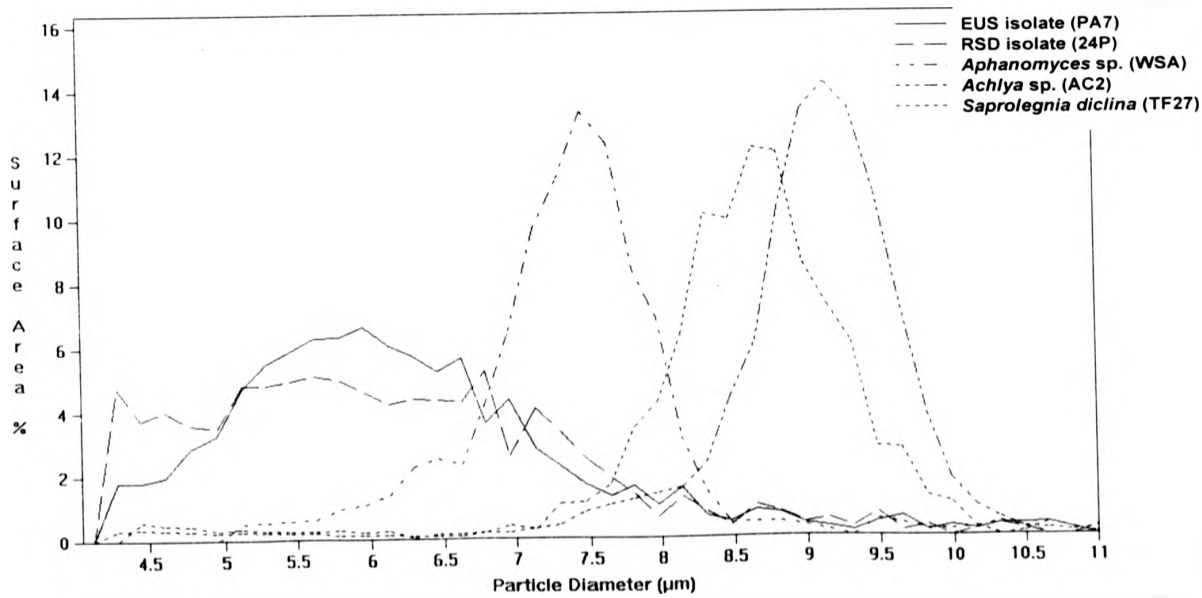


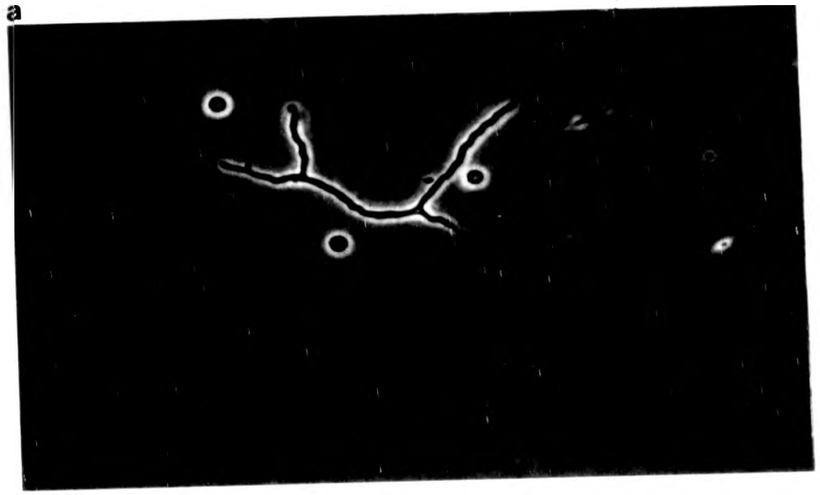
Plate 6.1

(a) Indirect germination of a starved germling of EUS isolate RF6 (x400)

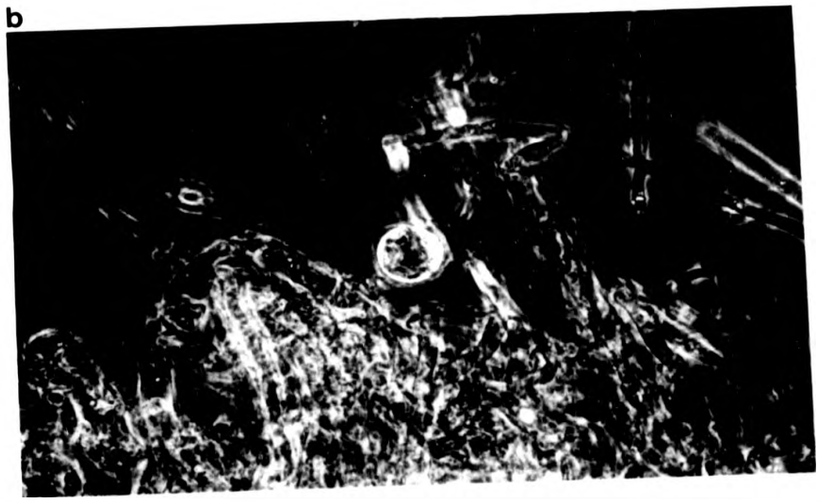
(b) Giant cyst of EUS isolate BH, associated with zoosporangia (x400)

(c) Giant cyst of EUS isolate RF6, associated with germlings (x400)

a



b



c

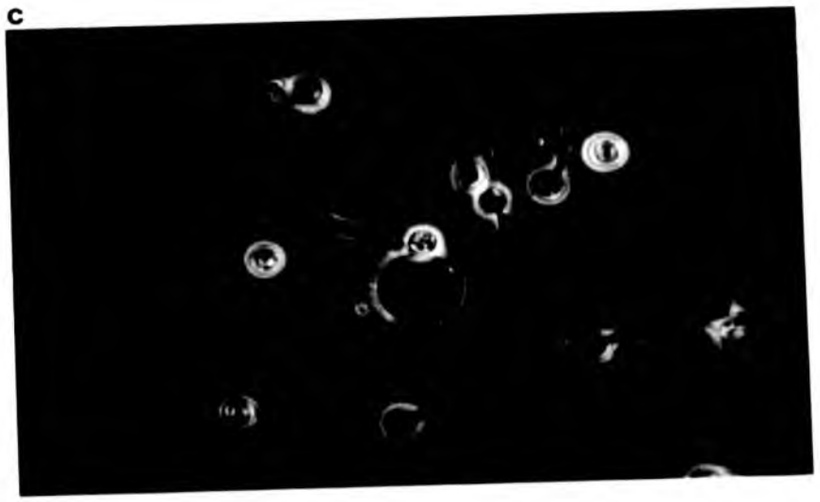


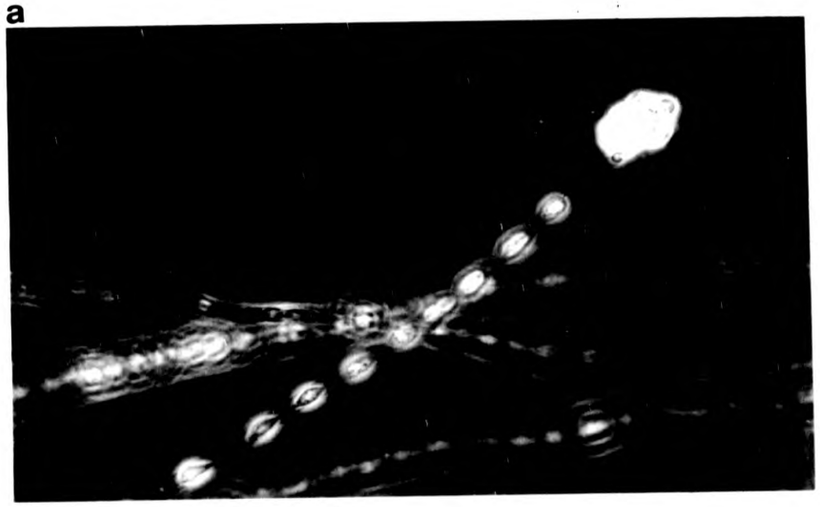
Plate 6.2

(a) Single row of primary zoospores of MG isolate NJM9030 connected by a fine strand of cytoplasm, being released from a terminal sporangium, and encysting as an apical cluster (x400)

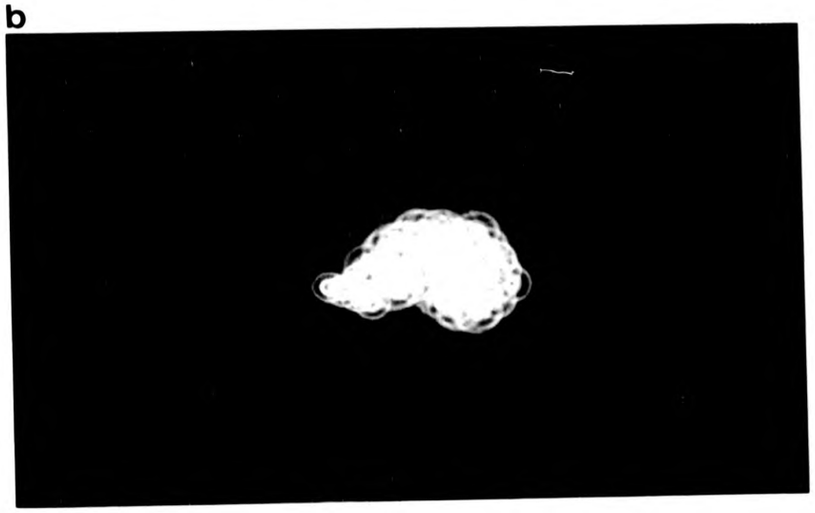
(b) Lateral evacuation tube on either a complex terminal sporangium, or an intercalary sporangium of EUS isolate PA7 (x400)

(c) As (b) for MG isolate NJM9030 (x400)

a



b



c

CHAPTER 7. ULTRASTRUCTURE AND IMMUNOCYTOCHEMISTRY

7.1 Introduction

Chapter 6 studied some of the possible behavioural adaptations of zoospores of *A. invadans* to a pathogenic lifestyle, and work described here examines zoospore ultrastructure and surface binding characteristics of *A. invadans* in order to define taxonomic markers particular to the species, and obtain information that may be relevant to pathogenesis.

Given that the heterokont, biflagellated zoospore is probably the most distinctive feature of the Oomycetes, species-specific characteristics of zoospore structure are also considered to be important in elucidating the phylogenetics of the class (Beakes, 1987; Hardham, Cahill, Cope, Gabor, Gubler and Hyde, 1994). Of the saprolegniacean Oomycetes, detailed ultrastructural information is available on *Saprolegnia ferax* (Heath and Greenwood, 1971; Lehnen and Powell, 1991); *Aphanomyces euteiches* (Hoch and Mitchell, 1972a; b); and *Aphanomyces astaci* (Olson, Cerenius, Lange and Söderhäll, 1984; Cerenius, Olson, Lange and Söderhäll, 1984). Comparison of homologous organelles in zoospores of different Oomycetes have provided important cladistically-valid taxonomic information, and the study of analogous features may provide information on the functional biology of these fungi. However it is important that the homology, or analogy, of these characters is carefully determined (Powell and Blackwell, 1995). Homologous single-membrane bounded kinetosome-associated organelles, or K bodies, occur in all orders of Oomycetes except for the Peronosporales, and are morphologically variable enough to be used as specific taxonomic markers in some species of *Aphanomyces* (Powell *et al*, 1985). These organelles were therefore of particular interest to the present study.

The tubule-filled cavity of *S. ferax* secondary zoospore K bodies has been shown to contain certain carbohydrate moieties (Lehnen and Powell, 1988) that bind to the

lectin WGA (Lehnen and Powell, 1993) and are released into the ventral groove during encystment (Lehnen and Powell, 1989), forming an adhesion pad that becomes progressively more polymerised (Durso, Lehnen and Powell, 1993). Burr and Beakes (1994) demonstrated that a saprophytic *Saprolegnia* isolate, E3 (considered to be *S. diclina*, although this is questioned by Molina *et al*, 1995), showed similar WGA-binding characteristics. In contrast, secondary zoospores of the fish-parasite, *S. parasitica* (TP41), showed only patchy dorsal binding with this lectin. Monoclonal antibodies raised against *S. parasitica* reacted with some vesicle fractions of TP41, but did not identify similar vesicles in E3 (Burr and Beakes, 1994). These interspecific variations in the immunocytochemistry of vesicles associated with encystment and host/substrate adhesion are considered to be indications of important ecological differences between closely-related saprolegniacean fungi. Similar immunocytochemical studies have been carried out among other Oomycete orders, namely the Peronosporales: *Phytophthora cinnamomi* (Hardham *et al*, 1994) and *Pythium aphanidermatum* (Estrada-Garcia, Green, Booth, White and Callow, 1989); and the Lageniales: *Lagenidium giganteum* (Berbee and Kerwin, 1993). Preliminary immunocytochemical investigations of *A. invadans* are described here using a variety of polyclonal (PAb) and monoclonal (MAb) antibodies and different carbohydrate-binding lectins.

7.3 Materials and methods

Most of the work described here was undertaken at Newcastle University, based on techniques described by Burr and Beakes (1994). The isolates studied are listed in Appendix 1. In all cases fungi were grown in drop cultures of GPY and induced to sporulate as in Section 2.2.5(d). Given the relatively delicate nature of *A. invadans* zoospores, problems were encountered with their fixation and centrifugation, and a variety of techniques were tested.

7.3.1 SEM

Sporulating mycelium was placed in fixative [2.5% glutaraldehyde (TAAB Lab Equip Ltd, Aldermaston, Berkshire) in 0.1 M sodium cacodylate buffer (TAAB Lab Equip Ltd), or half this concentration] for 1 hour. Fungus was brought through 2 washes of cacodylate buffer and left in buffered 1% OsO_4 for 1 hour. After 2 further washes, zoospores were attached to glass coverslips, dehydrated in a graded acetone series and critical-point dried. Cover slips were then mounted on aluminium stubs, sputter coated with gold, and observed on a Cambridge Stereoscan 240 SEM.

7.3.2 TEM

Sporulating mycelium, or suspensions of motile or vortex-encysted zoospores, were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer or 50 mM PIPES buffer (or $^1/_2$, $^1/_4$, or $^1/_8$ this concentration of fixative) for 30 min. Mycelium was transferred through 2 changes of buffer before post-fixation in 1% OsO_4 in cacodylate buffer for 1 hour. Similarly, zoospore suspensions were centrifuged twice at between 800 - 3000 xg for 5 - 20 min followed by addition of buffer, before further centrifuging and dropwise addition of buffered 1% OsO_4, and were then left at room temperature for 1 hour. Mycelium was then transferred to buffer and left overnight; and zoospore suspensions centrifuged, resuspended in buffer, and left overnight. Zoospore suspensions were then concentrated by centrifugation and pipetted onto 0.2 μm nitrocellulose filters (Whatman). A few drops of molten agarose (1%) was added and allowed to solidify at 4°C for 1 min. The agarose was then peeled off the filter and cut into 2 mm blocks. Mycelium, or zoospores embedded in agarose were then dehydrated in an acetone series (10 min in 20%, 40%, 60%, 70%, 80%, 90%, and 3 x 100% acetone) and infiltrated with resin (1 hour in 1:2, 1:1, 3:1, and 2 x 1:0 resin:acetone) (medium-hard Transmit resin, TAAB Lab Equip Ltd). Blocks were placed in 0.75 ml plastic polymerisation tubes, filled with resin and polymerised at 60°C for 24 hours. Silver-grey sections were cut using a Reichert Ultracut ultramicrotome. These were collected on formvar-coated copper grids, stained in aqueous uranyl acetate and lead citrate, and examined using a Philips 301 electron microscope.

7.3.3 Immunocytochemistry

Suspensions of mixed motile, encysted and germinating zoospores were fixed in either 4% formaldehyde in 50 mM PIPES buffer (to allow better access of antibodies and lectins to intracellular contents: A.H. Hardham, unpublished manual) or 0.2% glutaraldehyde in 4% formaldehyde in 50 mM PIPES buffer (to give good structural preservation of membranes, with low levels of aldehyde-induced fluorescence). After 30 min, cells were centrifuged at 1000 xg for 5 min, resuspended in 50 mM PIPES, centrifuged again, resuspended in 50 mM PIPES and 75 mM glycine, centrifuged again, and finally resuspended in 100 µl phosphate buffered saline (PBS: 0.02 M $NaH_2PO_4.2H_2O$, 0.02 M $Na_2HPO_4.2H_2O$, 0.15 M NaCl, pH 7.2). Ten microlitre aliquots were dispensed into multiwell slides (Flow Labs Inc) and dried at 40°C for 1 hour. Slides were kept in the dark at 4°C and stained with either antibodies or lectins (listed in Figure 7.1) within 2 weeks. Ten microlitres of antibody or lectin preparations (as described below) were added to the cells and incubated in a moist chamber at 37°C for 45 min. Lectin-stained slides were then mounted using DAKO antifade fluorescent mounting medium (DAKO Corporation, Carpinteria, CA, USA). Antibody-stained slides were washed 3 times by adding 10 µl PBS for 3 min and then wicking this away with filter paper. Monoclonal fluorescein isothiocyanate (FITC)-conjugated secondary antibody [10 µl, diluted 1:30 in PBS with 1% bovine serum albumin (BSA)] was then added (anti-rabbit against polyclonal primary antibodies, and anti-mouse against monoclonal primary antibodies). Slides were washed a further 2 times and mounted in DAKO antifade medium. A DNA-specific stain, DAPI (4',6-Diamidino-2-phenylindole), was added to the final PBS wash of some slides at 1/1000 so that nuclei and mitochondria could also be observed by UV fluorescence microscopy. All slides were examined under a Leica DMRB research microscope and pictures recorded photographically using Tmax 1600 ASA film uprated to 3200.

The three polyclonal rabbit antisera were prepared as detailed in Chapter 8. These comprised antisera raised against mycelial extract of an EUS isolate (αPA7), mycelial

extract of a saprophytic *Aphanomyces* (αF3SA), and an *A. invadans*-specific 10 kDa band eluted from an electrophoretic gel (αband). The anti-*S. parasitica* monoclonal (HB4) (courtesy of G.W. Beakes) had been raised against mixed primary and secondary zoospores and cysts of isolate TP41 as described in Burr and Beakes (1994). Anti-*Phytophthora* monoclonals (Vsv1, Lpv1, Cpa2) (courtesy of A.H. Hardham) had been raised against *P. cinnamomi* spores as described in Hardham *et al* (1986; 1991). A commercially available anti-tubulin monoclonal (αTAT) was also tested. Non-immune mouse serum was used a negative control. Anti-*P. cinnamomi* MAbs were used at 10 μg/ml, and all other primary antibodies were diluted 1/10 in PBS with 1% BSA.

FITC-labelled lectins (Sigma) were used at 20 μg/ml in PBS containing 0.5% BSA. $CaCl_2$ (0.1 mM) and $MnCl_2$ (0.01 mM) were added to Con A preparations. Inhibitory saccharides (0.1 M) were added to lectin preparations to block binding sites and act as negative controls (αmannose was used against Con A; N-acetyl-D-galactose against SBA; and N-acetyl D-glucose against WGA).

7.3 Results

7.3.1 SEM

Scanning EM photos show that the primary zoospore cysts form strong clusters comprising variable numbers of cysts (usually over 20). Although papillae can be observed on some cysts, these are not as pronounced or consistent as in the truly papillate *Aphanomyces* species and probably develop prior to release of secondary zoospores (Plate 7.1). In concordance with other members of the genus *Aphanomyces* (Scott, 1961), SEM observations showed that both primary and secondary cysts of *A. invadans* do not possess cyst coat ornamentation.

7.3.2 TEM

Problems were encountered achieving adequate fixation and centrifugation of EUS, MG and RSD isolates (low osmolarity fixative and centrifugation speeds were

necessary to prevent cell lysis), and therefore not enough samples were successfully examined for ultrastructural comparisons between pathogenic isolates. However, observations of the fine structure of primary cysts of *A. invadans* isolate 33P revealed certain features that distinguished it from other species examined. In particular, the cyst coat appeared to lack a thick, electron dense (encystment vesicle derived) outer wall layer, typical of saprolegniacean fungi (Plates 7.2 and 7.3). Peripheral vesicle components were also not clearly identified in the cells examined.

K bodies were not identified in secondary zoospores and cysts, although few well-fixed samples were examined. K bodies were also difficult to locate in primary cysts but a few samples showed putative immature K bodies with different substructure to that observed in *A. astaci, A. laevis* and saprophytic *Aphanomyces*, WSA (Plate 7.5).

Plate 7.4 shows a secondary cyst of saprophytic *Aphanomyces*, WSA, with an obvious outer wall layer and K body.

7.3.3 Immunocytochemistry

A pictorial representation of the antibody- and lectin- binding characteristics of each isolate tested is given in Figures 7.2 and 7.3, and some example photographs are shown in Plate 7.6.

Polyclonal antisera (αPA7, αF3SA, αband) all showed a high degree of cross-reactivity (Plate 7.6, 1-3). Anti-band PAb showed slightly less intense binding than αPA7 or αF3SA, but no specific component of cells could be identified as the site of binding. The anti-*Saprolegnia* MAb, HB4, bound strongly to certain zoospore vesicle fractions and the cyst coat of *S. parasitica* as previously described by Burr (1991), but showed much less reactivity with *Aphanomyces* spp (Plate 7.6, 4b). Of particular interest was the strong positive binding of *P. cinnamomi* MAb, Vsv1, to patches on zoospores of *A. invadans* (Plate 7.6, 5b). Vsv1 showed a possible slight reaction with an *Aphanomyces* saprophyte (WSA), and no reaction with *S. parasitica* (TP41). Other

P. cinnamomi MAbs, Lpv1, cross-reacted with *A. invadans* to a lesser extent than Vsv1, and Cpa2 showed no significant binding to any of the isolates tested. Anti-tubulin (αTAT) sera bound strongly to zoospore surfaces and flagellae (Plate 7.6, 6b), but the resolution was not good enough to observe the microtubule structure of the cells. DAPI staining revealed that all zoospores and cysts of saprophytic *Aphanomyces* (WSA and F3SA) were uninucleate, whereas EUS and RSD preparations included giant cysts that usually contained 4-6 nuclei (Plate 7.6, 4c).

Of the lectins, soybean agglutinin (SBA) showed no significant binding to any of the isolates. Con A binding was less intense and more variable in *A. invadans* isolates than *S. parasitica,* and conversely, WGA binding was stronger in *A. invadans* isolates than *S. parasitica*. WGA binding was also variable between saprophytic *Aphanomyces* isolates, appearing to be associated with the flagellae and possible ventral vesicle fractions in WSA, and showing similar characteristics to *S. parasitica* in F3SA.

7.4 Discussion

Initial difficulties were experienced locating structures that could represent K bodies in *A. invadans*. This, combined with the lack of a typical saprolegniacean dense outer cell wall, and the ability of *A. invadans* zoospores to cross-react with *P. cinnamomi* MAbs, aroused the possibility of a taxonomic relationship with the Peronosporales. However, putative K bodies were subsequently observed in primary cysts of *A. invadans* isolate 33P, and slight cross-reactions of Vsv1 MAb with another *Aphanomyces* sp (WSA) indicated that, although *A. invadans* shows some atypical ultrastructural and immunocytochemical features, there is no strong evidence against its placement in the genus *Aphanomyces*. Further molecular studies described in Chapter 11 confirm this, although future work should include a peronosporalean fungus for comparative purposes.

The putative K body illustrated in Plate 7.5(a) has a substructure distinct from all the morphotypes described by Powell and Blackwell (1995). It does not show the helical fibres typical *A. euteiches* (Hoch and Mitchell, 1972a; Sadowski and Powell, 1990), the regular

paracrystalline rods of *A. astaci* (Cerenius *et al*, 1984; Olson *et al*, 1984; Powell *et al*, 1985) nor the cavity containing scattered thick fibres characteristic of *A. laevis* (Cerenius *et al*, 1984; Olson *et al*, 1984; Plate 7.5b). It could also be distinguished from K bodies commonly observed in various zoospore stages of the saprophytic *Aphanomyces* WSA (Plate 7.5b). The *A. invadans* K body may therefore represent a useful taxonomic marker for distinguishing the species, however, further work is needed to characterise this organelle and determine any variation in structure during development. Holloway and Heath (1977) commented that K1 bodies found in primary cysts of *Saprolegnia ferax* were morphologically variable depending on stage of development, and Powell and Blackwell (1995) considered that knowledge of the ontogeny of the organelle is required before it can be regarded as truly homologous to K bodies in other species. As only immature K bodies have been observed in *A. invadans*, no morphological description can be given as yet for this species.

It was previously considered unlikely for MAbs to vesicle fractions of Peronosporales or Saprolegniales to cross-react with each other (Hardham *et al*, 1994; Beakes, Burr, Wood and Hardham, 1995). The finding here that Vsv1 shows patchy reactivity with *A. invadans* is therefore significant. Vsv1 was previously shown to cross-react with *Pythium* spp (Cope, Webb, O'Gara, Philip and Hardham, 1996) but not to non-peronosporalean fungi. The small ventral vesicles to which Vsv1 react in *Phytophthora* spp are involved in cyst adhesion during encystment, and are considered functionally analogous to K bodies in saprolegniaceans (Hardham, 1995). The distribution of the Vsv1-reacting fluorescent patches throughout the *A. invadans* cells, however, does not correspond to the location of typical saprolegniacean K bodies. The lack of Vsv1-reactivity with cysts (Plate 7.6, 5b), also indicates that binding is not with a component that is released on encystment. These features are consistent with the characteristics of saprolegnian large peripheral or fibrillar vesicles (Burr, 1991), however, ultrastructural observations of immunogold-labelled sections are needed before the binding site of any of the antibodies or lectins can be confidently stated.

The MAb, Lpv1, binds to the large peripheral vesicle fraction of *P. cinnamomi*, and is considered specific to that genus (Cope *et al*, 1996). However, slight patchy binding was observed here on zoospores of *A. invadans*. Cpa2 binds to the small dorsal vesicle fraction of *P. cinnamomi* which also reacts to the same fractions as SBA (Hardham *et al*, 1994). This is consistent with present work as neither Cpa2 nor SBA showed any significant binding with any of the isolates investigated here.

Western blots combining mycelial extracts with lectins are described in Chapter 8. As with the present immunocytochemical studies, Con A and WGA showed significantly more binding to *A. invadans* than SBA (=GMA). The lack of reactivity of SBA with all the saprolegniacean species studied here was similarly found for *Saprolegnia* by Burr (1991) and distinguishes these species from *Phytophthora* spp (Hardham *et al*, 1994). Burr and Beakes (1994) showed that the binding characteristics of lectin WGA distinguished *S. parasitica* (TP41) from the saprophyte, E3, and this character was shown to be similarly variable between species of *Aphanomyces* studied here. Given the high level of reactivity of WGA with *A. invadans* zoospore vesicles, it would be interesting to test the ability of this lectin to induce encystment in *A. invadans*.

Particularly long boathook spines on the secondary cysts of fish-parasitic *Saprolegnia* isolates are thought to facilitate passive entanglement with fish surfaces and indicate that these cysts are the main infective spore (Pickering, Willoughby and McGrory, 1979; Beakes *et al*, 1994). *A. invadans*, as with other *Aphanomyces*, is shown to lack cyst wall ornamentation, and further work is required to determine the relative importance of active zoospore-, or passive cyst-, encounters with fish. Burr and Beakes (1994) suggested that WGA-binding material is involved in adhesion in *Saprolegnia,* and it can be speculated that given the high level of WGA-binding material on the surface of *A. invadans* propagules, that this may actually provide a important means of attachment to hosts, either by passive encounter, or following zoospore chemotaxis and induced encystment.

Previous studies using polyclonal antibodies raised against the pea pathogen, *Aphanomyces euteiches,* have showed little or no cross-reactivity with plant pathogenic *Phytophthora* spp, and have been used to detect *A. euteiches* in infected plant and soil samples (Kraft and Boge, 1994; Petersen, Olson and Rosendahl, 1996). The use of antibodies to probe for *A. invadans* in water samples would, however, have to be much more specific to differentiate the pathogen from the various saprophytic *Aphanomyces* spp present. Anti-*A. invadans* polyclonals used here are clearly not adequate, as they show significant cross-reactivity with zoospores of *Aphanomyces* saprophytes, *Saprolegnia*, and even *Lagenidium* (G.W. Beakes, pers. comm.). Hardham *et al* (1986; 1991) developed MAbs against *P. cinnamomi* that are species- or even strain- specific, and Cahill and Hardham (1994a; b) showed that these were capable of detecting 250 - 5000 zoospores of *P. cinnamomi* per ml of soil suspension using a dipstick assay technique. The development of such a technique for *A. invadans* would provide a means of studying the ecology of the fungus, and determining whether a particular water body is potentially infective.

Plate 7.1

(a) Scanning electron micrograph of *A. invadans* (33P) showing cluster of at least 12 encysted primary zoospores. Cyst diameter = 5.6 - 5.7 μm

(b) Scanning electron micrograph of saprophytic *Aphanomyces* (TF54) showing cluster of at least 8 encysted primary zoospores. Cyst diameter = 4.9 - 5.8 μm

a

b

Plate 7.2

Transmission electron micrograph of *A. invadans* (33P) showing a section through a cluster of at least 35 encysted primary zoospores (1 cm = 1.9 μm)

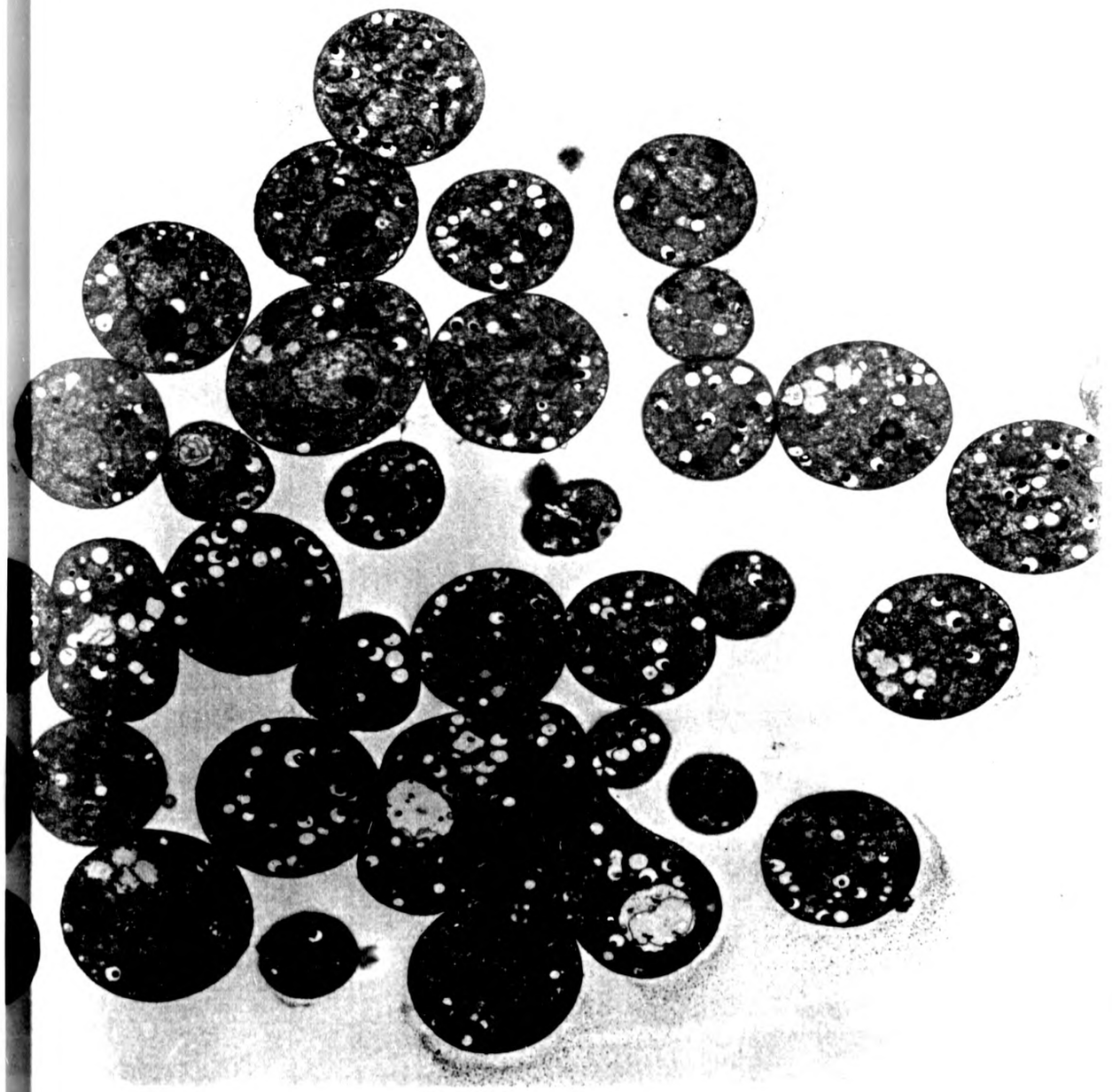


Plate 7.3

Transmission electron micrograph of *A. invadans* (33P) showing a section through an encysted primary zoospore (1 cm = 270 nm). D = dense body vesicle, F = fibrillar or peripheral vesicle, L = lipid, M = mitochondrion, N = nucleus. Arrow indicates thin outer wall

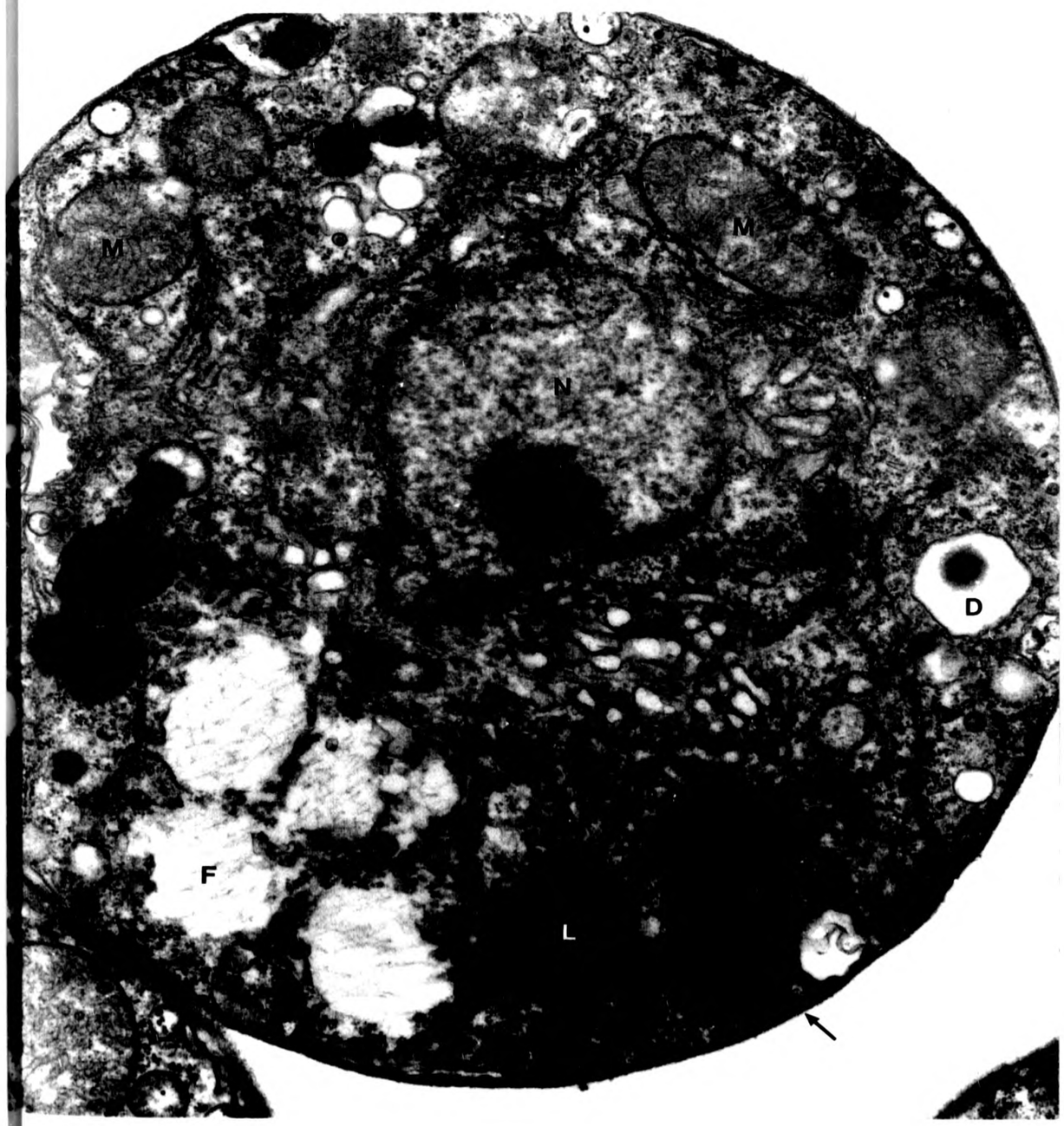


Plate 7.4

Transmission electron micrograph of saprophytic *Aphanomyces* (WSA) showing a section through an encysted secondary zoospore (1 cm = 345 nm). D = dense body vesicle, K = K body, L = lipid, M = mitochondrion. Arrow indicates thick outer wall

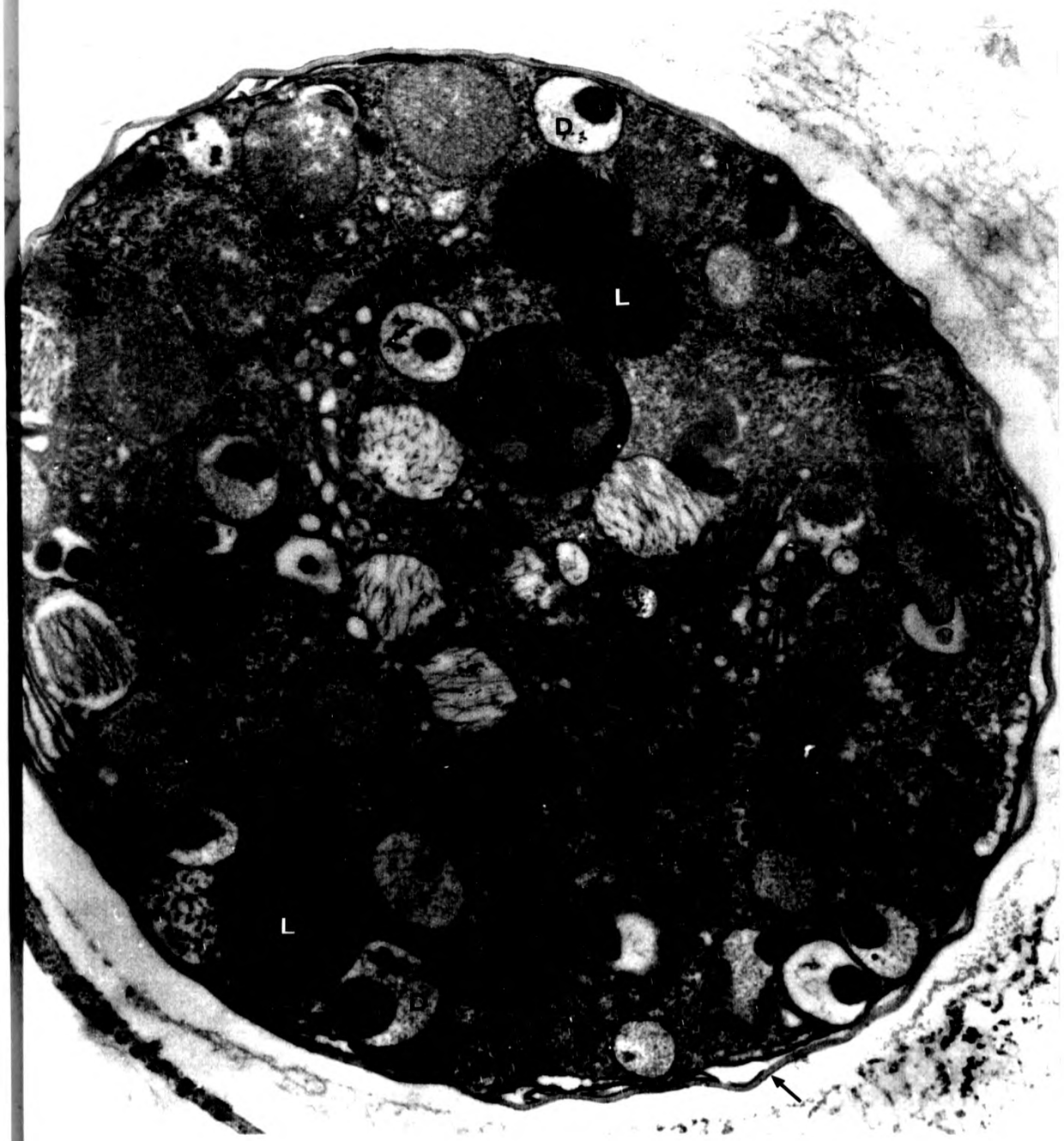


Plate 7.5

Transmission electron micrographs showing variable structure of K bodies in:

(a) Above - *A. invadans* (33P) primary cyst (1 cm = 154 nm)

(b) Below, left - saprophytic *Aphanomyces* (WSA) secondary cyst (1 cm = 147 nm)

(c) Below, right - *Aphanomyces laevis* primary cyst (1 cm = 265 nm)

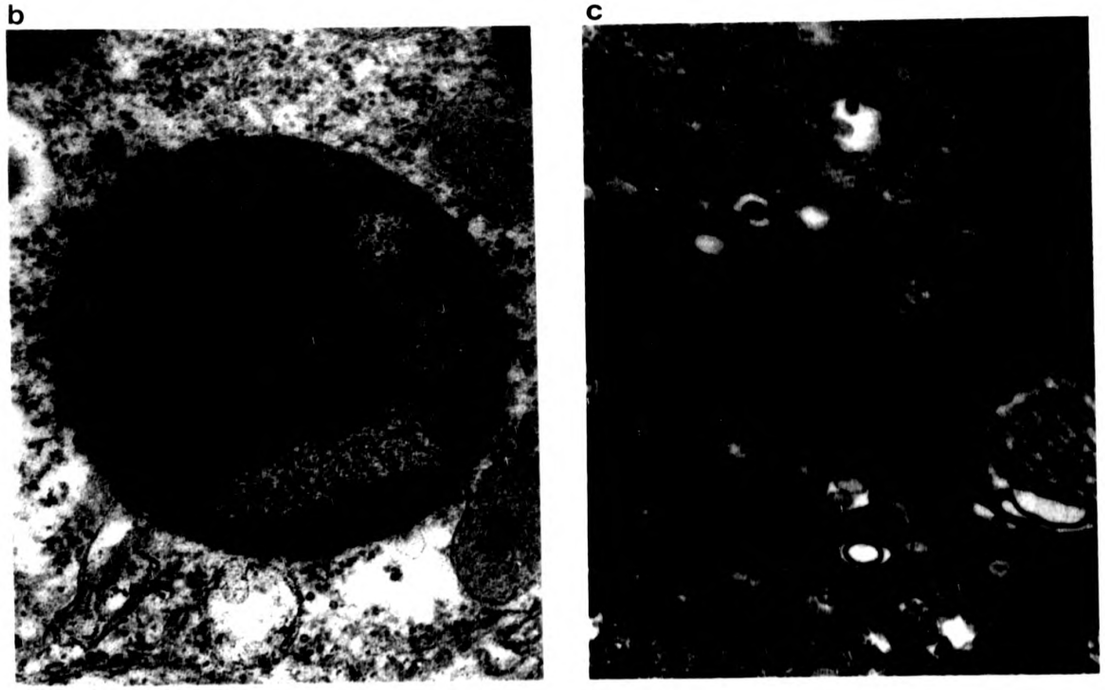


Figure 7.1

Primary antibodies and lectins used in this investigation

Antibody /Lectin code	Origin	Type	Specificity
αPA1 (see chapter 8)	*A. invadans* mycelial extract	Polyclonal	To zoospore, cyst and hyphal surfaces
αband (see chapter 8)	*A. invadans* 10 kDa electroeluted band	Polyclonal	To zoospore, cyst and hyphal surfaces
αF3SA (see chapter 8)	saprophytic *Aphanomyces* mycelial extract	Polyclonal	To zoospore, cyst and hyphal surfaces
HB4/HB2 Burr and Beakes (1994)	*S. parasitica* cysts and zoospores	Monoclonal	To cyst coat material (Con A reacting)
Vsv1 Hardham *et al* (1994)	*P. cinnamomi* cysts and zoospores	Monoclonal	To small ventral vesicle fraction - forms adhesive pad on cysts
Lpv1 Hardham *et al* (1994)	*P. cinnamomi* cysts and zoospores	Monoclonal	To large peripheral vesicle fraction, storage protein
Cpa2 Hardham *et al* (1994)	*P. cinnamomi* cysts and zoospores	Monoclonal	To small dorsal vesicle fraction - forms peripheral matrix on encystment
αTAT	tubulin	Monoclonal	To tubulin, an acidic protein that makes up microtubules
Con A Concanavalin A	Sigma Chemicals	Lectin	To mannose and glucose molecules
SBA soybean agglutinin	Sigma Chemicals	Lectin	To N-acetyl-D-galactose molecules
WGA wheat-germ agglutinin	Sigma Chemicals	Lectin	To N-acetyl-D-glucosamine molecules

Figure 7.2(a) **Antibody-binding to zoospores and cysts**

ANTIBODY	ZOOSPORE	CYST AND GERMTUBE
EUS (PA1, 33P) and RSD (24P) isolates		
flagella cyst coat αPA7 (α *A. invaderis*) zoospore germtube	++++ ++++	++++ ++++
flagella cyst coat HB4 (α *S. parasitica*) zoospore germtube	- (+) on contents of lysed cells	(+) -
flagella cyst coat Vsv1 (α *P. cinnamomi*) zoospore germtube	- ++++ surface patches	++ patchy -
flagella cyst coat Lpv1 (α *P. cinnamomi*) zoospore germtube	- + faint patches	- -
flagella cyst coat Cpa2 (α *P. cinnamomi*) zoospore germtube	- -	(+) -
***Saprolegnia parasitica* TP41**		
flagella cyst coat αPA7 (α *A. invaderis*) zoospore germtube	+++ ++ all over surface	++ patchy ++ patchy, variable
flagella cyst coat HB4 (α *S. parasitica*) zoospore germtube	- +++ surface patches	++++ -
flagella cyst coat Vsv1 (α *P. cinnamomi*) zoospore germtube	- -	- -
flagella cyst coat Cpa2 (α *P. cinnamomi*) zoospore germtube	- -	- -

Figure 7.2(b) **Antibody-binding to zoospores and cysts**

ANTIBODY	ZOOSPORE	CYST AND GERMTUBE
Saprophytic *Aphanomyces* WSA		
flagella cyst coat αPA7 (α *A. invaderis*) zoospore germtube	+++ +++	+++ +++
flagella cyst coat HB4 (α *S. parasitica*) zoospore germtube	- + patches	+ faint -
flagella cyst coat Vsv1 (α *P. cinnamomi*) zoospore germtube	- + 1 or 2 faint patches	- -
flagella cyst coat Lpv1 (α *P. cinnamomi*) zoospore germtube	- -	- -
flagella cyst coat Cpa2 (α *P. cinnamomi*) zoospore germtube	- (+)	(+) -
Saprophytic *Aphanomyces* F3SA		
flagella cyst coat αPA7 (α *A. invaderis*) zoospore germtube	++ ++	+++ +++
flagella cyst coat HB4 (α *S. parasitica*) zoospore germtube	- + faint	+ faint -
flagella cyst coat Cpa2 (α *P. cinnamomi*) zoospore germtube	- (+)	(+) -

Figure 7.3(a) **Lectin-binding to zoospores and cysts**

LECTIN	ZOOSPORE	CYST AND GERMTUBE
EUS (PA1, 33P) and RSD (24P) isolates		
Con A flagella cyst coat zoospore germtube	- ++ variable	++ variable -
SBA flagella cyst coat zoospore germtube	- -	- -
WGA flagella cyst coat zoospore germtube	(+) +++	+++ also empty cyst cases +++
***Saprolegnia parasitica* TP41**		
Con A flagella cyst coat zoospore germtube	++ +++	+++ -
SBA flagella cyst coat zoospore germtube	- -	- -
WGA flagella cyst coat zoospore germtube	- ++ in patches	- ++++ decreases towards the tip

Figure 7.3(a) **Lectin-binding to zoospores and cysts**

LECTIN	ZOOSPORE	CYST AND GERMTUBE
EUS (PA1, 33P) and RSD (24P) isolates		
Con A	flagella - cyst coat zoospore ++ variable germtube	++ variable -
SBA	flagella - cyst coat zoospore - germtube	- -
WGA	flagella (+) cyst coat zoospore +++ germtube	+++ also empty cyst cases +++
***Saprolegnia parasitica* TP41**		
Con A	flagella ++ cyst coat zoospore +++ germtube	+++ -
SBA	flagella - cyst coat zoospore - germtube	- -
WGA	flagella - cyst coat zoospore ++ in patches germtube	- ++++ decreases towards the tip

Figure 7.3(b) **Lectin-binding to zoospores and cysts**

LECTIN	ZOOSPORE	CYST AND GERMTUBE
Saprophytic *Aphanomyces* WSA		
Con A flagella cyst coat zoospore germtube	+ ++	++ patchy -
SBA flagella cyst coat zoospore germtube	- -	- -
WGA flagella cyst coat zoospore germtube	+++ +++ 1 or 2 patches	++ 1 or 2 patches, variable (+)
Saprophytic *Aphanomyces* F3SA		
Con A flagella cyst coat zoospore germtube	- ++ 1 or 2 patches	++ -
SBA flagella cyst coat zoospore germtube	- -	- -
WGA flagella cyst coat zoospore germtube	- +++ in patches	- +++

Plate 7.6

Corresponding photographs using (a) normarski interference light, (b) FITC-fluorescence, and (c) DAPI-fluorescence microscopy illustrating:

- **non-specificity of polyclonal antisera (PAbs)**

1 **αF3SA PAb binding with *Saprolegnia parasitica* (TP41) secondary zoospore (0.2% glut, 4% form)**

2 **αPA7 PAb binding with saprophytic *Aphanomyces* (F3SA) secondary zoospores (0.2% glut, 4% form)**

3 **(b) αband PAb binding with RSD isolate (24P) germling (4% form), and (c) DAPI stain showing location of 2 nuclei**

- **lack of cross-reactivity of α*Saprolegnia parasitica* MAb with *A. invadans***

4 **(b) HB4 not binding to a zoospore and cysts of EUS isolate (PA1) (0.2% glut, 4% form), and (c) DAPI stain revealing 4 nuclei in one giant cyst**

- **cross-reactivity of α*Phytophthora cinnamomi* MAb with *A. invadans***

5 **Vsv1 binding to zoospores, but not cysts, of EUS isolate (33P) (4% form)**

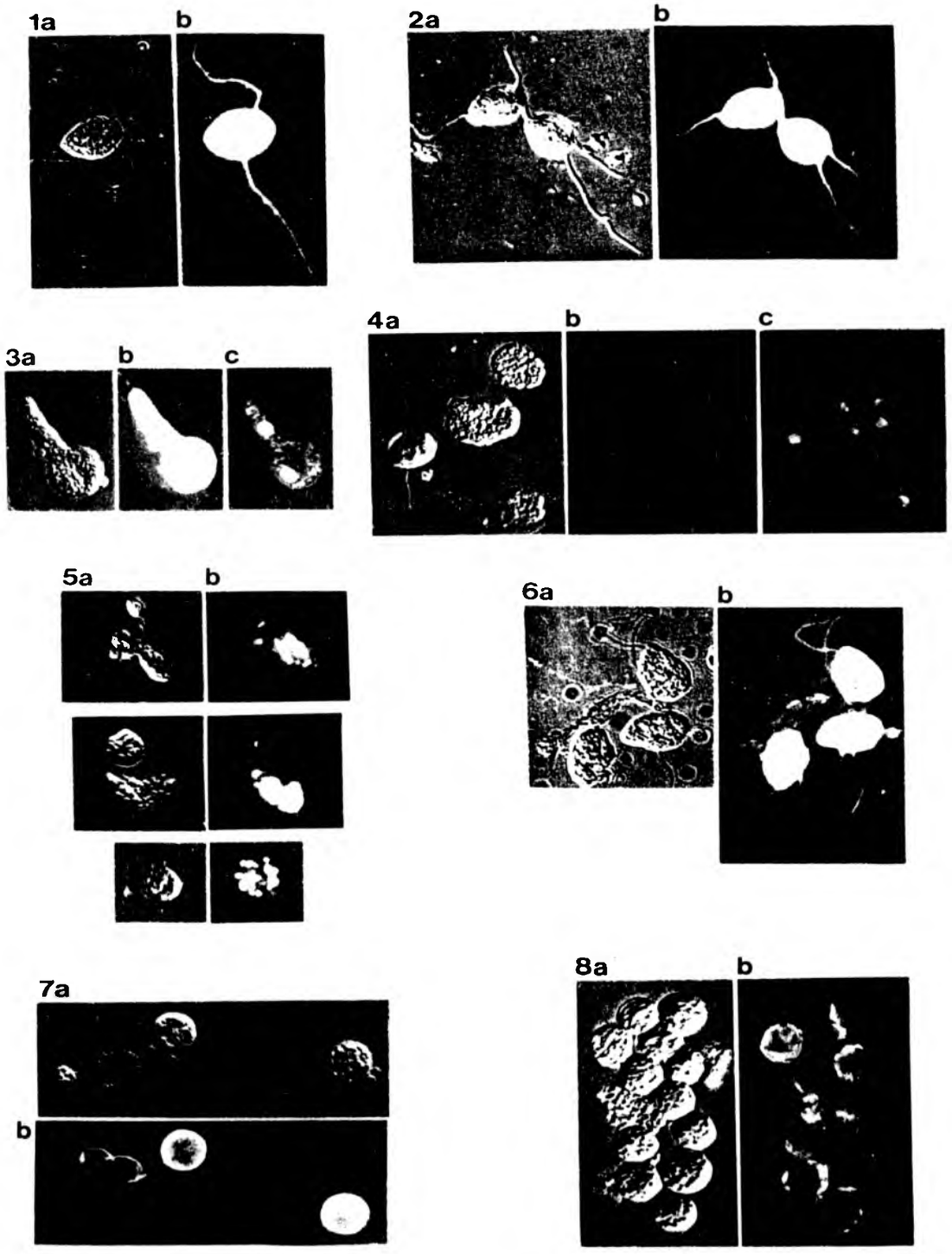
- **reactivity of αtubulin MAb with *A. invadans***

6 **αTAT binding to saprophytic *Aphanomyces* (WSA) secondary zoospores (0.2% glut, 4% form)**

- **WGA binding with *Aphanomyces* spp**

7 **WGA binding to cysts, and empty "ghost" cysts, of EUS isolate (33P) (4% form)**

8 **WGA showing patchy binding to cysts of saprophytic *Aphanomyces* (WSA) (0.2% glut, 4% form)**



CHAPTER 8. GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS[3]

8.1 Introduction

This study attempts to compare the EUS, RSD and MG pathogenic *Aphanomyces* isolates and a variety of other Oomycete fungi as listed in Appendix 1, in terms of their sodium dodecyl sulphate polyacylamide gel electrophoresis (SDS-PAGE) profiles; and to characterise strain-specific bands using various stains, lectins and polyclonal antibodies.

Callinan *et al* (1995a) compared Philippines EUS and Australian RSD isolates and other *Aphanomyces* species in terms of their SDS-PAGE banding patterns. Although these were not clear for all isolates, EUS and RSD isolates showed some similarities in banding patterns, and appeared distinct from *A. cochloides, A. laevis* and *A. euteiches*.

Other studies have employed protein electrophoresis as a means of establishing interspecific and intraspecific relationships between Oomycete fungi (Kaosiri and Zentmyer, 1980; Erselius and De Vallavieille, 1984; Bielenin, Jeffers, Wilcox and Jones, 1988; Brasier *et al,* 1993; Zhang, Zheng, Li, Ann and Ko, 1994; Latorre, Perez, Wilcox and Torres, 1995). Hansen (1987) considered protein electrophoresis to be one of the most valuable tools used in the speciation of *Phytophthora* fungi as the banding pattern obtained on polyacrylamide gels represents the diverse products of 50 or more genes. Even where *Phytophthora megasperma* isolates were indistinguishable by classical taxonomic means, protein banding patterns were capable of separating an alfalfa-pathogenic group from other legume and Douglas fir isolates (Hansen *et al*, 1987).

Barr (1983b) stated of the *Aphanomyces*, "this genus is ripe for thorough taxonomic study using such aids as electrophoresis or serology". Peduzzi and Bizzozero (1977) also suggested that serological tests using polyclonal antibodies may provide a useful criterion

[3]The information presented in this Chapter is published in Lilley, J.H., Thompson, K.D. and Adams, A. (1997) Characterization of *Aphanomyces invadans* using electrophoretic and Western blot analysis. Diseases of Aquatic Organisms (in press)

for the identification of saprolegniacean species. Although lack of specificity has been a problem in subsequent studies using polyclonal antisera against Oomycetes, a degree of serological profiling has been demonstrated in several cases (Krywienczyk and Dorworth, 1980; Bullis, Noga and Levy, 1990; White, Lyons, Wakeham, Mead and Green, 1994a).

Lectins have been used in the intensive study of the surface components of Oomycete zoospores, cysts and germlings (Hardham, 1989; Lehnen and Powell, 1993; Burr and Beakes, 1994) from which, evidence in support of particular taxonomic groupings have been obtained (Beakes *et al*, 1994). However, no studies have been found on the use lectins in Western blots to distinguish fungi, as described here.

8.2 Materials and methods

8.2.1 Preparation of fungal extracts

A list of the fungi used in the present study are given in Appendix 1. To obtain protein-rich fungal extracts, 45 ml zoospore suspensions were produced in Petri dishes as described in Section 2.2.5(b) and these were added to an equal volume of double strength GPY broth. Germlings were allowed to develop for 1-3 days at 22°C depending on the growth rate of the isolate, so that thin mycelium mats formed on the bottom of each Petri dish. Growth medium was decanted and the samples washed in 500 ml sterile distilled water. Samples were filtered through sterile Whatman 541 filter paper and excess water removed using dry sterile filter paper. Fungal mats were ground in liquid nitrogen using a pestle and mortar. The resulting powder was then homogenized in 1 ml Wood's (1988) extraction buffer (85 mM Tris HCl, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.198g/l ascorbic acid and 1g/l glycerol at pH 7.5) with the addition of 5 µM phenylmethylsulphoxylfluoride (PMSF) (Sigma). The homogenate was centrifuged twice at 13,000 xg for 5 min and the protein concentration of the extracts were estimated by ultraspectrophotometric readings at OD_{280}. These were adjusted to 5 mg/ml with sample buffer and the extracts were frozen at -70°C. Prior to adding sample buffer, a 100 µl aliquot was taken of each extract and stored at -70°C for subsequent protein digestion according to Hitchcock and Brown (1983). This

involved incubating the aliquots with 100 µl of a 10 µl/ml solution of proteinase K (Sigma) for 1 hour at 60°C before diluting in sample buffer.

8.2.2 Preparation of extracellular products (ECP)

Media (500 mls GPY) in which the fungus had been cultured were collected, placed in dialysis tubing with a molecular weight cutoff of 10 kDa (Fisons) and concentrated using polyethylene glycerol (8 kDa, Sigma). Concentrates were dialysed using three changes of two litres PBS over 24 hours at 4°C.

8.2.3 SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970) using precast acrylamide separating gels (4-20%) (BioRad). The gels were subjected to electrophoresis for 45 min at 200 V, then stained with either in 0.1% (w/v) Coomassie blue R250 (Sigma), silver stain (BioRad) or Schiffs reagent (Merck).

8.2.4 Electroelution of 10 kDa band

An *A. invadans*-specific band (from isolate PA7) of molecular weight 10 kDa was excised from a 12% (w/v) polyacrylamide slab gel and electroeluted using a Hoefer electroeluter at 100 V for 1 hour according to manufacturers specifications.

8.2.5 Immunisation schedule

Three New Zealand White rabbits, obtained from the animal house, University of Stirling, were immunised with mycelium extracts from either (a) a saprophytic *Aphanomyces* isolate F3SA, (b) *A. invadans* PA7 or (c) the electroeluted band from PA7. The extracts (300 µg protein) were mixed 1:1 with Freunds complete adjuvant (Sigma) and 1.5 ml was delivered subcutaneously into two sites. The rabbits received two further subcutaneous booster injections of fungus extract in Freunds incomplete adjuvant 4 and 8 weeks later. A final injection of 1 ml fungus extract in sterile saline was given intravenously on week 12. The rabbits were then bled out by cardiac puncture 10 days later.

826 Western blotting

The polyclonal antisera were used to screen each isolate for antigenic bands by Western blot analysis. Samples were applied to each lane and subjected to electrophoresis as described above. The samples were transferred from the gel to a sheet of nitrocellulose membrane by a wet blotting system (Hoefer) at 50 V for 1 hour. Following transfer, the nitrocellulose membrane was washed with two changes of high salt wash buffer (HSW: 0.02 M Tris, 0.5 M NaCl, 0.1% Tween-20, 0.01% methiolate, pH 7.8) and blocked for 2 hours with 1% (w/v) BSA in distilled water. The membrane was then washed twice in HSW and antisera (diluted 1/100 in PBS) were applied to the nitrocellulose membrane and incubated for 1 hour at 4°C. After two washes in HSW, goat anti-rabbit IgG-Horseradish Peroxidase (HRP) conjugate (SAPU: Scottish Antibody Production Unit, Carluke, UK) (diluted 1/100 in 0.5% (w/v) casein in PBS) was applied to the membrane and left for a further hour. Unbound conjugate was removed from the membrane by washing twice in HSW followed by one wash with Tris buffer saline (TBS: 10 mM Tris, 0.15 M NaCl, pH 7.5). The blot was developed with 4-chloro-1-naphthol (BioRad) and the reaction stopped by washing with distilled water for 10 min.

Lectins were used to examine carbohydrate moieties in each of the fungal extracts. For these studies the nitrocellulose membranes were incubated for 1 hour with lectins labelled with biotin (Sigma) (see Table 8.1) diluted to 20 µg/ml in low salt wash buffer (LSW: 0.02 M Tris, 0.038 M NaCl, 0.05% Tween-20, 0.01% methiolate, pH 7.4) in place of the rabbit polyclonal antisera. The blots were washed and incubated for 1 hour with streptavadin-peroxidase (SAPU) diluted 1/100 in LSW buffer. Finally, the membranes were washed five times with HSW buffer and the reaction developed as described above.

827 Immunohistochemistry (IHC)

The technique used was based on the method of Adams and Marin de Mateo (1994). Fixed blocks of muscle from snakehead fish experimentally infected with *A. invadans* (TA1) during experiments described in Chapter 3, were embedded in paraffin wax and slides of 5 µm cross-sections were prepared. The tissue sections were dewaxed, encircled

with a wax PAP pen (Merck) and fixed for 10 min with methanol containing 10% v/v hydrogen peroxide to bleach endogenous peroxidases. The slides were washed three times with TBS. Normal goat serum diluted in TBS (10% v/v) was added to the slides which were then incubated for a further 20 min. The serum was poured off, the slides were placed in a moist chamber and each of the three rabbit sera (1/100 in TBS) were added to the sections for 1 hour at 20°C. Normal rabbit serum was used as a negative control. The slides were washed as above. Goat anti-rabbit-HRP conjugate (1/50 in TBS) was added to the slides for 1 hour and the slides washed as previously described. To visualize the reaction, slides were incubated for 10 min with 3'3 diaminobenzidine tetrahyrochloride (DAB) (Sigma) in the presence of hydrogen peroxide (H_2O_2) [100µl of 1% H_2O_2 to 0.5 ml (1.5 mg/ml DAB) and 5 ml TBS]. The reaction was stopped by immersing the slides in tap water. The slides were counterstained with haematoxylin for 3-4 min then dehydrated and mounted. Positive tissue appeared brown in colour under a light microscope.

8.2.8 Indirect fluorescent antibody technique (IFAT)

IFAT was carried out on TA1 tissue sections according to Neelam, Thompson, Price, Tatner, Adams, Ellis and Stevens (1995) using 1/100 dilutions of the rabbit antisera. Normal rabbit serum was used as the negative control. A 1/100 dilution of FITC-donkey anti-rabbit IgG (SAPU) was used as the secondary antibody.

8.3 Results

8.3.1 SDS-PAGE

Coomassie blue stained SDS-PAGE gels gave very similar banding patterns for all the EUS, RSD and MG isolates (Figure 8.1) and these were distinct from all other fungi tested. Bands shown by these isolates consistently visualized using Coomassie blue occurred at 48, 56 and 61 kDa. However, banding patterns were generally rather faint, therefore the more sensitive silver stain was used to further highlight bands. Using silver stain, the intensity and number of bands visualized were greater for the EUS, RSD and MG pathogens compared with the other fungi. In order to compare isolates from different gels a pictorial representation of the silver stain bands was constructed (Figure 8.2a). Bands

specific to all the EUS, RSD and MG isolates were located at approximately 10, 84, 195 and 240 kDa. The 10 kDa band was electroeluted and its presence in the resulting sample verified using SDS-PAGE (Figure 8.2b). This sample was used to prepare the third polyclonal antiserum (αband). While the bands above remained constant, molecular weights of other bands were inconsistent when gels were stained with the silver stain reagent, even between different gels run using the same fungal extracts. Silver staining also showed similarities between other fungal isolates, with clear groups being identified among some of the saprophytic *Aphanomyces* (TF5, TF41, F3SA, SSA and T1SA), two of the *Achlya* isolates (S2AC and AC2) and two *Saprolegnia* isolates (TF29 and TF31).

Silver-stained proteinase K-treated samples and Schiffs-stained gels revealed high levels of the low molecular weight carbohydrate around 10 kDa in *A. invadans* (as shown for PA7 in Figure 8.3a, lanes 1 and 2 respectively) and at approximately 5 and 14 kDa in most of the saprophytic *Aphanomyces* (as shown for F3SA in Figure 8.3b, lanes 1 and 2). These bands can also be identified on the untreated silver-stained gels (Figure 8.2a). Another carbohydrate band of note revealed by Schiffs staining was the 100 kDa band which was apparent on EUS (Figure 8.3a, lane 2), RSD and MG isolates (data not shown) but not on any of the other fungi.

8.3.2 Western blotting (i) Lectins

Figure 8.3 also shows the bands of PA7 and F3SA recognised by each lectin. The bands described above could not be positively identified by lectin staining studies, although the 100 kDa band visualized using Schiffs stain may equate with a band of similar size revealed by the lectin LEA on EUS, RSD and MG isolates. The relative affinities of each lectin for the fungal carbohydrates varied, as indicated by the time taken for bands to develop in 4-chloro-1-naphthol. This ranged from 10 sec for Con A to 14 hours for LEA.

There was again remarkable consistency between EUS, RSD and MG isolates in terms of the bands revealed by lectin-binding on the Western blots. Figure 8.4a illustrates this consistency with regard to HGA. The lectin UEA-1 gave a very similar banding pattern to

HGA. Out of the nine lectins tested, only ECA revealed any differences between *A. invadans* isolates with 36/1P and 10D showing possible additional bands (Figure 8.4b, lanes 5 and 6). For all the EUS, RSD and MG isolates a band at approximately 45 kDa was recognised by Con A, ECA, HGA, BS-1, LEA and UEA-1. WGA recognised a region between 33 and 123 kDa, producing an area of continuous staining.

For F3SA and a few apparently similar saprophytes, 2 bands were generally recognised at 55 and 90 kDa for all lectins tested except LEA. Similar banding patterns were obtained with GMA, BS-1, UEA-1 and AHA among the saprophytes.

8.3.3 (ii) Polyclonal antisera

Western blot analyses showing the response of the three polyclonal antisera against fungal extracts are presented in Figure 8.5. The anti-saprophyte (αF3SA) and anti-*A. invadans* (αPA7) sera showed a high degree of cross-reactivity with all isolates tested, with both antisera recognising similar bands on a given isolate (Figure 8.5a and b). However, the αF3SA serum recognised a band at 45 kDa on PA7 and a band at 42 kDa on F3SA which the αPA7 serum only faintly recognised. There was also a substantial amount of staining of low molecular weight material in the F3SA extract by the αF3SA serum which was not recognised by the αPA7 serum. Both the αF3SA and αPA7 sera recognised the 10 kDa band of PA7 and were also able to recognise this band in the pathogenic MG isolate NJM9030 (RSD isolates were not tested with these antisera).

Antiserum raised against the PA7 electroeluted band (αband) recognised only two bands found solely in extracts of fungi from the EUS, RSD or MG group (Figure 8.5c). However neither band appeared at the molecular weight of the original 10 kDa band, but instead at around 50 kDa.

The reaction of the polyclonal antisera with fungal ECP is shown by Western blots in Figure 8.6. There was little response of either the αF3SA or the αPA7 sera with the ECP from the saprophyte F3SA (Figure 8.6a), however, both antisera reacted strongly with the

ECP of the *A. invadans* isolate PA7 (Figure 8.6b). Six major bands were observed at about 20, 35, 45, 50, 60 and 85 kDa in the PA7 ECP. Anti-PA7 band serum did not react with ECP of either PA7 or F3SA (Figure 8.6c).

8.3.4 Histochemical analysis

Fungal hyphae in tissues of snakehead fish infected with *A. invadans* isolate TA1 were positively labelled with both αF3SA and αPA7 when the secondary antibody was conjugated with either HRP (Figure 8.7a and b respectively) or FITC (Figure 8.8a and b), with the αF3SA serum eliciting the stronger response of the two. Hyphae were only very faintly labelled with αPA7 band serum (Figures 8.7c and 8.8c), while no reaction occurred with the negative control (Figures 8.7d and 8.8d).

8.4 Discussion

EUS, RSD and MG pathogenic *Aphanomyces* isolates were shown to be very similar in terms of their protein and carbohydrate components, as indicated by SDS-PAGE banding patterns visualised using various stains, lectins and polyclonal antibodies. Furthermore, these techniques distinguished this group of pathogens from all the other Oomycete fungi that were previously shown to be non-pathogenic to EUS-susceptible fish (Chapter 3). The results are consistent with other studies in this report which also indicate that the EUS, RSD and MG isolates constitute a single species.

Protein profiles, as revealed by Coomassie blue and silver staining, were complex and difficult to reproduce consistently. However, it was possible to identify specific bands that could act as taxonomic markers for *A. invadans*, although they differed between the two stains. It may be the case that the three bands identified in the Coomassie blue-stained gels of *A. invadans* equate with the three major bands in EUS and RSD samples that are shown between the 43 and 87 kDa reference markers in Callinan *et al* (1995a). The latter workers, however, grew their fungi for 15 days at 30°C, whereas in the present study, mycelium was grown from zoospores for a maximum of 3 days at 22°C to ensure a high proportion of protein-rich growing tips. This difference in the age of the samples would

affect the biochemical composition of the cells, and thus may be reflected in the different SDS-PAGE profiles. Chen, Hoy and Schneider (1991) warned that in the case of *Pythium,* soluble protein profiles were affected by storage and culture conditions. This is also an important consideration when comparing different fungi of greatly varying growth rates. In the present study, growth times were adjusted to between 1-3 days so as to produce similar yields for each fungus. Despite this adjustment, the slower-growing EUS, MG and RSD isolates still showed more intense coloration than the other fungi with both Coomassie blue and silver stain.

Silver stain reacts with carbohydrate as well as protein and in order to highlight the carbohydrate bands an initial protein digest treatment was performed on each sample. This treatment revealed very few bands, suggesting that the majority of bands in non-treated silver-stained samples were associated with protein. Proteinaceous glycoconjugates would have been broken down by the proteinase K digest and the resulting products may constitute at least part of the low molecular weight bands observed in the samples. Sadowski and Powell (1990) used silver methenamine to show that the plasma membranes of *Aphanomyces euteiches* zoospores were rich in glycoproteins and thus the membranes may be the main source of this material in the mycelial extracts tested here. Schiffs, a general carbohydrate stain, also showed few bands, but these did reveal differences between the EUS, MG and RSD isolates and the other fungi.

Other workers have used isozyme profiles to characterise *Aphanomyces* species (Larsson, 1994) and other Oomycetes (Wang and LeJohn, 1974a; b; c; Beakes and Ford, 1983; Oudemans and Coffey, 1991). During the present study a preliminary attempt was made to obtain esterase profiles for some isolates. This was unsuccessful and as new fungal extracts had to be made, this work was abandoned due to lack of time.

Western blots using lectins gave a more sensitive analysis of the specific carbohydrate moieties in each sample, and provided a robust technique for distinguishing EUS, MG and RSD samples from other fungi. Only one lectin (ECA) showed any differences between

the pathogens, although evidence from Chapter 11 shows that the pathogens form a very genetically homogenous group, and this result would therefore require further verification before it is used as a means of strain-typing.

Some lectins with different carbohydrate specificities revealed similar banding patterns, which may be explained in that each of the bands visualised were composed of complex polysaccharides with different residues accessible to several different lectins.

In their study of the lectin-binding properties of Oomycete propagules, Burr and Beakes (1994) showed that Con A-binding material (mannose and glucose) was associated with the glycocalyx of zoospores of a variety of Oomycete species. The high reactivity of Con A with all the fungi tested here suggests that there are significant amounts of these saccharides in the mycelium of saprolegniaceous species as well.

The polyclonal antisera, αF3SA and αPA7, showed a great lack of specificity, with αF3SA reacting more strongly with PA7 than homologous antiserum. These antisera also reacted strongly with bands from *Achlya* and *Saprolegnia* samples (data not shown). This is consistent with the results of Bullis *et al* (1990; 1996), which showed that mouse anti-*Saprolegnia parasitica* polyclonal and monoclonal sera cross-reacted, albeit to a lesser extent, with a UM-*Aphanomyces* isolate used here (84-1240), and even some non-Oomycete fungi. However, the antisera prepared in this study was useful in highlighting antigenic fungal bands on Western blots. These were consistent for all EUS, MG and RSD isolates and distinct from other *Aphanomyces* spp tested. The 10 kDa band was clearly identified on EUS, MG and RSD samples by both the αF3SA and the αPA7 rabbit antisera. This band was also identified by artificially and naturally infected snakehead fish sera (see Chapter 9). Thus, the 10 kDa band has been shown to be immunogenic in fish and rabbits. However, the antiserum prepared by injecting the electoeluted 10 kDa band into rabbits did not recognize the 10 kDa band by Western blot. Instead, two bands at around 50 kDa were recognised on EUS, MG and RSD samples. These bands were also faintly visible on silver-stained gels of the electroeluted 10 kDa band (Figure 8.2b) and

their appearance may be due to conformational changes to the 10 kDa band as a result of electroelution. Nonetheless, these bands were specific to EUS, MG and RSD isolates as αband serum did not cross-react with other fungus samples.

The serum raised against mycelium extract from a saprophytic *Aphanomyces* (αF3SA) did not recognise ECP secreted by homologous fungus but did recognise the same secreted products from PA7 as αPA7. This suggests that *A. invadans* isolate PA7 secreted greater quantities of ECP than the saprophyte F3SA, which may represent the release of proteolytic enzymes relevant in the pathogenesis of *A. invadans*. The molecular weights of the bands in the ECP recognised by the antisera did not directly correspond to bands found in the mycelium extract. However, the antisera were raised against mycelium extract and therefore bands recognised in the ECP should correspond to components found in the mycelium extract. If this is indeed the case, the difference in molecular weight of the bands revealed between the two samples may be explained by ECP components being altered in some way, such as being cleaved on secretion. The fact that the αF3SA serum recognised the PA7 ECP again illustrates the non-specific nature of the antisera. However, the lack of any reaction with the αband serum suggests that the electroeluted band is not secreted into the ECP or was lost on dialysis during sample preparation.

In his study of histological staining techniques of *S. parasitica*- and *A. astaci*- infected tissue, Southgate (1983) found the latter to be significantly more difficult to stain, probably, it was speculated, due to the smaller diameter of the hyphae which presented insufficient cell wall cellulose for most techniques to work. The only technique that demonstrated *A. astaci* hyphae in infected tissues was Grocott's silver stain, a long and involved procedure, and in conclusion, he strongly advised the development of alternative fluorescence antibody techniques. The rabbit antisera used in the present study proved a very effective diagnostic tool for identifying *A. invadans* hyphae in fish tissue, particularly by IFAT, and compares favourably with Grocott's stain in terms of ease of use. It would be interesting to use this technique on sections of UM-affected fish tissue to compare reactivity of the invasive fungus involved in that disease with that of *A. invadans*. A UM isolate was shown

here to have very different protein and carbohydrate profiles from *A. invadans*, but it is possible that this is not actually the true pathogen involved in that disease (Chapter 3).

Raising monoclonal antibodies against *A. invadans* hyphal material would provide a means of developing a more specific probe for use in the immunohistochemical diagnosis of EUS. Lectins were used on sections of infected fish tissue in ths study, in an attempt to obtain a more specific stain for *A. invadans*. This technique has been used for other fish diseases (Marin de Mateo, Adams, Richards, Castagnaro and Hedrick, 1993) but the lectins tested here gave no discernible reactivity.

Table 8.1 **Lectins used in the study**

Lectin (*origin*)	Abbreviation	Carbohydrate specificity
Concanavalin A (*Canavalia ensiformis*)	Con A	terminal α-D-mannosyl and α-D-glucosyl residues
Wheat germ agglutinin (*Triticum vulgaris*)	WGA	N-acetyl-β-D-glucosaminyl residues and N-acetyl-β-D-glucosamine oligomers
Coral tree agglutinin (*Erythrina cristagalli*)	ECA	D-galactose and D-galactosides
Horse gram agglutinin (*Dolichos biflorus*)	HGA	terminal N-acetyl-α-D-galactosaminyl residues
Soybean agglutinin (*Glycine max*)	GMA (=SBA, Chapter 7)	N-acetyl-α-D-galactosamine
(*Bandeiraea simpicifolia*)	BS-1	terminal α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues
Tomato agglutinin (*Lycopersicon esculentum*)	LEA	N-acetyl-β-D-glucosamine oligomers
Gorse seed agglutinin (*Ulex europaeus*)	UEA-1	L-fucose
Peanut agglutinin (*Arachis hypoaea*)	AHA	D-galactose

Figure 8.1

Coomassie-stained SDS-PAGE gel (4-20%) of various isolates of the EUS pathogen (lanes 2 to 7) and RSD pathogen (lanes 8 and 9) showing almost identical polypeptide band patterns.

Lanes: (1) and (10) are BioRad low molecular weight markers; (2) RF6; (3) G2PA; (4) PA7; (5) BH; (6) 36/1P; (7) 10D; (8) 4P and (9) 24P

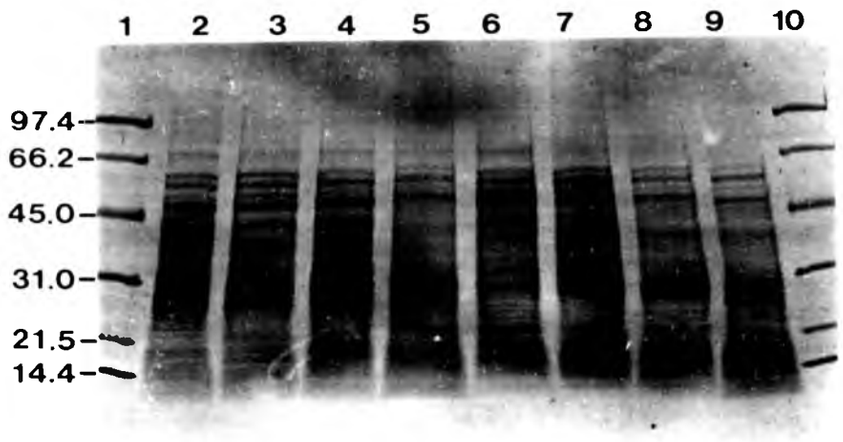


Figure 8.2

(a) Pictorial representation of silver-stained SDS-PAGE gels (4-20%) of selected isolates. Top row comprises EUS, RSD and MG pathogens; UM fungus (84-1240) and *A. astaci* (FDL457 & FDL458). Bottom row comprises *Aphanomyces*, *Achlya* and *Saprolegnia* saprophytes

(b) Silver-stained electroeluted 10 kDa band (on 4-20% SDS-PAGE gel)

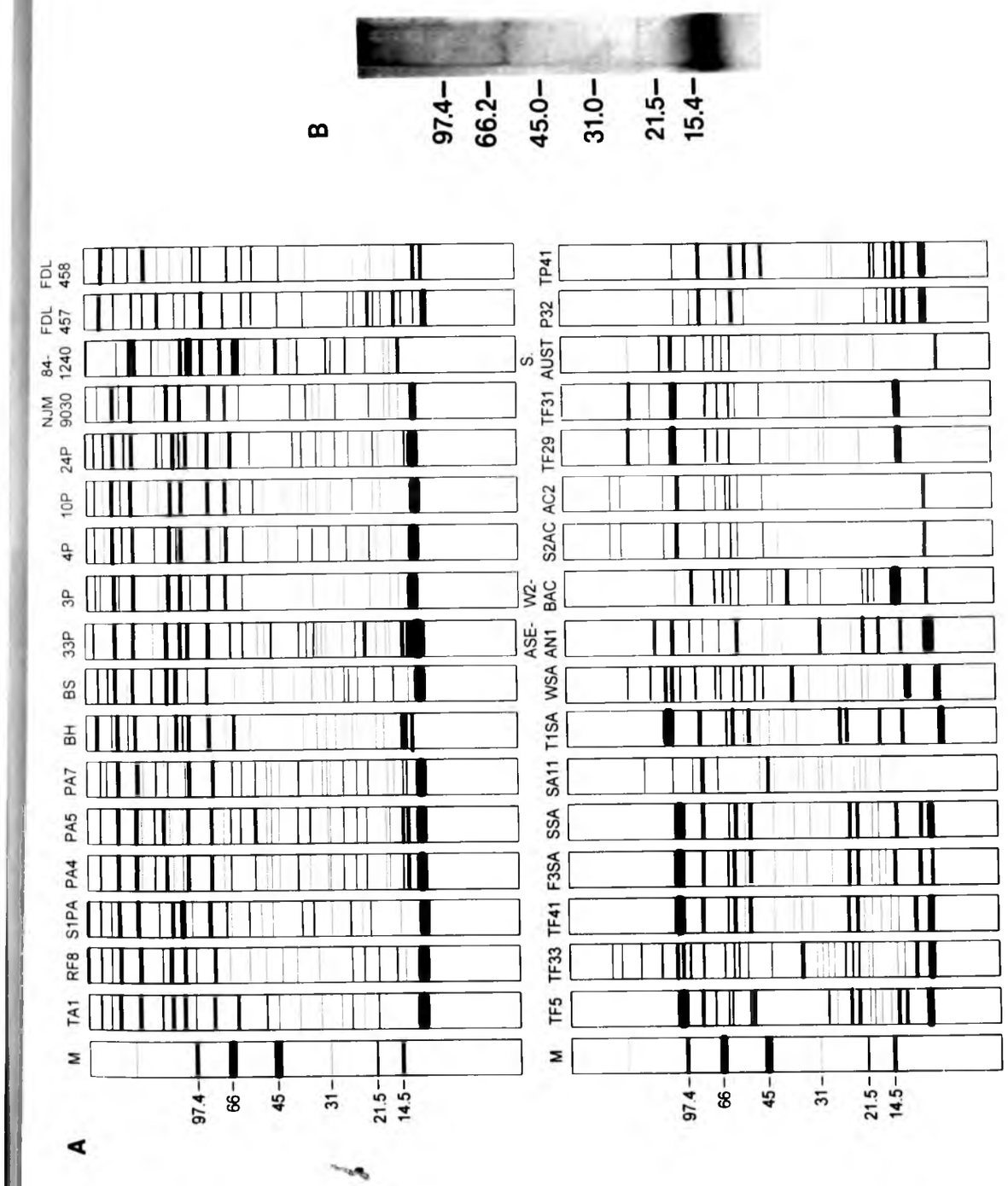


Figure 8.3

Polysaccharide banding patterns of SDS-PAGE gels (4-20%)

(a) *Aphanomyces invadans* isolate PA7

(b) Saprophytic *Aphanomyces* isolate F3SA

Lanes: (1) proteinase K-treated silver-stained gel; (2) Schiffs-stained gel; (3-11) Western blots stained with various lectins. BioRad low-range markers are indicated on the left of lanes 1-2, and BioRad broad-range markers are indicated on the right of the lanes 3-11.

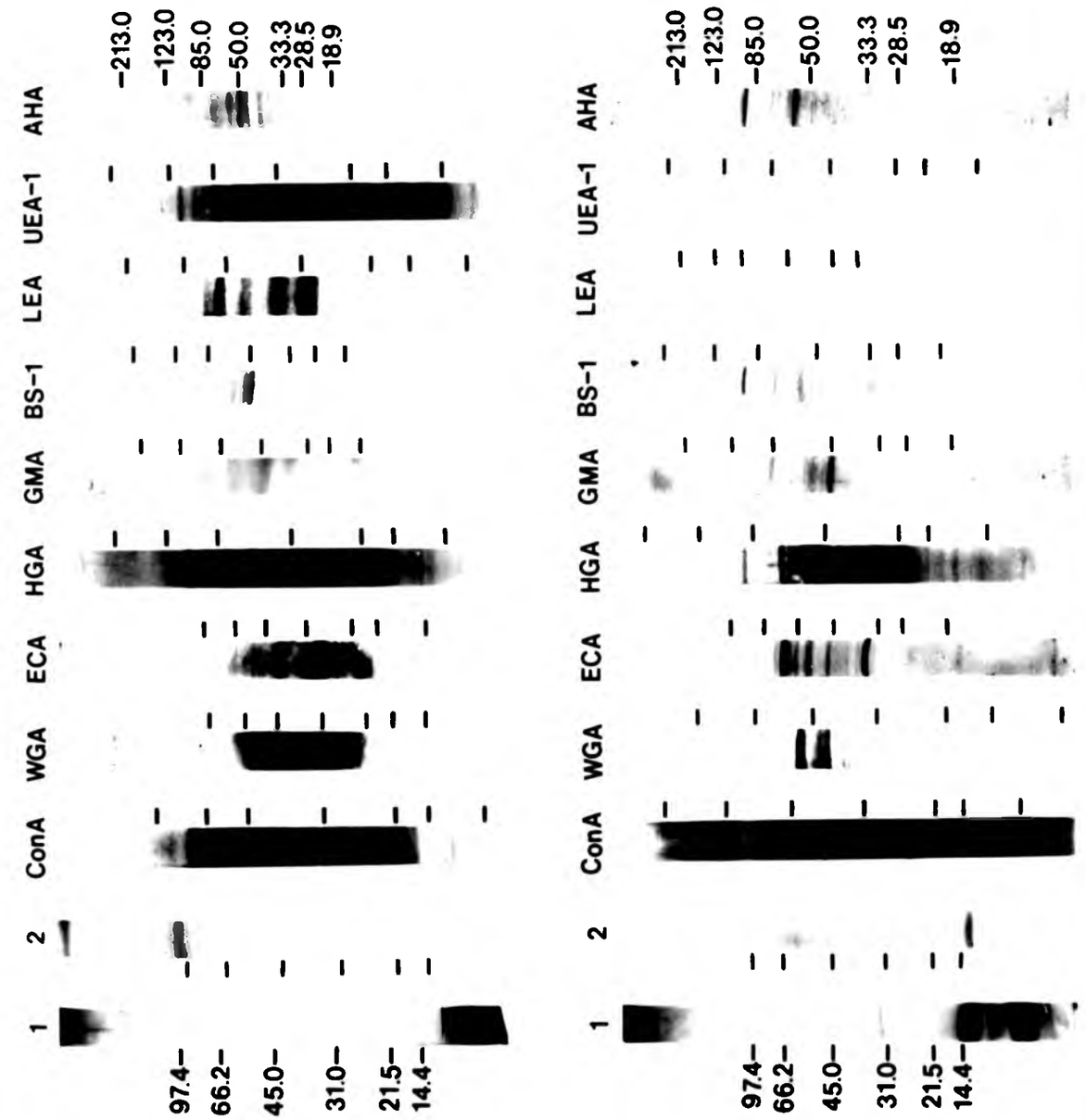


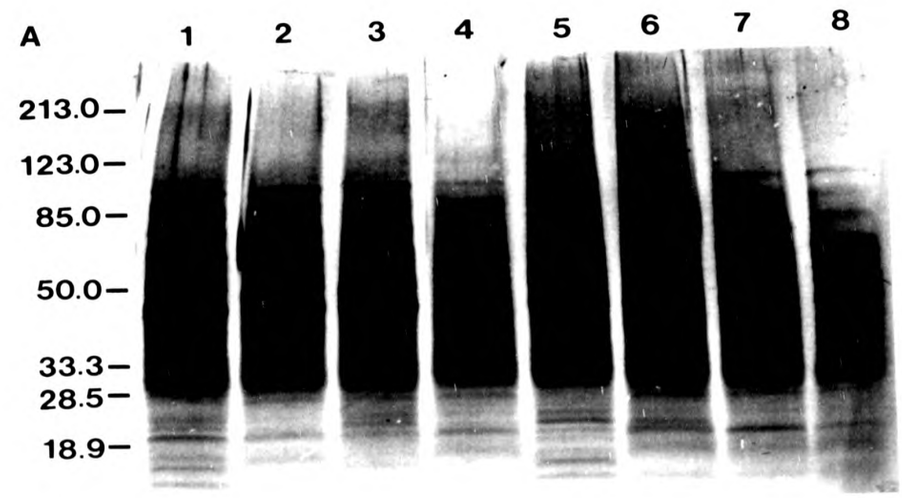
Figure 8.4

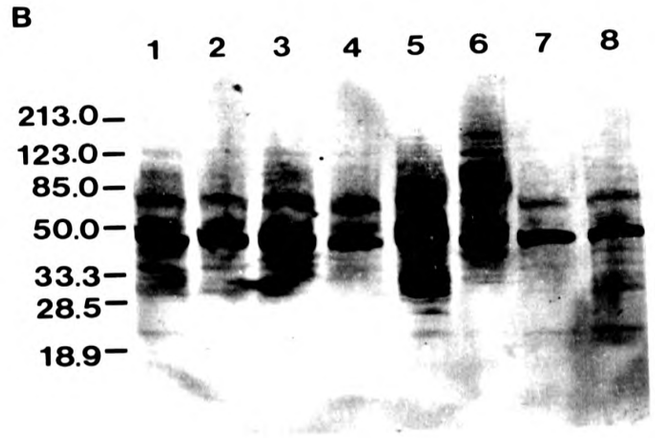
Western blots of various isolates of the EUS pathogen (lanes 1 to 6) and the RSD pathogen (lanes 7 and 8) stained using:

(a) HGA

(b) ECA

Lanes: (1) RF6; (2) G2PA; (3) PA7; (4) BH; (5) 36/1P; (6) 10D; (7) 4P and (8) 24P





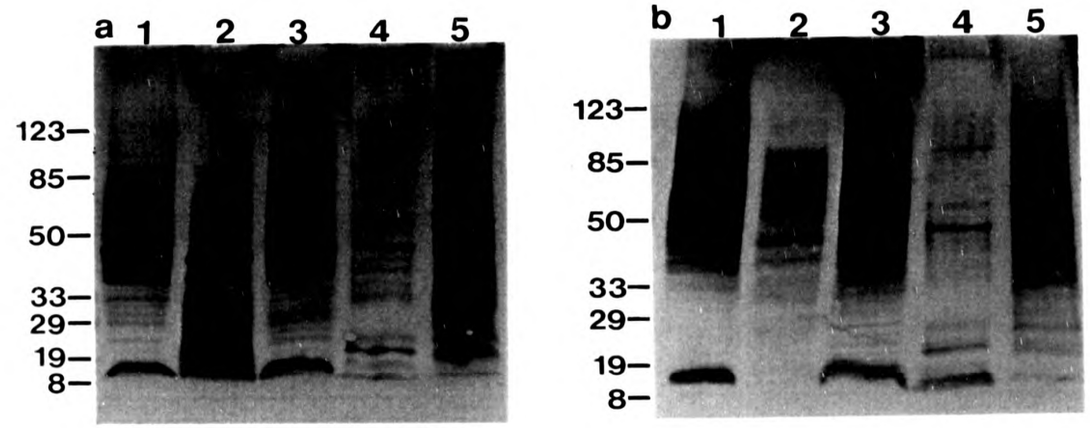


Figure 8.5

Western blot analyses showing the response of various polyclonal antisera with different fungal extracts.

(a) Rabbit anti-saprophyte (αF3SA)

(b) Rabbit anti-*A. invadans* (αPA7)

(c) Rabbit anti-PA7 electroeluted band (αband)

Lanes: Mycelial extracts from (1) *A. invadans* isolate PA7; (2) saprophytic *Aphanomyces* F3SA; (3) MG isolate NJM9030; (4) *A. astaci* isolate FDL458 and (5) UM isolate 84-1240.

Figure 8.6

The response of polyclonal antisera with fungal extracellular products (ECP) by Western blot analysis.

(a) Rabbit anti-saprophyte (αF3SA)

(b) Rabbit anti-*A. invadans* (αPA7)

(c) Rabbit anti-PA7 electroeluted band (αband)

Lanes: (1) ECP from *A. invadans* isolate PA7; (2) ECP from saprophytic *Aphanomyces* F3SA.

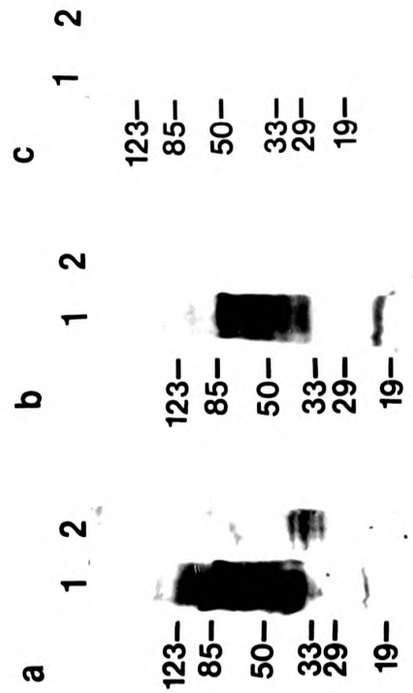


Figure 8.7

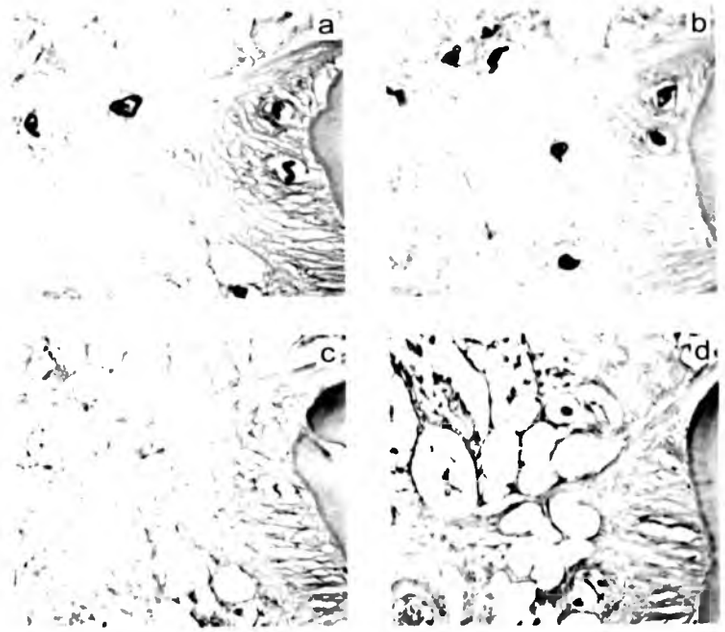
The reaction of rabbit polyclonal antisera with tissues from snakehead fish infected with *A. invadans* (isolate TA1) by immunohistochemistry. The plates show the reactions obtained with:

(a) Rabbit anti-saprophyte (αF3SA)

(b) Rabbit anti-*A. invadans* (αPA7)

(c) Rabbit anti-PA7 electroeluted band (αband)

(d) Negative control (normal rabbit serum)



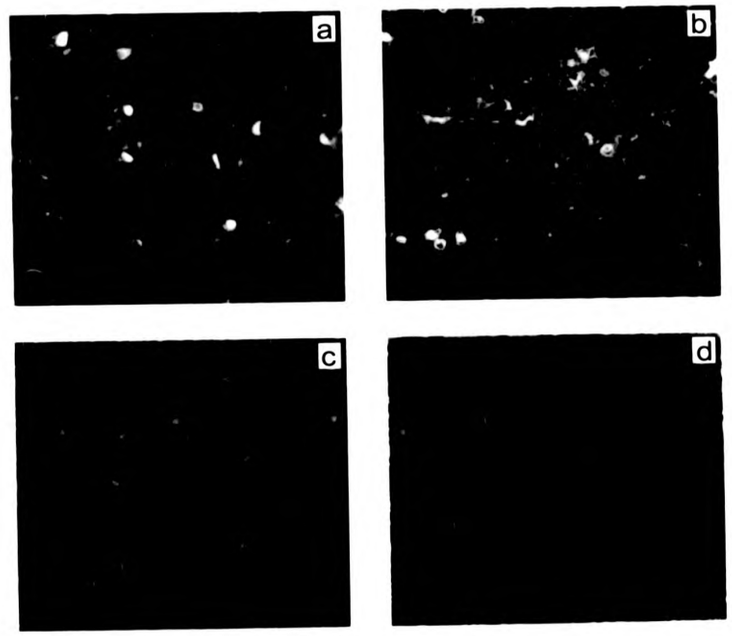


Figure 8.8

The reaction of rabbit polyclonal antisera with tissues infected with *A. invadans* (isolate TA1) by the indirect fluorescent antibody technique. The plates show the reactions obtained with:

(a) Rabbit anti-saprophyte (αF3SA)

(b) Rabbit anti-*A. invadans* (αPA7)

(c) Rabbit anti-PA7 electroeluted band (αband)

(d) Negative control (normal rabbit serum)

CHAPTER 9. IMMUNE RESPONSE OF SNAKEHEADS[4]

9.1 Introduction

Very little work has been published on aspects of the immune response of fish to *A. invadans*, or to saprolegniacean fungi as a whole. A short overview of this subject is given here.

9.1.1 Non-specific response of fish to fungi

In an attempt to halt the invasion of *A. invadans* through the internal tissues of fish, macrophages surround and envelop invading mycelium by forming granulomas (Chinabut *et al,* 1995). This distinctive chronic inflammatory response is one of the defining features of EUS (Roberts *et al,* 1994a), MG (Miyazaki and Egusa, 1972), RSD (Callinan *et al,* 1989), and UM (Noga *et al,* 1988; 1989). Where other saprolegniaceans have been observed penetrating beneath the dermis of fish, no such cellular response is induced (Puckeridge *et al,* 1989; Bly *et al,* 1992). *Ichthyophonus hoferi*, a systemic fish pathogen usually referred to as a fungus (Roberts, 1989), but probably more closely related to the choanoflagellates (Spanggaard, Skouboe, Rossen and Taylor, 1996), is also characteristically encapsulated by macrophages, multinucleate giant cells and granulomas (Miyazaki and Jo, 1985).

Multinucleate giant cells can be derived from the fusion of macrophages encapsulating a foreign body (Secombes, 1985), and have been shown to do so *in vitro* in response to *A. invadans* spores (K.D. Thompson, in manuscript). There does seem to be a distinct difference in the ability of various fish species to produce multinucleate giant cells in response to invasive *Aphanomyces* infection (Table 9.1). Despite extensive observation of snakehead and mullet histopathology in relation to EUS and RSD infection, no giant cells have been reported, however common carp and rainbow trout artificially inoculated with *A. invadans* commonly form giant cells, and all three known cell types have been observed

[4]The information presented in this Chapter is published in Thompson, K.D., Lilley, J.H. Chinabut, S. and Adams, A. (1997) The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis*. Fish and Shellfish Immunology (in press)

(foreign body type, intermediate and Langhans) (Table 9.1). Giant cell formation does not, however, seem to be an indicator of the resistance of a species, as some highly susceptible species (ayu and puntius) also form giant cells, although Wada *et al* (1996) gave ratios of the type of inflammatory response around hyphae to show that the giant cells of ayu were not as active as those of common carp.

Besides encapsulation, the other main function of the cellular response is phagocytosis (Wilson, 1976; Secombes, 1985). Recent work has shown that cultured rainbow trout head kidney macrophages are able to phagocytose *A. invadans* zoospores *in vitro,* but that the density of spores in the inoculum affects the success of this activity (K.D. Thompson, in manuscript; Khan, 1997). Fungal hyphae, however, are known to be poorly degraded by phagocytes, and the primary function of the cellular response to *A. invadans*, as in other animal mycoses, is probably containment (Faisal and Hargis, 1992).

The cellular response of fish to pathogenic *Aphanomyces* infection varies according to temperature (Chinabut *et al,* 1995) and fish species (Wada *et al,* 1996; Khan, 1997). Wada *et al* (1996) suggested that the greater susceptibility of ayu to infection by MG fungus than common carp was a result of a slower and less efficient cellular defence mechanism.

Table 9.1 Multinucleate giant cell formation in fish naturally or experimentally affected by EUS, RSD, MG and UM agents

Fish species	Giant cell type	Dis-ease	Reference
Susceptible fish			
Brevoortia tyrannus (menhaden)	gc	UM	Noga *et al* (1988)
Carassius auratus (goldfish)	fb	MG	Miyazaki (1994)
Carassius carassius (crucian carp)	fb	MG	Miyazaki (1994)
Channa argus (snakehead)	-	MG	Miyazaki & Egusa (1973c; cited by Wada *et al*, 1994)
Channa maculata (Formosan snakehead)	-	MG	Miyazaki (1994)
Channa striata (striped snakehead)	-	art	Chinabut *et al* (1995)
Clarias gariepinus (catfish)	gc	art	Callinan (pers. comm.)
Colisa lalia (dwarf gourami)	-	MG-like	Wada *et al* (1994)
Mugil sp (mullet)	-	MG	Miyazaki (1994)
Mugil cephalus (mullet)	-	RSD	Callinan *et al* (1989)
Plecoglossus altivelis (ayu) sw-raised fish	mostly fb	MG	Miyazaki (1994)
Plecoglossus altivelis (ayu) fw-raised fish	mostly Lang	MG	Miyazaki (1994)
Puntius schwanenfeldi (rosy barb)	fb	art	Khan (1997)
Scatophagus argus (red scat)	gc	RSD	Pearce (1990)
Tridentiger obscurus obscurus (trident goby)	fb	MG	Miyazaki (1994)
Moderately susceptible fish			
Onchorynchus mykiss (rainbow trout)	fb, Lang, inter	art	Khan (1997)
Resistant fish			
Cyprinus carpio (common carp)	fb, Lang, inter	art	Sharifpour (1997)
Oreochromis niloticus (Nile tilapia)	fb	art	Khan (1997)

- = no giant cell reported in histopathological description
gc = unspecified giant cells
fb = foreign body giant cells
Lang = Langhans giant cells
inter = intermediate giant cells
art = artificial challenge with *A. invadans* spores
sw = seawater
fw = freshwater

Non-cellular, non-specific defence mechanisms in fish have been comprehensively reviewed by Alexander and Ingram (1992). Of the various types of non-immunoglobulin proteins and glycoproteins listed, some lytic molecules and agglutinins were considered to have particular significance in the control of fungal infections. As saprolegniaceans lack chitin, the lytic molecules, lysozyme and chitinase, probably have a limited effect, but proteases identified from the mucus of fish (Hjelmeland, Christie and Raa, 1983) may be of some relevance. Of the agglutinins, some lectins may be involved in the resistance of fish eggs to infection by pathogenic fungi (Balakhnin, Dudka and Isaeva, 1990, cited by Alexander and

Ingram, 1992). Snakeheads (*Channa punctata*) have been studied in this regard, and serum, mucus and egg extracts have all yielded a particular calcium dependent (C-type) lectin (Manihar *et al*, 1990; 1991). Szalai, Norcum, Bly and Clem (1992) isolated a homologue of the human precipitin, C-reactive protein, from channel catfish and called it phosphorylcholine-reactive protein (PRP). They subsequently suggested that low temperatures may reduce levels of serum PRP and increase susceptibility of channel catfish to *Saprolegnia* infection (Szalai *et al*, 1994). Fish also possess enzyme-inhibitors that are known to inhibit bacterial extracellular proteases (Ellis, 1987) and may be active against *A. invadans* ECP. Ellis and Stapleton (1988) considered that proteases of *Aeromonas salmonicida* could also be activated by components in fish serum and that this correlated with the susceptibility of different species to infection.

Durán, Rodríguez Aparicio, Reglero and Pérez Díaz (1987) examined changes in levels of some serum enzymes indicative of tissue damage, as a result of *Saprolegnia* infection in brown trout, and showed that glutamate-oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT), thought to be associated with liver cell damage, had eight times their normal activity in infected trout. Surprisingly creatine phosphokinase (CPK) activity, considered to be associated with muscle damage, did not change.

Components of the innate and acquired immune systems of fish are all known to occur in the surface mucus (Shephard, 1994), but studies on the activity of mucus against fungi are somewhat conflicting. Roberts and Bullock (1980) reported fungi occurring among the skin biota of healthy fish, and Willoughby and Pickering (1977) showed that mucus taken from salmonid fish provided an effective germination and growth medium for *Saprolegnia* spores. Conversely, possible neutrophils or lymphocytes (Wood, Willoughby and Beakes, 1986; Willoughby, 1989) and an inhibitory morphogen (Wood *et al*, 1988) in the mucus of salmonid fish were shown to be active against *Saprolegnia*. It is possible that the continuous secretion and sloughing of mucus is the most important means of preventing the establishment of fungal growth (Shephard, 1994).

9.1.2 Acquired immune response of fish to fungi

There are very few reports in the literature of the humoral response of fish against fungi, and none dealing with *A. invadans* infection. Hodkinson and Hunter (1970) demonstrated what they considered to be anti-*Saprolegnia* antibodies occurring in Atlantic salmon, but these were not necessarily associated with a corresponding fungus infection. Alexander and Ingram (1992) pointed out that it is not certain whether these antifungal precipitins are immunoglobulins, and whether they constitute a primary or secondary immune response. Richards and Pickering (1979) demonstrated a possible increase in the electrophoretic mobility of slower moving proteins in the serum of *Saprolegnia*-infected brown trout, and hypothesised that this may be due to immunoglobulin production in response to the fungus. Sohnle and Chusid (1983), however, investigated both cellular and humoral responses of rainbow trout to unidentified saprolegniacean fungi, and showed that a significant inflammatory response was mounted by the fish but neither precipitating antifungal antibodies nor plasma factors capable of inhibiting fungal growth were found. Faisal and Hargis (1992) demonstrated a significantly higher level of lymphocyte activity in "ulcer disease syndrome" affected menhaden (probably equivalent to UM), than healthy fish, and hypothesised that cell-mediated hypersensitivity may have an important role in the course of that disease.

In the present study, Western blot analysis of various striped snakehead (*Ch. striata*) sera was used to investigate antibody production against *A. invadans*.

9.2 Material and methods

9.2.1 Preparation of fungi

A. invadans isolate, PA7, was used in fish inoculations. Zoospore suspensions were produced as in Section 2.2.5(b). The zoospores were prepared as vaccinates by microwaving them for 10 seconds, after which time they were no longer motile. Both the spore suspensions and homogenised mycelium mats (used to produce the zoospores) were used to immunise the fish. Mycelium extracts of an EUS (PA7), MG (NJM9030),

RSD (3P), *A. astaci* (FDL458), and saprophytic *Aphanomyces* (F3SA) isolate were prepared for gel electrophoresis (SDS-PAGE) as described in Section 8.2.1.

9.2.2 Inoculation of fish and collection of sera

Snakehead fish (500g), obtained from Suphanburi Province, Thailand, were maintained at the Aquatic Animal Health Research Institute (AAHRI), Bangkok. Three groups of fish were used in the study. The first group (n=8) were non-vaccinated healthy control fish. The second group (n=5) received a primary intramuscular injection of microwaved *A. invadans* (isolate PA7) spores (1 ml, containing 5000 spores/ml), followed by an intraperitoneal injection of microwaved PA7 spores mixed with homogenised mycelium (1 ml, containing 5000 spores/ml mixed with 1 mat of mycelium) 10 days later. The third group, consisting of non-vaccinated healthy fish, were challenged with an intramuscular injection of motile PA7 zoospores (1 ml, containing 5000 spores/ml). The first two groups of fish were maintained at a water temperature of 27°C, while the third group was maintained at 20°C throughout the challenge period. Blood was collected from the caudal vein of fish on day 30 of the study. Blood samples were also collected from 7 snakehead fish infected with EUS during a natural outbreak of the disease in Pichit Province, Thailand. These fish had also been sampled for fungal, viral and bacterial analysis, and both *A. invadans* (isolate 96PA) and rhabdoviruses (Kanchanakhan, 1996b) were recovered.

9.2.3 Western blot analysis

Sera from the fish of each group were pooled and screened by Western blot analysis, using a similar method to that described in Chapter 8. Fungal extracts were subjected to SDS-PAGE (Section 8.2.3) and blotted onto nitrocellulose membranes (Section 8.2.6). These were washed with two changes of Tris buffered saline with Tween-20 (TTBS: 0.05 M Tris, 0.15 M NaCl, 0.1% v/v Tween-20 pH 7.6) and non-specific binding sites were blocked for 2 hours with 1% BSA. Membranes were then washed twice in TTBS, incubated in fish sera (1/10 dilution in 0.2 M PBS, pH 7.3) for 1 hour at 20°C, washed again, and incubated for 1 hour at 20°C, this time with rabbit anti-snakehead IgM (diluted 1/100 in PBS) (courtesy of Jitkasem Chanphong, AAHRI). Membranes were then washed

again, and goat anti-rabbit IgG-HRP conjugate (SAPU) (diluted 1/100 in TTBS) was applied for 1 hour. Unbound conjugate was removed from the membranes by washing twice with TTBS and once with PBS. The assay was developed by incubating with chromogen (6 mg DAB, Sigma) dissolved in 10 ml of substrate buffer (20 mM Tris hydrochloride, 500 mM NaCl, 30 ml H_2O_2, pH 7.5) until bands appeared. The reaction was stopped with distilled water.

9.3 Results

The response of the various snakehead sera to the different *Aphanomyces* mycelium extracts (Figure 9.1) reflected the similarities found between the protein profiles of the EUS, MG and RSD isolates shown in Chapter 8. Sera from healthy fish elicited a weak non-specific reaction with all mycelium extracts, particularly with bands present at around 40 and 55 kDa in the extracts of the EUS, MG and RSD isolates (PA7, NJM9030 and 3P) (Figure 9.1a, lanes 1, 3 and 4, respectively). Sera from fish immunised with the spores and mycelium from isolate PA7 produced a stronger reaction with these extracts than was observed with healthy fish sera, particularly with the 40 and 55 kDa bands (Figure 9.1b). This sera also identified a band at 37 kDa, unique to the mycelium extracted from saprophytic *Aphanomyces* isolate, F3SA (lane 2), while only an undefined region of staining between 36 and 80 kDa was observed with the sera against *A. astaci* (FDL458) (Figure 9.1b, lane 5). As well as recognising the bands mentioned above, antisera from both the experimentally challenged fish, and fish from a natural EUS outbreak (Figure 9.1c and d), identified a low molecular weight band at around 10 kDa. This band was not detected by the immunised sera from group two fish immunised with microwaved fungi and was not apparent on the profiles of the saprophytic isolate of *Aphanomyces* (F3SA) or with *A. astaci* (FDL458).

9.4 Discussion

The 10 kDa band common to all the EUS, MG and RSD pathogens, that was identified by sera from snakeheads naturally and artificially infected with *A. invadans,* is considered to be the same band that was recognised by rabbit polyclonal antisera (Chapter 8). In

Chapters 7 and 8, immunocytochemistry of *A. invadans* propagules and immunohistochemistry of infected fish tissue using polyclonal antiserum raised against the 10 kDa band failed to identify any specific source of the antigen; however Western blot analysis showed that it was not associated with the ECP (Chapter 8). The results given here suggest that the 10 kDa band is not immunogenic to snakeheads when it is presented as a vaccinate in the form of microwaved fungal particles, but is immunogenic when the fungus is actually growing within the fish. A possible explanation for the differences in the antigenicity of this band may be due to alterations in its structural conformation during vaccine preparation compared to *in situ* fungal growth within the fish. These findings have serious implications for the development of vaccines against EUS. It is possible that protective antibodies are produced against antigens present on growing fungus, but it would be very difficult to use these as a vaccine if the fungus needs to be alive for a protective response to be elicited and if conformational changes occur as a result of processing the dead fungus. However, Hodkinson and Hunter (1971) showed that the growth medium in which fungi are grown prior to the preparation of antigen extracts is important to eliciting a response in fish. This was demonstrated by showing that anti-*Saprolegnia* precipitins in salmon sera only gave a positive reaction against *Saprolegnia* mycelium extracts from fungus that had previously been grown in salmon extract medium.

It is not known whether snakehead sera containing antibodies against *A. invadans* are protective against infection, although this can be tested by using sera from challenged fish for passive immunisations of naive snakeheads. An attempt to vaccinate snakeheads using PA7 mycelial extract, followed 5 weeks later by PA7 challenge, did not result in any increased survival in vaccinated fish and a similar inflammatory response was observed in both vaccinated and non-vaccinated fish (K.D. Thompson, unpublished data). It would be interesting to determine whether cytotoxic T lymphocytes are involved in the cellular response of vaccinated fish to *A. invadans* challenge.

Aside from the 10 kDa band specific to EUS, MG and RSD isolates, sera from snakeheads infected with *A. invadans* also recognised various other bands on all the fungal extracts, giving an indication of the cross-reactive nature of at least some of the antibodies. This is a common feature of polyclonal antisera raised against fungi, and may be due to the presence of very similar epitopes on a variety of fungal species and genera (Chapter 8; Wilson, 1976; Bullis *et al,* 1990). Bands revealed on all extracts by the serum from healthy fish may be a result of the fish having been previously exposed to fungi ubiquitous in the environment. The 37 kDa band in the F3SA extract and the 55 and 40 kDa bands in the PA7 extract, recognised here by the snakehead sera, were also recognised by sera from rainbow trout infected with PA7 (K.D. Thompson, unpublished data; Skliris, 1995). Clear 10 kDa bands have not as yet been confirmed against PA7 extract using infected trout sera.

Further studies on the humoral defences of fish to infection by *A. invadans* should also consider the role of such factors in the mucus, which is clearly important in preventing primary infection. Given that the non-specific cellular response of fish appears to be important in EUS infections, the use substances such as glucans and adjuvants, to stimulate this mechanism may offer an alternative means of protecting fish from *A. invadans* invasion.

Reports of the relative susceptibility of different fish populations may also provide an indication of the development of immunity against EUS. Cruz-Lacierda and Shariff (1995) demonstrated that naïve snakeheads, flown to the Southeast Asian Fisheries Development Centre (SEAFDEC), Iloilo, from a Philippine island where EUS had never been reported, consistently developed EUS when exposed to ulcerated fish, or water from an EUS-enzootic environment (Laguna de Bay), without the need to artificially damage the skin. However, similar experiments using healthy snakeheads from Suphanburi, Thailand, where EUS has recurred annually since 1982, failed to consistently reproduce EUS (Roberts *et al,* unpublished data). This finding, along with the observation that the severity of the disease generally subsides following initial

outbreaks (Lilley *et al*, 1992), suggests there is a possibility that fish may develop a degree of resistance to subsequent infection. Healing of EUS lesions is sometimes observed (Subasinghe, 1993), possibly as a result of a rise in temperature (Chinabut *et al*, 1995), and this would provide surviving fish with the opportunity of developing acquired immunity. Alternatively, the initial mass mortalities caused by EUS may have selected for resistant individuals that have reduced the susceptibility of subsequent populations. If it is the case, it may be possible to use selective breeding as a means of reducing losses due to EUS in fish farms (Fjalestad, Gjedrem, and Gjerde, 1993). With regards to the crayfish plague, Unestam and Weiss (1970) demonstrated some evidence for heightened resistance in noble crayfish (*As. astacus*) following an initial challenge with *A. astaci*. Alderman *et al* (1987) however, believed that there was no evidence of resistance in European crayfish populations and doubted that crayfish ever survived a proper challenge to develop acquired immunity.

Figure 9.1

The response of snakehead sera against extracts of mycelium from *Aphanomyces* spp by Western blot analysis.

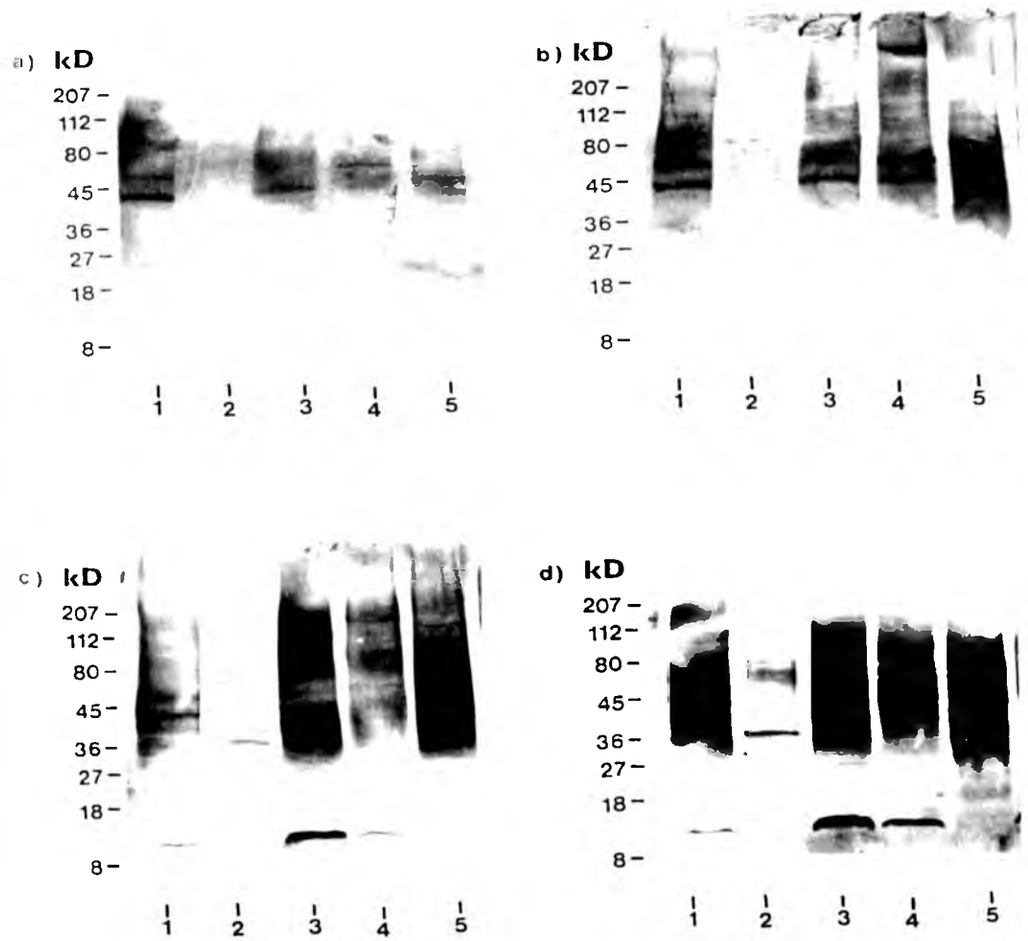
(a) Healthy fish serum

(b) Serum from fish immunised with spores and mycelium from *A. invadans* isolate PA7

(c) Serum from fish experimentally challenged with PA7

(d) Serum from fish suffering from a natural EUS outbreak

Lanes: Mycelial extracts from (1) *A. invadans* isolate PA7; (2) saprophytic *Aphanomyces* F3SA; (3) MG isolate NJM9030; (4) RSD isolate 3P and (5) *A. astaci* isolate FDL458



CHAPTER 10. PYROLYSIS MASS SPECTROMETRY (PYMS)[5]

10.1 Introduction

Pyrolysis mass spectrometry (PyMS) is an analytical technique that can be used to obtain biochemical fingerprints of whole micro-organisms (Magee, 1993). Briefly, the complex organic material of the sample is thermally degraded (pyrolyzed) in an inert atmosphere. Curie point PyMS, as used here, employs a ferro-magnetic foil as a sample carrier which is heated and maintained at its Curie point by means of high-frequency alternating magnetic field. The resulting vapour or pyrolysate is bombarded with low-energy electrons which generate molecular and fragment ions. These are separated by a quadrupole mass spectrometer on the basis of their mass:charge ratio (m/z) and displayed in the form of quantitative mass spectra.

Pyrolysis followed by gas chromatography (PyGC) has been used for the identification and characterization of filamentous fungi, including *Aspergillus* (Stretton, Campbell and Burns, 1976) and *Penicillium* (Söderström and Frisvad, 1984). However the use of mass spectrometry as a separation method following pyrolysis provides greater resolution (>150 variables) than PyGC (~40 variables) and has been increasingly used in bacterial systematics (Shute, Berkeley, Norris and Gutteridge, 1985; Winstanley, Magee, Limb, Hindmarch, Spencer, Whiley, Beighton and Hardie, 1992; Magee, Randle, Gray and Jackson, 1993; Manchester, Toole and Goodacre, 1995). Fungal studies using PyMS have been mainly restricted to yeasts (Windig and Haverkamp, 1982; White, Sisson, Freeman and Cookson, 1994b). Weijman, van Eijk, Roeijmans, Windig, Haverkamp and Turkensteen (1984) evaluated PyMS as a method of diagnosing potato-gangrene caused by *Phoma;* and Niemann, Van der Bij, Brandt-der Boer, Boon and Baayen (1991) used this technique to demonstrate different levels of lignin degradation in carnations by *Fusarium* and *Phialophora*. However there are no reports using PyMS as an aid to classification in filamentous

[5] A paper based on work described in this chapter, entitled "Characterisation of *Aphanomyces invadans* using pyrolysis mass spectrometry (PyMS)" has been sumitted to Mycological Research for publication

fungi. The present study aimed to evaluate PyMS as a method of discriminating different levels of Oomycete taxa: between isolates of *A. invadans*, species of *Aphanomyces* and genera of Saprolegniaceae.

10.2 Materials and methods

This work was undertaken with the use of facilities at the Biomedical Mass Spectrometry Unit, Medical School, Newcastle upon Tyne.

Forty five fungal isolates were tested as listed in Appendix 1. Fungal colonies were grown in Petri dishes of GPY liquid media (Appendix 2a). The same batch of GPY was used throughout the experiment. Asian isolates were grown at room temperature (20-24°C) and UK and USA isolates were grown at 12°C. For each isolate, three squares of mycelium, approximately 2 mm^2, were cut from the edges of actively growing colonies and used to inoculate a Petri dish containing GPY media. Halfway through the total growth period, the resulting three mycelial mats were again cut into 2 mm^2 squares. The total incubation time was calculated from known growth rates of the isolates to produce end wet weights of approximately 0.3g, and ranged between three days for the saprophytic *Aphanomyces* spp to eight days for *A. astaci*. Ideally, identical growth conditions should be adopted for each isolate, but in this case that would result in mycelium at very different stages of growth. The present regime was used so that each fungal culture was rich in actively growing hyphal tips.

The resulting mycelial mat was washed in sterile distilled water and filtered through cheesecloth. This was repeated four times. The mycelial mat was then homogenized in liquid nitrogen and stored at -20°C. Preliminary work had shown that unhomogenized, washed mycelium produced less consistent results, possibly because of a lack of uniformity in the age of hyphae within the fungal preparation. Duplicate preparations of four isolates (S1PA, PA7, 10P and WSA) were prepared to check the reproducibility of different preparations of the same isolate.

Thawed homogenated material formed a paste, a small amount of which was smeared thinly on to alloy foils (50% iron : 50% nickel). The foils were inserted into pyrolysis tubes and oven-dried for a few minutes. It was found that excessive drying often resulted in the sample dropping off the foil. Three replicate tubes were prepared from each fungal homogenate. The samples were loaded on a RAPyD 400 pyrolysis mass spectrometer (Horizon Instruments Ltd, Heathfield, East Sussex) and pyrolyzed for 3 sec at a Curie point of 530°C. Preliminary runs were carried out to evaluate the amount of homogenated material required on the foil to give total ion counts of between three and ten million.

10.3 Results and discussion

Sample mass spectra are given in Figures 10. And 10.2. Canonical variate analysis (CVA) was performed on each spectrum and the average value for the three replicates was calculated. A 3-D representation of these results (Figure 10.3) shows that the majority of *Aphanomyces* species could be easily distinguished from a wide scatter of *Achlya* and *Saprolegnia* outgroup species. Further CVA using the *Aphanomyces* species alone (Figure 10.4) succeeded in discriminating EUS, MG and RSD pathogens from the saprophytic isolates. The variation within the group of saprophytes is probably an indication that at least two species are involved.

A dendrogram produced by group-average hierarchal cluster analysis (HCA) (Figure 10.5) gave a more clear-cut distinction between EUS, MG and RSD pathogens and the saprophytes by forming two main groups separated at a similarity index of 61%. The first main group clustered the EUS pathogen with RSD and MG isolates. Other studies described in this report indicate that these fungi all represent a single species. The PyMS evidence tended to support this hypothesis, although two isolates (33P and 4P) clustered with the main group at a much lower similarity index. Also of interest was the proximity that the crayfish plague fungus, *Aphanomyces astaci,* showed to the EUS-RSD-MG group.

UM *Aphanomyces* isolates were shown to be distinct from *A. invadans,* and instead clustered with saprophytic *Aphanomyces* to form the second group. This is consistent with the suggestion by Dykstra *et al* (1989) that at least one of the UM-isolates is *Aphanomyces laevis.*

Duplicate cultures of one isolate (WSA) clustered together at only 91% similarity, indicating that any of the detailed relationships at this level or above were unreliable. Therefore PyMS probably lacks the sensitivity to resolve intraspecific differences in these fungi. Indeed the isolates representing some species, *A. astaci* and *A. laevis* in particular, failed to group convincingly. However in the clustering of the EUS-RSD-MG group of fungi and their separation from non-pathogenic *Aphanomyces* species, the system proved highly discriminatory.

Figure 10.1 Sample mass spectrum for *Aphanomyces invadans* isolate RF8 (average of three replicates)

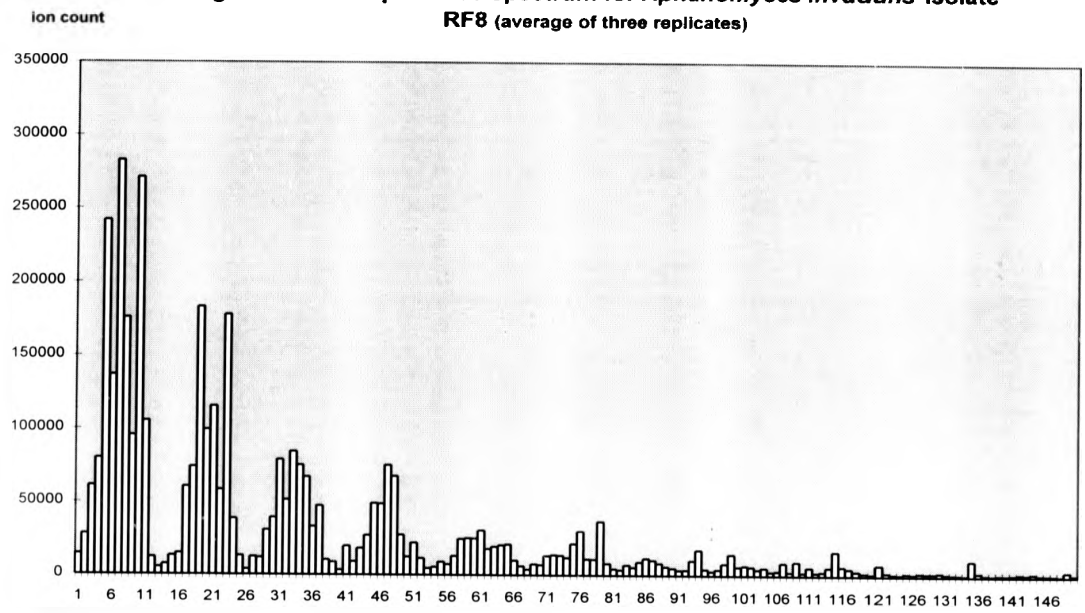


Figure 10.2 Sample mass spectrum for *Saprolegnia diclina* isolate E3 (average of three replicates)

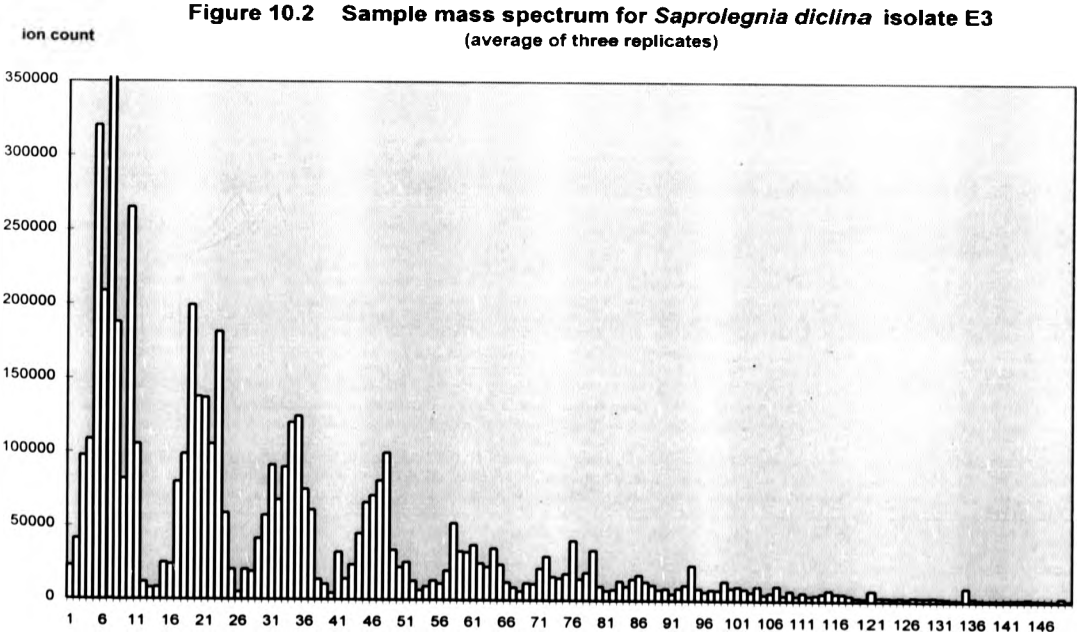


Figure 10.3 Canonical variate analysis (CVA) of pyrolysis mass spectra of all fungus isolates. Markers represent the average of three replicates.

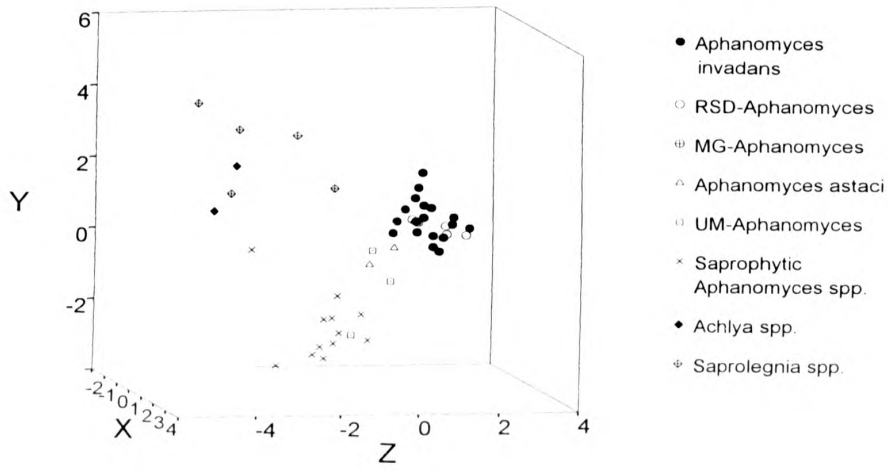


Figure 10.4 CVA of pyrolysis spectra of *Aphanomyces* species only. Markers represent the average of three replicates

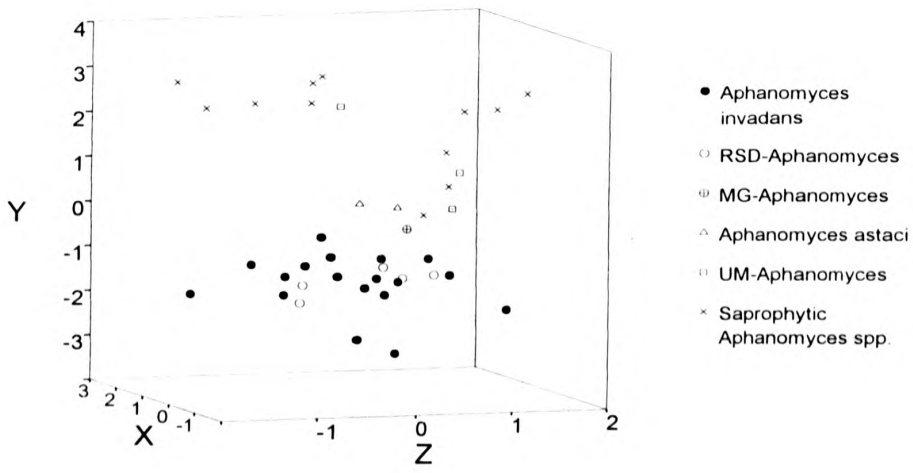
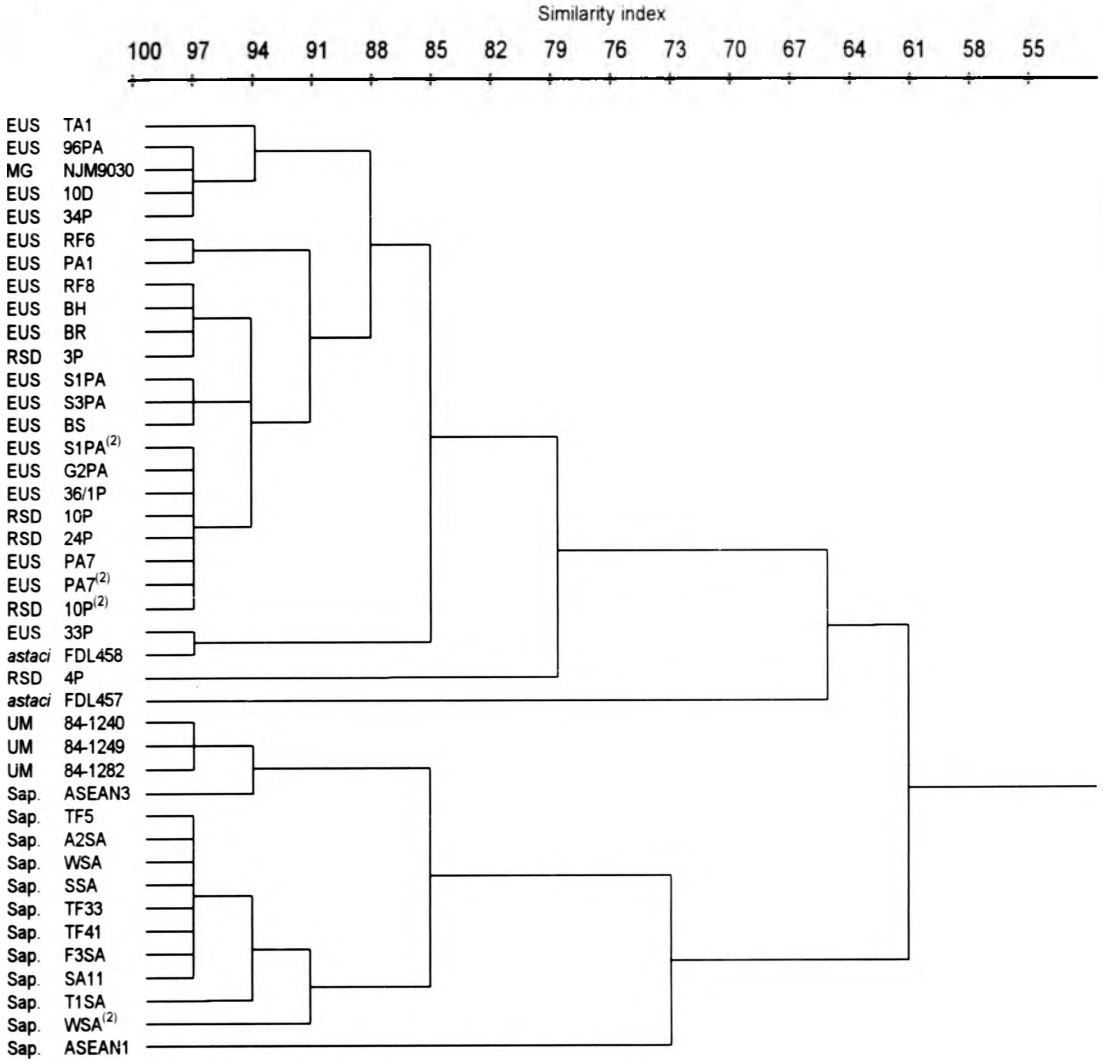


Figure 10.5 Hierarchal cluster analysis (HCA) dendrogram of the same data set as in Figure 10.4. Includes EUS, MG and RSD *Aphanomyces*, *A. astaci*, UM *Aphanomyces* and various saprophytic *Aphanomyces* species (Sap.). S1PA$^{(2)}$, PA7$^{(2)}$, 10P$^{(2)}$ and WSA$^{(2)}$ are duplicate cultures of S1PA, PA7, 10P and WSA respectively



CHAPTER 11. RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD)[6]

11.1 Introduction

Molecular data sets generated using polymerase chain reaction (PCR) technology, are rapidly becoming an essential part of any detailed fungal taxonomic study (Metzenberg, 1991; Foster, Kozak, Loftus, Stevens and Ross, 1993). Various PCR-based methods of genetic fingerprinting have been used to provide information on interspecific (Molina *et al*, 1995; Briard, Dutertre, Rouxel and Brygoo, 1995; Cooke and Duncan, 1997), and intraspecific (Hantula, Dusabenyagasani and Hamelin, 1996; Majer, Mithen, Lewis, Vos and Oliver, 1996; Sequerra, Marmeisse, Valla, Normand, Capellano and Moiroud, 1997) relatedness in fungi. Such information is increasingly being combined with morphological data to construct total evidence phylogenies (McLaughlin, Barres and Szabo, 1995; Tehler, 1995; Untereiner, Straus and Malloch, 1995) or mapping characters of phylogenetic significance (Chen and Hoy, 1993; Mitchell, Roberts and Moss, 1995; Crawford, Bassam, Drenth, MacLean and Irwin, 1996).

Random amplification of polymorphic DNA (RAPD) has proved a particularly sensitive technique for establishing detailed, intraspecific relationships between fungal isolates (Cooke, Kennedy, Guy, Unkles and Duncan, 1996; Fegan, Manners, MacLean, Irwin, Samuels, Holdom and Li, 1993; Hodge, Sawyer and Humber, 1995). In the case of the crayfish plague fungus, *Aphanomyces astaci*, Huang, Cerenius and Söderhäll (1994) used RAPD analysis to categorise three groups of Swedish isolates, and show that new strains are being introduced and affecting indigenous crayfish populations. Diéguez-Uribeondo *et al* (1995) later showed that a new Spanish *A. astaci* isolate represented another, more distantly related group.

[6]Information presented in this Chapter is published in Lilley, J.H., Hart, D., Richards, R.H., Roberts, R.J., Cerenius, L. and Söderhäll, K. (1997) Pan-Asian spread of single fungal clone results in large scale fish-kills. Veterinary Record 140, 11-12. A paper based on the *Aphanomyces astaci* data entitled "RAPD evidence for the origin of crayfish plague in Britain" has been submitted to Aquaculture for publication.

This study uses RAPD-PCR analysis to examine how close the relationship is between EUS, MG and RSD isolates. Twenty isolates from 6 countries were studied, along with 2 UM isolates, 6 *A. astaci* isolates and 6 saprophytic *Aphanomyces* spp.

Given that no UK isolates of *A. astaci* have been previously characterised using RAPD-PCR, this study offered a valuable additional opportunity to type the two UK isolates obtained for the various comparative studies (FDL457 and FDL458) according to the groups established by Huang *et al* (1994). It was envisaged that this would provide evidence of the origin of the crayfish plague in Britain.

11.2 Materials and methods

This work was carried out at the Department of Physiological Mycology, University of Uppsala, Sweden.

11.2.1 Fungi

The isolates used in this study are indicated in Appendix 1. In addition to the fungi used in several studies here, additional *A. astaci* isolates were obtained from the culture collection of the Department of Physiological Mycology, Uppsala. These comprised J1, PI and Kv (representing RAPD groups A, B and C respectively, as described by Huang *et al,* 1994) and Pc (group D, Diéguez-Uribeondo *et al,* 1995).

11.2.2 DNA preparation

Genomic DNA was extracted using the following procedure for all isolates except *Aphanomyces astaci*. About 50 mg of mycelium grown in GPY broth was homogenised in 11 ml lysis buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 2% SDS). Proteinase K was added to a final concentration of 1 mg/ml and incubated overnight at 37°C with shaking. The sample was then chilled on ice for 10 min. Five millilitres of saturated NaCl was added to the tube which was mixed and then chilled for another 5 min. Precipitated protein was pelleted by centrifugation at 2000 xg for 15 min at 4°C.

The supernatant was transferred to a fresh tube and centrifuged again to ensure removal of the precipitate. RNase A was added to a final concentration of 20 μg/ml and the tube incubated for 30 min at 37°C. Two volumes of 100% ethanol was added to the sample which was then mixed and stored at -20°C overnight. The sample was centrifuged at 2000 xg for 15 min at 4°C. The resulting DNA pellet was washed with 10 ml ice-cold 75% ethanol and centrifuged again for 5 min. The pellet was vacuum-dried and resuspended in 200 μl distilled water.

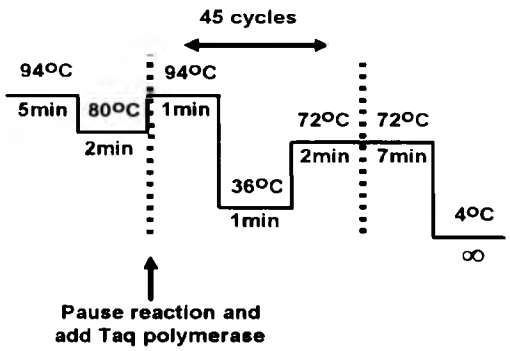
DNA preparations of *A. astaci* isolates were made from cultures grown in PG-1 broth using the Nucleon II kit by following the supplied procedure for filamentous fungi. Briefly, mycelium was ground in liquid nitrogen and scraped into 2 ml lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% SDS). Three microlitres of 10 mg/ml RNase A was added and the sample incubated at 37°C for 30 min. Then 1.5 ml 5 M sodium perchlorate was added to the sample which was mixed for 15 min and incubated at 65°C for 25 min. Ice-cold chloroform (5.5 ml) was then added and the solution mixed for 10 min and centrifuged at 800 xg for 1 min. About 800 μl of a silica suspension was added and the tube centrifuged at 1400 xg for 3 min. The clear DNA-containing phase was recovered to which equal volume of 99% cold ethanol was added and the resulting precipitate was centrifuged at 5000 xg for 5 min. The DNA pellet was washed in 70% ethanol and centrifuged again. The final pellet was vacuum-dried and resuspended in 100 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

11.2.3 RAPD-PCR

Each 50 μl reaction tube contained 5-50 ng genomic DNA, 20 pmol primer, 5 μl 10X USB buffer (Amersham), 1.5 mM $MgCl_2$, 200 mM of each dNTP and 1.5 units of AmpliTaq DNA polymerase. Fourteen random 10-mer primers were obtained from Operon Technologies and used in the RAPD analyses. The nucleotide sequences of each primer are given below.

A3	5'-AGTCAGCCAC
A4	5'-AATCGGGCTG
A6	5'-GGTCCCTGAC
A7	5'-GAAACGGGTG
A10	5'-GTGATCGCAG
A12	5'-TCGGCGATAG
A18	5'-AGGTGACCGT
A19	5'-CAAACGTCGG
A20	5'-GTTGCGATCC
B1	5'-GTTTCGCTCC
B2	5'-TGATCCCTGG
B4	5'-GGACTGGAGT
B5	5'-TGCGCCCTTC
B10	5'-CTGCTGGGAC

PCR reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 using a similar temperature cycle as described by Huang *et al* (1994). This is illustrated below. Amplification products were separated on 1.4% agarose gels stained with ethidium bromide and visualised under UV illumination.



1124 Cluster analysis

The presence or absence of fragments given by each isolate was marked by hand, and the data analysed using the "Dice" similarity measure in the statistics package, SPSS for Windows Release 601 (Dice, 1945; SPSS, 1988). This is a binary matching coefficient that gives double weight for matching bands. It is identical to the formula of Nei and Li (1979), which is used in most RAPD studies of fungi, including those of

Huang *et al* (1994) and Diéguez-Uribeondo *et al* (1995) on *A. astaci*. Nei and Li's similarity coefficient (F) is given as:

$$F = 2n_{XY}/(n_X + n_Y)$$

where n_X and n_Y are the numbers of fragments in samples X and Y respectively and n_{XY} is the number of fragments shared by the two samples.

11.3 Results

11.3.1 A total of 321 bands, averaging 80.7 fragments per isolate, were used to calculate similarity coefficients (F). The mean similarity coefficient (F ± SD) comparing all the EUS, MG and RSD isolates was 0.95 ± 0.03 (Figure 11.1). The 6 saprophytic *Aphanomyces* spp, 6 *A. astaci* isolates and 2 UM-*Aphanomyces* isolates in combination gave an average similarity coefficient of 0.15 ± 0.05 compared with the EUS, MG and RSD isolates. A dendrogram generated from this data using the neighbour joining method of Saitou and Nei (1987) is given in Plate 11.1. Two sample gels showing polymorphism fragments generated using two random primers are given in Plate 11.2.

11.3.2 Crayfish plague

When the *A. astaci* isolates are analysed separately, a total of 118 band positions, giving an average of 56.7 bands per isolate, was used to calculate similarity coefficients (Figure 11.1). The British isolates FDL457 and FDL458 were found to be almost identical, with an F value of 0.98. In comparison to the other strains the British isolates most resembled PL, the isolate representing group B, with an average similarity coefficient of 0.90. Average F values between the British isolates and group A, C and D fungi were 0.58, 0.72 and 0.34 respectively. Therefore the British isolates were found to be most similar to the group C representative (PL). A sample gel illustrating the similarity of the polymorphic DNA fragments of British isolates with PL, using two random primers, is given in Plate 11.3.

11.4 Discussion

Given that RAPDs have been shown to be very sensitive in distinguishing strains of saprolegniacean fungi (Huang *et al*, 1994; Diéguez-Uribeondo *et al*, 1995; 1996), the results of this study indicate an extreme lack of genetic diversity between all the EUS, MG and RSD isolates. Therefore it is concluded that, not only do they represent a single species, but they may be considered "clonets" by the definition given by Tibayrenc (1996). Plate 11.1 shows that all the Philippine isolates clustered together, but from present data this cannot be considered a significant divergence from the other isolates, given the low level of variation involved. As the isolates are shown to be conspecific, it is recommended in Chapter 12 that the name *Aphanomyces invadans* Willoughby *et al.* 1995 (as *A. invaderis*) is used for all the EUS, MG and RSD ulcerative disease isolates.

The isolate NJM9201 obtained from a diseased dwarf gourami imported to Japan from Singapore has been previously shown to have similar growth (Hatai *et al,* 1994) and pathogenicity (Rha, Sinmuk, Wada, Yuasa, Nakamura, Hatai and Ishii, 1996) characteristics to MG isolates, but is still considered by those authors to be a distinct fungus. Present work shows that this isolate is the same species as the MG fungi, and all the other *A. invadans* isolates.

The genetic homogeneity between all the *A. invadans* isolates may be associated with observations that it lacks any sexual reproductive structures (Chapter 4). However, RAPD studies on *A. astaci*, which is similarly asexual, yielded four distinguishable groups from 15 European isolates (Huang *et al*, 1994; Diéguez-Uribeondo *et al*, 1995). These showed an average between-group similarity of 0.25 ± 0.08, and the average within-group similarity (0.84 ± 0.11) was also lower than for the *A. invadans* isolates (data recalculated from Diéguez-Uribeondo *et al*, 1995). This indicates that there have been several introductions of *A. astaci* to Europe over a number of years whereas *A. invadans* has achieved its colonisation of Asia in one

relatively rapid episode. This correlates with the accounts of the spread of EUS outbreaks described in Chapter 1.

The time span over which *A. invadans* appears to have spread across Asia (from Japan in 1971 to Pakistan in 1996) is not unreasonable for a fungal pathogen. *A. astaci* spread from Italy to colonise most of Europe between the Netherlands and Russia over a similar time span in the last century (Alderman, 1996). In another study that employed RAPD-PCR to trace the origin of a pathogenic fungus, Hajek, Hodge, Liebherr, Day and Vandenburg (1996) showed that a single genotype of the weevil pathogen *Zoophthora phytonomi* appears to have spread to much of Eastern North America over a period of 8 years.

Further comparisons can be drawn with the clonal genotypes of different *Phytophthora* spp that cause epidemics in particular host plant species. For example, Hantula, Lilja and Parikka (1997) showed by means of random amplified microsatellites (RAMS) that all 20 isolates of *Phytophthora cactorum* originating from strawberry in 6 countries in Northern Europe were clonal. Likewise, Goodwin, Cohen and Fry (1994) used mating types, allozymes and Southern analyses of over 300 isolates of the Irish potato famine fungus, *Phytophthora infestans*, from 20 countries to demonstrate that of a number of original genotypes, it was probably a single genetic individual that was transported from Mexico to the United States, Europe and the rest of the world causing epidemics of potato blight from the 1840s onwards. Further work on different *P. infestans* genotypes showed that following migration, mutation rates at pathogenicity loci are high, resulting in several pathotypes within each genotype investigated (Goodwin, Sujkowski and Fry, 1995).

In the case of EUS, it is not possible to determine whether *A. invadans* was introduced to Japan, or arose through changes in an endemic genotype. However, given the established nature of the fish importation industry in Japan, the former would seem the more plausible theory. Japan imports fish even from isolated areas,

such as some Pacific islands, and it can be theorised that the pathogen may have evolved in such an environment alongside a natural, resistant host. Further discussion of the mechanism of spread of *A. invadans* is given in Chapter 12.

These results correspond with other molecular studies on the present isolates undertaken by workers at Glasgow University. In those studies, restriction fragment length polymorphism (RFLP) analysis and nucleotide sequences of the rRNA gene also showed no differences between any of the EUS, MG and RSD isolates (D. Hart, pers. comm.). A dendrogram constructed from the RFLP data is reproduced here as Plate 11.4. As indicated by work described in Chapters 4 and 10, *A. invadans* is shown to be most closely related to *A. astaci* of all the fungi compared, and the saprophytic *Aphanomyces* are shown to represent several different species. Four different groups of saprophytic *Aphanomyces* were identified by RFLP analyses: the UM isolate (84-1240), isolate SA11, *A. laevis* (ASEAN1, ASEAN3) and the other saprophytic *Aphanomyces* isolates. Of the latter group, T1SA, TF54, F3SA and SSA were identical, whereas TF41, A2SA and TF5 each showed minimal variation. It is interesting to note that the UM isolate, thought to be *A. laevis* (Dykstra *et al,* 1989), is shown to be distinct from Asian *A. laevis* isolates. Yeh (1989) has previously shown RFLP analyses to be effective in distinguishing *A. astaci*, *A. stellatus, A. cochlioides* and *A. eutiches.*

Crayfish plague

Despite its long history in most of Europe, the crayfish plague was only first reported in Britain in 1981 (Alderman, 1993). This coincided with the wave of imports of signal crayfish, *Pacifastacus leniusculus,* which began in 1976. Although this species is known to act as a vector for the disease (Alderman, Holdich and Reeve, 1990), no direct evidence could be supplied to link the events. Indeed it was conjectured that the outbreaks may simply be a recrudescence of a disease that had been present in Britain for as long as in mainland Europe. However, the indication here that English stocks of *A. astaci* belong to a group only isolated since 1970 and which originate

from imported signal crayfish in Sweden, provides strong evidence that the disease was introduced to Britain with similar shipments.

The Spanish strain of *A. astaci* (group D) is known to be very different physiologically from other strains and may also differ in terms of pathogenicity (Diéguez-Uribeondo *et al*, 1995). Therefore this raises the possibility of further introductions of new, more pathogenic strains of the fungus and mitigates against a relaxation in restrictions on the movement of crayfish.

Figure 11.1 **Similarity matrix generated from RAPD data**

	TA1	RF6	RF8	S3PA	G2PA	PA1	PA4	96PA	BR	BH	36/1P	10D	30P	33P	34P	4P	10P	24P	NJM9030	NJM9201	84-1240	84-1282	TF41	TF54	F3SA	WSA	SA11	ASEAN3	FDL457	FDL458	J1	PL	KV
TA1																																	
RF6	.94																																
RF8	.98	.95																															
S3PA	.99	.95	.98																														
G2PA	.99	.98	.99	.99																													
PA1	.99	.95	.98	.98	.98																												
PA4	.98	.95	.97	.99	.98	.99																											
96PA	.95	.93	.95	.96	.95	.96	.96																										
BR	.98	.96	.98	.98	.98	.98	.97	.96																									
BH	.97	.94	.97	.97	.95	.99	.99	.90	.96																								
36/1P	.98	.95	.98	.98	.97	.98	.97	.97	.99	.94																							
10D	.92	.88	.91	.93	.92	.93	.93	.91	.92	.96	.93																						
30P	.93	.90	.92	.94	.94	.93	.93	.91	.93	.94	.94	.96																					
33P	.92	.89	.91	.93	.93	.92	.93	.92	.92	.97	.93	.99	.97																				
34P	.91	.88	.90	.92	.92	.93	.92	.91	.91	.96	.92	1.00	.96	.97																			
4P	.98	.93	.96	.97	.98	.96	.95	.93	.96	.97	.96	.94	.95	.94	.92																		
10P	.99	.94	.98	.99	.98	.98	.98	.95	.98	.97	.98	.93	.95	.93	.93	.98																	
24P	.99	.94	.98	.99	.98	.98	.98	.95	.98	.97	.98	.93	.95	.93	.93	.98	1.00																
NJM9030	.98	.94	.98	.98	.99	.97	.97	.94	.96	.96	.96	.91	.92	.92	.90	.96	.97	.97															
NJM9201	.98	.91	.97	.97	.98	.97	.96	.93	.97	.96	.96	.89	.92	.91	.89	.96	.97	.97	.97														
84-1240	.17	.14	.15	.17	.10	.14	.17	.12	.18	.14	.16	.13	.12	.14	.17	.15	.17	.17	.17	.12													
84-1282	.16	.13	.14	.16	.09	.13	.16	.11	.17	.11	.15	.12	.10	.13	.16	.14	.16	.16	.16	.10	.96												
TF41	.18	.18	.18	.18	.17	.16	.16	.16	.18	.24	.18	.18	.18	.18	.18	.18	.18	.18	.19	.19	.13	.11											
TF54	.16	.14	.16	.16	.14	.13	.14	.12	.16	.21	.15	.16	.16	.16	.16	.16	.16	.16	.17	.17	.20	.15	.55										
F3SA	.22	.19	.21	.21	.19	.21	.19	.18	.22	.17	.22	.20	.20	.21	.20	.22	.22	.22	.21	.22	.11	.12	.47	.40									
WSA	.06	.11	.06	.06	.06	.06	.06	.06	.06	.11	.06	.10	.11	.06	.10	.06	.06	.06	.06	.06	.10	.10	.06	.18	.17								
SA11	.07	.10	.09	.09	.08	.08	.09	.09	.09	.09	.09	.11	.11	.09	.11	.07	.09	.09	.08	.08	.00	.00	.14	.11	.12	.00							
ASEAN3	.06	.03	.06	.06	.05	.04	.06	.06	.06	.06	.06	.07	.07	.06	.07	.06	.06	.06	.07	.07	.09	.09	.13	.06	.08	.00	.04						
FDL457	.17	.22	.17	.17	.21	.18	.17	.18	.17	.23	.18	.23	.22	.19	.24	.19	.18	.18	.18	.17	.13	.14	.12	.14	.12	.15	.13	.00					
FDL458	.20	.26	.20	.20	.24	.21	.20	.21	.20	.27	.21	.25	.24	.20	.26	.22	.21	.21	.21	.20	.15	.15	.13	.15	.15	.15	.19	.00	.98				
J1	.15	.19	.15	.15	.19	.16	.15	.15	.15	.20	.15	.21	.20	.17	.20	.16	.16	.16	.15	.16	.11	.11	.15	.13	.11	.00	.15	.11	.57	.59			
PL	.16	.22	.16	.16	.20	.17	.16	.18	.15	.22	.17	.23	.22	.19	.22	.17	.17	.17	.16	.17	.13	.14	.11	.14	.14	.15	.17	.00	.88	.92	.58		
KV	.13	.18	.13	.13	.16	.13	.13	.13	.12	.14	.12	.17	.16	.14	.17	.14	.14	.14	.14	.14	.09	.09	.09	.13	.08	.00	.13	.00	.73	.71	.63	.71	
PC	.09	.13	.09	.09	.13	.10	.09	.09	.09	.17	.09	.12	.13	.11	.12	.11	.09	.09	.08	.09	.14	.11	.10	.10	.11	.00	.14	.03	.36	.32	.29	.35	.36
	TA1	**RF6**	**RF8**	**S3PA**	**G2PA**	**PA1**	**PA4**	**96PA**	**BR**	**BH**	**36/1P**	**10D**	**30P**	**33P**	**34P**	**4P**	**10P**	**24P**	**NJM9030**	**NJM9201**	**84-1240**	**84-1282**	**TF41**	**TF54**	**F3SA**	**WSA**	**SA11**	**ASEAN3**	**FDL457**	**FDL458**	**J1**	**PL**	**KV**

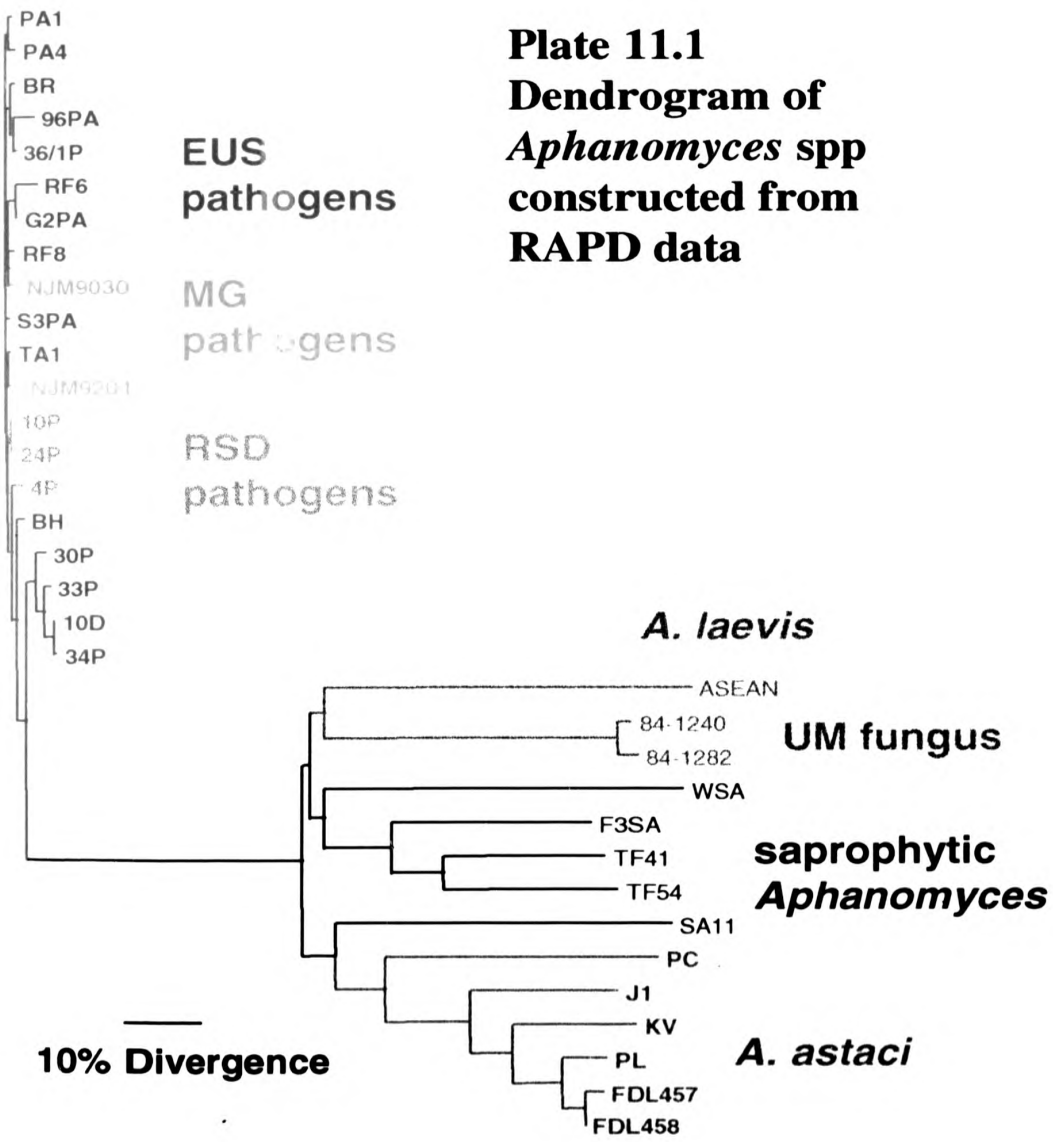


Plate 11.1
Dendrogram of *Aphanomyces* spp constructed from RAPD data

Plate 11.2

RAPD band profiles using primers (a) above - A19; and (b) below - A7 (Operon Technologies). Markers denote 1200, 800 and 400 base-pairs.

Lanes for (a) correspond to EUS pathogens: (1) TA1, (2) RF6, (3) RF8, (4) S3PA, (5) G2PA, (6) PA1, (7) PA4, (8) 96PA, (9) BR, (10) BH, (11) 36/1P, (12) 10D, (13) 30P, (14) 33P, (15) 34P; RSD pathogens: (16) 4P, (17) 10P, (18) 24P; MG pathogens: (19) NJM9030, (20) NJM9201; UM fungus: (21) 84-1240; saprophytic *Aphanomyces:* (22) TF41; and *A. astaci:* (23) FDL457, (24) PC

Lanes for (b) are the same except that (22) is saprophytic *Aphanomyces* isolate F3SA

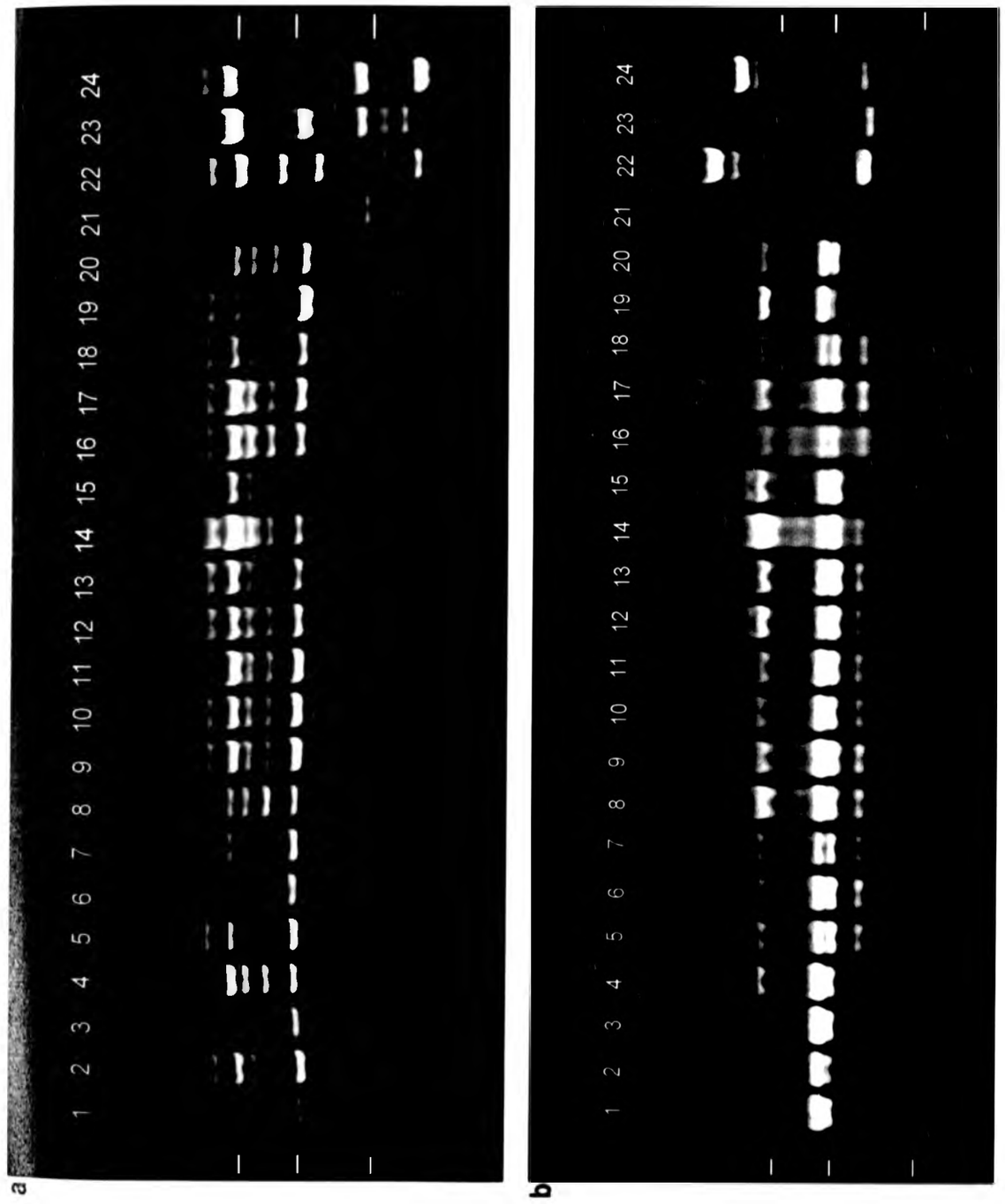
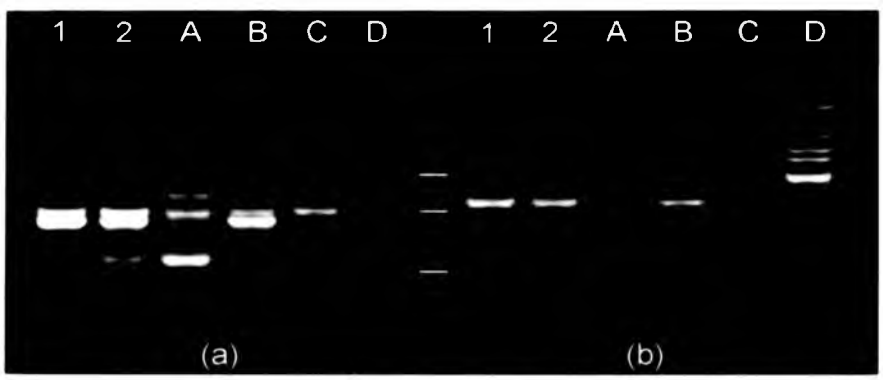


Plate 11.3

RAPD band profiles using primers (a) A10 and (b) B01 (Operon Technologies). Centre markers denote 1200, 800 and 400 base-pairs. Lanes 1 and 2 are UK isolates FDL457 and FDL458. Lanes A, B, C and D are isolates J1, PL, KV and PC respectively, representing *A. astaci* groups A, B, C and D.



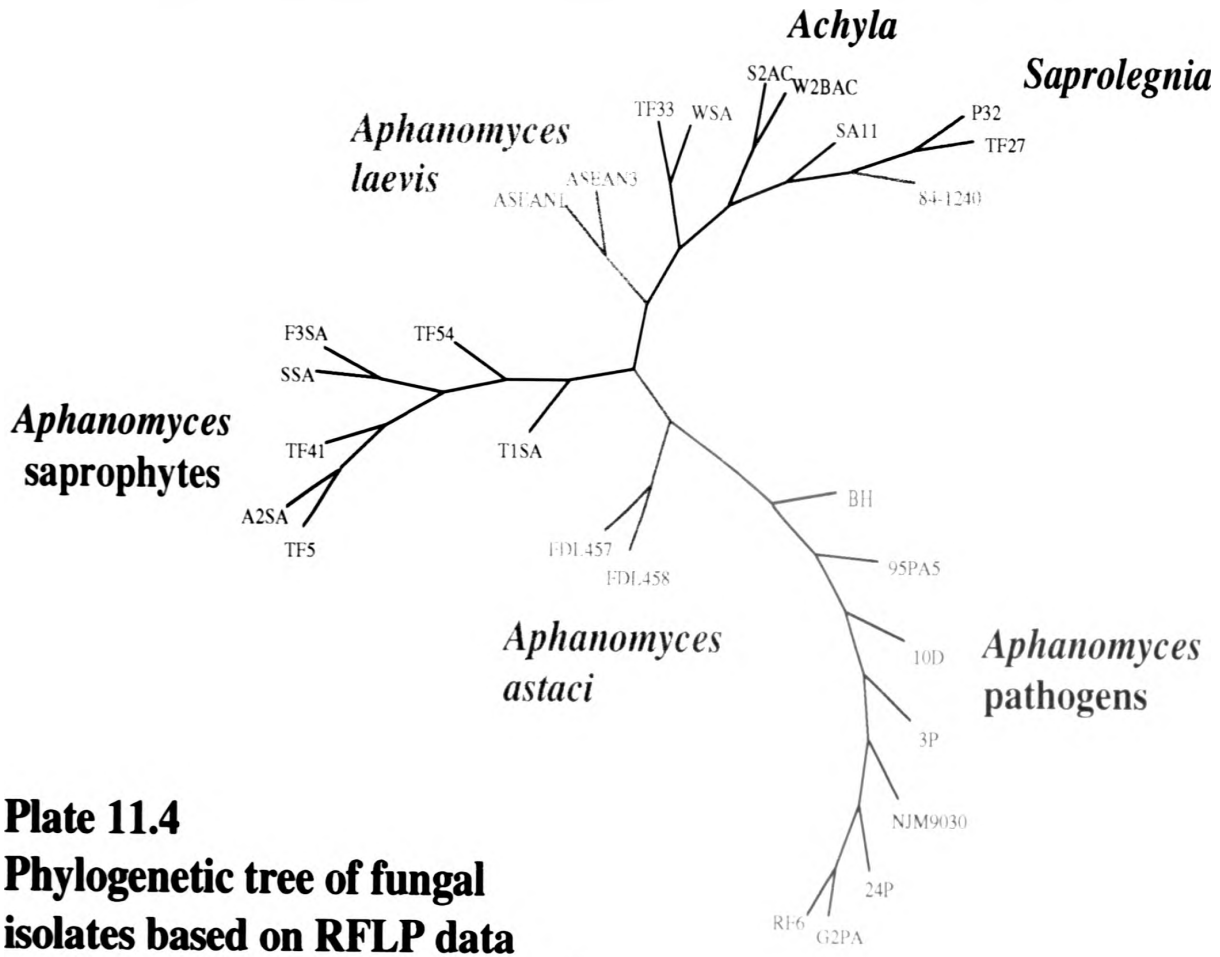


Plate 11.4
Phylogenetic tree of fungal isolates based on RFLP data
(information supplied by D. Hart, Glasgow University)

CHAPTER 12. GENERAL DISCUSSION

12.1 The contribution of the present work towards study objectives

12.1.1 Taxonomical relationships of *Aphanomyces invadans*

Comparative molecular, morphological, biochemical and behavioural studies described here have shown that EUS, MG and RSD pathogenic *Aphanomyces* isolates represent the same fungal species, named *A. invadans* (see below). Any variation in morphology described by different workers (Table 2.1) may be due to culture conditions or observational differences. The differences between *A. invadans* and all other *Aphanomyces* spp compared here indicate that it is not merely a pathogenic *forma specialis* (Holub, Grau and Parke, 1991) or pathotype (Brasier and Rayner, 1987), but a species in its own right. Dendrograms constructed from growth data (Chapter 4), PyMS data (Chapter 10), and RFLP analyses (D. Hart, reprinted in Chapter 11) show that *A. invadans* is most closely related to the crayfish plague fungus, *A. astaci,* out of the fungi tested. Despite its unusual cell wall structure and ability to cross-react with anti-*Phytophthora* monoclonal antibodies (Chapter 7), there is enough evidence to retain *A. invadans* within the genus *Aphanomyces*.

As mentioned in the Introduction (Section 1.1), the EUS *Aphanomyces* pathogen will be listed for the first time in the forthcoming edition of the Index of Fungi [1997, 6(13): 706], published by the International Mycological Institute (IMI) (J. David, pers. comm.). It will be given as *Aphanomyces invadans* Willoughby *et al.* 1995 (as "*invaderis*"). Although the description of *A. invaderis* by Willoughby *et al* (1995) is considered to be validly published, the name is not etymologically correct, and will therefore be changed to *A. invadans*. For this reason, this thesis, and most of the resulting publications, have adopted the name *A. invadans*. Based on evidence described in this thesis, MG and RSD pathogens will be included within the species. As mentioned in Section 2.1.3, *A. piscicida* does not conform to ICBN protocol, principally because any taxon of the rank of family or below requires a Latin description (Korf, 1995) and this was not supplied in Hatai (1980). This name will therefore be mentioned in the Index of Fungi (1997) as an invalid synonym of *A.*

invadans and listed as *Aphanomyces piscicida* Hatai, 1980, *nom. inval.* Art. 36.1. The holotype specimen of *A. invadans* (RF6) is presently being maintained at the Freshwater Biological Association, Windermere, as well as at the Institute of Aquaculture, University of Stirling. When referring to studies on fish disease caused by *A. invadans*, Egusa and Masuda (1971) should be cited as the first report of the disease; Hatai *et al* (1977a) as the first isolation of the fungus; and Willoughby *et al* (1995) as the describers of the species.

Given that the same pathogenic fungus is responsible for EUS, MG and RSD, there is a strong argument for adopting the same name for all of these diseases. Of the names presently used, mycotic granulomatosis (MG), first proposed by Miyazaki and Egusa (1972), is considered the most appropriate as it describes the defining feature of the disease. Although the name EUS is widely known in Southeast and South Asia, it has been used to describe a number of unrelated disease conditions, and adoption of a more specific name may avoid some of the confusion that has resulted. In addition, the description of the disease given by Roberts *et al* (1994a) (See Section 1.1) is not wholly accurate as work in Australia has indicated that outbreaks there do not require a "complex aetiology"; and, although the disease has predominated in tropical and subtropical areas, it is known to be capable of affecting cold water fish like *Tor* sp in the Himalayan valley regions of Nepal and rainbow trout can be experimentally infected (Khan, 1997). Any new name or definition for EUS would, however, have to be deliberated by a consultation of regional experts.

The relationship of EUS to UM remains unclear. The *Aphanomyces* fungus considered by Dykstra *et al* (1986) to be an invasive pathogen of UM, is shown here to be quite unlike *A. invadans*, being more closely related to *A. laevis* (Chapters 10 and 11). In addition, there has been no evidence of its ability to grow in internal tissues of fish (Chapter 3; Noga, 1993a). Faisal and Hargis (1992) considered the inability to reproduce the disease with these isolates to be an indication that some other (bacterial) aetiology is responsible. However, these results cannot be considered to be evidence against a primary fungal

aetiology, until a fungus (or fungi) capable of penetrating internal tissues can be identified. Successful reproduction of typical UM mycotic granulomas by introducing a fungus (or fungi) directly into the tissue (ie by intramuscular injection) should be a priority, before the question of whether another primary biological aetiology is required for infection, can be addressed.

12.1.2 Characterisation of *A. invadans*

The work undertaken for this study purposefully did not focus on any one aspect of the characterisation of *A. invadans*. This is because speciation of asexual saprolegniacean fungi has involved a wide variety of different approaches. Given that very little work has been done on the biological characteristics of *A. invadans*, it was hoped that use of a variety of techniques adopted by previous workers for other saprolegniaceans (eg EM, immunocytochemistry, SDS-PAGE, RAPD-PCR), and novel methodologies for this group of fungi (eg PyMS, lectin histochemistry), would identify the areas that can be focused on in further studies. The use of various fungal growth characteristics on agar plates was also considered an important aspect of the present work as simple cultural techniques would provide the easiest and most rapid means of identifying cultures in affected areas. Barr (1983a) stated that "a simple test such as a response to temperature may be reliable and is usefully applied as an aid for routine identification of certain species of zoosporic fungi".

It is recommended that the diagnosis of candidate *A. invadans* cultures should proceed by initially identifying the typical sporulation characteristics of *Aphanomyces* spp using light microscopy. A preliminary screening for *A. invadans* isolates may be done by measuring growth rates in comparison to values given in Table 4.2. For example, failure to grow at 37°C, and on SDA, CMA and MEA would distinguish *A. invadans* from Asian saprophytic strains. Fungal cultures should be confirmed as *A. invadans* by injecting 100 to 1000 zoospores, in 0.1 ml APW, intramuscularly in EUS-susceptible fish (preferably *Channa striata*) at 20°C and observing the pathology after 1-2 weeks. Investigation of possible strain differences in *A. invadans* can be most easily achieved using DNA fragment banding profiles generated by RAPD-PCR. Molecular primers have been designed by

Hart (pers. comm.) that provide a *A. invadans*-specific probe based on the internally transcribed spacer (ITS) region between the 18S and 5.8S rRNA genes. It is hoped that once this molecular probe has been rigorously tested, it would available for use in the diagnosis of *A. invadans* DNA from fish and water samples using a PCR-based hybridisation technique.

Apart from measuring growth rates on nutrient agar, other culture experiments, chemical susceptibility tests and zoospore behaviour studies revealed specific information about the biology of the *A. invadans*, but are unlikely to provide a useful means of characterising the fungus. With further study, ultrastructural and immunocytochemical observations made here, may provide species-specific taxonomic markers for *A. invadans* (eg the identification of a putative K body with distinctive substructure), but difficulties in processing samples for this work limit the usefulness of these techniques in providing diagnostic features. SDS-PAGE banding profiles were considered too complex for the diagnosis of *A. invadans*, but Western blot analyses with antisera or particular lectins, would be capable of characterising individual isolates as *A. invadans*. The development of monoclonal antibodies would further improve the specificity of Western blots as well as immunocytochemistry. PyMS was shown to be a relatively rapid method for distinguishing a large number of isolates into different species, but the technique lacked sufficient reproducibility, and several reference strains would be always be required for it to be of any value.

A. invadans can be easily identified histologically in fish tissue by its ability to penetrate muscle and visceral tissues, and the associated granulomatous and degenerative changes. User-friendly immunohistochemical stains are an effective means of identifying hyphae, and accompanied by H&E stained tissue sections, provide a useful tool in the diagnosis of *A. invadans* infection. This technique could also be further enhanced by the development of monoclonal antibodies.

12.1.3 The role of *A. invadans* in EUS

This study has clearly established that it is not the case that multiple opportunistic fungal species are responsible for the development of EUS lesions in fish; but rather that the specific fungus, *A. invadans,* is the cause of typical EUS mycotic granulomas. It is apparent, however, that in most cases of EUS, some other environmental (Callinan *et al,* 1996) or biological (Kanchanakhan, 1996b; Subasinghe, 1993) agent is required to cause initial dermatitis in fish, thereby enabling *A. invadans* to penetrate the skin barrier.

The extreme genetic homogeneity between all the *A. invadans* isolates provide strong evidence that this is the agent that has spread across Asia resulting in new outbreaks of EUS, and not, as has been widely believed, some other biological agent that has spread, predisposing fish to infection by a long-term opportunistic fungal resident. The spread of *A. invadans* in some areas can be accounted for by flood events like the serious inundations in Bangladesh in 1988. However, many outbreaks of EUS can only be explained by the massive cross-border movement of fish for the aquaculture or ornamental fish industries. Indeed, one isolate tested here (NJM9201) was obtained in Japan from a shipment of dwarf gouramies imported from Singapore (Hatai *et al,* 1994). Potential dangers extend beyond Asia as trials have demonstrated that *A. invadans* can also produce severe pathological changes in salmonid species (K.D. Thompson, pers. comm.; Bonner, 1997).

As the present work indicates, the unrestricted trade in aquatic animals is already having a major negative impact on aquaculture, fisheries and indigenous aquatic biological diversity through the transmission of disease. Several programmes involving the Food and Agriculture Organisation (FAO) and Office International des Epizooties (OIE) are being undertaken to develop effective regional health certification and quarantine guidelines (Anon, 1996), and this study lends support to the importance and urgency of these initiatives.

Roberts *et al* (1993) (and Noga, 1993a, in the case of UM) have commented that dead fungus is seen in the lesions of fish and sporulating mycelium is never seen, implying that no mechanism of fish to fish transmission could be found and that there may be some environmental source of infections. The infectivity studies described in Chapter 3 do not contradict this hypothesis. However, Callinan (pers. comm.) has shown SEM photographs of artificially infected fish clearly demonstrating the emergence of the fungus through the skin and its subsequent sporulation. This, along with the fact that *A. invadans* has not been isolated from water bodies not experiencing an active EUS outbreak; and the obvious difficulties *A. invadans* would have competing with vigorous saprophytic fungi outside a fish host; all suggest that diseased fish, and not environmental sources, are the main source of infective material for EUS. Clearly further studies on ecological aspects of the fungus are required, and suggestions for such work are given in Section 12.3 below.

12.2 Main conclusions

(i) A single specific fungus, recently named *Aphanomyces invadans,* is responsible for the mycotic granulomas characteristic of epizootic ulcerative syndrome (EUS).

(ii) *A. invadans* represents part of the same clonal lineage as *Aphanomyces* isolates from Australian red spot disease (RSD) and from Japanese mycotic granulomatosis (MG) (also known as *Aphanomyces piscicida*). Given that *A. invadans* has been given a formal published description, this name is adopted here to describe all these fungi. There is therefore a strong argument for adopting the same name for EUS, MG and RSD; and of these, mycotic granulomatosis (MG) is considered the most appropriate.

(iii) Fungal isolates from American ulcerative mycosis (UM), crayfish plague, saprolegniasis and saprophytic infections of aquatic animals are distinct species from *A. invadans* and unable to reproduce EUS. However, there is a possibility that the true UM pathogen is not among the isolates so far characterised.

(iv) *Aphanomyces invadans* is sexually sterile; slow-growing; thermolabile; incapable of growing on SDA, CMA and MEA media; and highly susceptible to several chemical treatments. Zoospores show particular chemotactic behaviour; are capable of limited polyplanetism, even in the presence of nutrient media; and germinate indirectly and have an abbreviated lifecycle in low nutrient backgrounds. Primary zoospore cysts appear to lack an outer layer to the cell wall and have a distinctive K body. Zoospores and cysts have distinctive lectin-binding characteristics and cross-react with a specific anti-*Phytophthora* MAb. *A. invadans* mycelial extracts can be distinguished from other fungi by means of electrophoretic banding profiles and pyrolysis mass spectrometry. Fingerprints generated by random amplification of polymorphic nuclear DNA are very consistent for geographically and chronologically distant *A. invadans* isolates.

(v) Striped snakeheads produce antibodies in response to infection with *A. invadans*. These react with a specific 10kDa band on *A. invadans* mycelial extracts, but it is not known whether they are protective against EUS.

12.3 Recommended future studies

Given that the importance of the fungal aetiology of EUS has only been realised in recent years there are still many avenues of research to pursue:

12.3.1 Treatment of EUS

A priority for further work is clearly to investigate methods of control of this damaging disease. Now that that *Aphanomyces invadans* has been established as the essential aetiological agent for EUS infection, studies can be targeted at treating this fungus, if possible in infected fish, but probably more feasibly, by devising strategic water treatments to prevent infection of fish. There are currently several untested treatments for EUS being recommended. The relative merits, or otherwise, of agricultural, slaked and quick lime need to be assessed. Accepted fungicidal agents are sometimes applied (potassium permanganate, salt, malachite green) but optimal dosages have yet to be determined using pond trials. Other remedies are used in local

circumstances and rarely publicised (eg homeopathic drugs: Mitra and Varshney, 1990; ash: De, 1991; neem leaves and turmeric: Anon, 1994b; banana latex: Sarkar, 1995). These should be evaluated. Fungicides that have been recommended for other Oomycete fungi (eg dinitroaniline herbicides: Bruin and Edgington, 1983; phosphonates: Coffey and Ouimette, 1988) should be assessed for activity against *A. invadans*. Novel antimicrobial treatments that may have some activity against fungi include microalgal extracts (Austin, Baudet and Stobie, 1992), tea tree oil and propolis (Kumar, 1997). Susceptibility to parasitism as a form of biological control is also of interest for possible future development. The ability of particular bacteria (Hatai and Willoughby, 1988; Petersen, Jegstrup and Olson, 1994; Bly, Quiniou, Lawson and Clem, 1997) and fungal parasites (Willoughby and Roberts, 1992b) to kill *Saprolegnia*, has been studied in this regard.

12.3.2 Fungal ecology, physiology and developmental morphology

Very little is presently known about the natural ecology of the fungus: its ability to persist outside a fish host and any other possible habitats or substrates which it can colonise, and with which it can be transmitted. Specifically, it is important to test how long a quarantined EUS-affected pond remains infective without further introduction of *A. invadans*, and how the presence of different potential substrates can affect this.

Various aspects of *A. invadans* zoospore physiology warrant further study with a view to arresting these processes to control fungal dispersal and infection. In particular, investigations are needed of the factors involved in sporulation; the length of time zoospores can remain infective after sporulation; the mechanisms of chemoattraction, encystment (using the lectin WGA in particular), and germination; and the identification of chemical attractants produced by fish.

Studies on the developmental ultrastructure and immunocytochemistry of *A. invadans* zoospores would yield information on possible adaptations for a fish-pathogenic life-style; and use of this information to draw comparisons with other Oomycete fungi

could help solve questions on the phylogenetic relationship of *A. invadans,* and *Aphanomyces* spp in general, to the Peronosporales.

The ECP is considered to be an important aspect *of A. invadans* pathogenicity and work could progress on the characterisation of the enzymes involved; their role in pathogenesis and fungal nutrition; ascertaining whether secretion is induced; and if so, further investigation could be made of the relevant RNA transcripts and gene sequences involved.

12.3.3 Fish immune systems and protective mechanisms involving the skin

Further work is required to establish the feasibility of inducing protective immunity in susceptible fish. The presence of a specific humoral response to the fungus in naturally and artificially infected snakeheads has been demonstrated here, and a passive immunisation experiment would test the ability of serum from such fish to confer immunity in other fish. This would give an indication of the potential for the possible future development of vaccines. The importance of the fishes cellular response in combating infection by *A. invadans* has been shown in histopathological studies of resistant common carp (Wada *et al,* 1996), and by *in vitro* macrophage studies undertaken by Thompson (pers. comm.). The possibility of enhancing cellular defences in susceptible fish by applying glucan or adjuvant preparations requires exploration. Fish mucus also contains substances with antimicrobial activity (Magarinos, Pazos, Santos, Romalde and Toranzo, 1995) and the importance of this mechanism in the resistance of fish to infection should be investigated.

12.3.4 Identification of other pathogens/risk factors

Given that the fishes skin needs to be breached before *A. invadans* can penetrate, other factors are clearly involved in the disease process. Acid water has been identified in some outbreaks in Australia and elsewhere as being capable of inducing infection (Callinan *et al,* 1995b; 1996a), and low temperature is known to affect the fish immune system, allowing the disease to progress fully (Chinabut *et al,* 1995).

However, in the absence of acid water, EUS outbreaks do occur, and there is a possibility that biological agents such as viruses (Kanchanakhan, 1996b) or parasites (Subasinghe, 1993), may have an involvement in the disease process in some areas. Further work is required to evaluate of the importance of these as risk factors in the disease process. Study of the sequential histological changes in the integument of susceptible fish during challenge experiments involving *A. invadans* and other agents capable of inducing dermatitis, would provide information on the interaction between the fish and the pathogen at the time of infection.

A full epidemiological survey of EUS outbreaks in particular areas would give an indication of the relative importance of the great number of factors reportedly involved in the disease process, and identify further risk factors.

12.3.5 Effective diagnosis and monitoring future spread

As indicated in Section 1.5, reports of new ulcerative diseases and mycoses of fish continue to occur. It is known that clonal lineages of other pathogenic Oomycete fungi have established a pan-global distribution (Goodwin *et al,* 1994), and given the recent spread of EUS, it is probable that without effective restrictions on the movement of fish, *A. invadans* will do the same. For such restrictions to be implemented, specific diagnostic techniques should be developed and informed quarantine guidelines drawn up. As knowledge of histological procedures is more widespread and probably more reliable than expertise in fungal isolation and characterisation, the diagnosis of EUS should primarily rely on histology; and the development of a MAb probe specific for *A. invadans* hyphae would provide a valuable immunohistochemical stain for use in this regard. It would also be desirable to use an *A. invadans*-specific probe to establish the true extent of the area colonised by the fungus, so that particular measures can be taken for fish within these areas. Given the extreme similarity in the pathology of UM to EUS, it would be of particular interest to ensure that the invasive fungal pathogen of UM is isolated and compared with *A. invadans*.

REFERENCES

Adams, A. and Marin de Mateo, M. (1994) Immunohistochemical detection of fish pathogens. In: Stolen, J.S., Fletcher T.C., Rowley A.F., Zelikoff, J.T., Kaattari, S.L. and Smith S.A. (eds), Techniques in Fish Immunology-3. Pp. 133-143. SOS Publication, Fair Haven, NJ.

Agersberg, H.P.K. (1933) Salient problems in the artificial rearing of salmonid fishes, with special reference to intestinal fungisitosis and the cause of white spot disease. Transactions of the American Fisheries Society 63, 240-250.

Ahmed, M. and Rab, M.A. (1995) Factors affecting outbreaks of epizootic ulcerative syndrome in farmed fish in Bangladesh. Journal of Fish Diseases 18, 263-271.

Alabi, R.O. (1971) Factors affecting seasonal occurrence of Saprolegniaceae in Nigeria. Transactions of the British Mycological Society 56(2), 289-299.

Alberts, V.A., Khan, S.S., Lim, D.V. and Te Strake D. (1989) Extracellular enzyme activity of some Saprolegniales from a Florida estuary. Mycologia 81(3), 460-463.

Alderman, D.J. (1976) Fungal diseases of marine animals. In: Jones, E.B.G. (ed.), Recent Advances in Aquatic Mycology. Pp. 223-260. Elek Science, London.

Alderman, D.J. (1982a) Fungal disease of aquatic animals. In: Roberts R.J. (ed.), Microbial Diseases of Fish. Pp. 189-242. Academic Press, New York.

Alderman, D.J. (1982b) *In vitro* testing of fisheries chemotherapeutants. Journal of Fish Diseases 5, 113-123.

Alderman, D.J. (1985) Malachite green: a review. Journal of Fish Diseases 8, 289-298.

Alderman, D.J. (1992) Malachite green and its alternatives as therapeutic agents. In: Aquaculture and the Environment. No.16, Pp. 235-244.

Alderman, D.J. (1993) Crayfish plague in Britain, the first twelve years. Freshwater Crayfish 9, 266-272.

Alderman, D.J. (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Revue Scientific et Technique de L'Office International des Epizooties 15(2), 603-632.

Alderman, D.J. and Polglase, J.L. (1984) A comparative investigation of the effects of fungicides on *Saprolegnia parasitica* and *Aphanomyces astaci*. Transactions of the British Mycological Society 83(2), 313-318.

Alderman, D.J. and Polglase, J.L. (1985) Disinfection for crayfish plague. Aquaculture and Fisheries Management 16, 203-205.

Alderman, D.J. and Polglase, J.L. (1986) *Aphanomyces astaci:* isolation and culture. Journal of Fish Diseases 9, 367-379.

Alderman, D.J. and Polglase, J.L. (1988) Pathogens, parasites and commensals. In: Holdich, D.M. and Lowery, R.S. (eds). Freshwater Crayfish. Biology, Management and Exploitation Pp. 167-212. Croom Helm, London.

Alderman, D.J., Polglase, J.L., Frayling, M. and Hogger, J. (1984) Crayfish plague in Britain. Journal of Fish Diseases 7, 401-405.

Alderman, D.J., Polglase, J.L. and Frayling, M. (1987) *Aphanomyces astaci* pathogenicity under laboratory and field conditions. Journal of Fish Diseases 10, 385-393.

Alderman, D.J., Holdich, D. and Reeve, I. (1990) Signal crayfish as vectors in crayfish plague in Britain. Aquaculture 86, 3-6.

Alexander, J.B. and Ingram, G.A. (1992) Noncellular nonspecific defence mechanisms of fish. Annual Review of Fish Diseases 2, 249-279.

Anon. (1994a) Antifungal susceptibility testing of yeasts. E test News 10, 1-4. AB Biodisk, Solna, Sweden.

Anon. (1994b) Ingenious treatment for fish disease. The Hindu, 9 November 1994.

Anon. (1996) Workshop proposes regional strategy for responsible movement and introduction of aquatic organisms. NACA Newsletter, Vol 13, April-June 1996. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Areerat, S. (1990) Control of EUS. Fish Health Section Newsletter, 1(2), 4-5. Asian Fisheries Society, Manila.

Austin, B., Baudet, E. and Stobie, M. (1992) Inhibition of bacterial pathogens by *Tetraselmis suecica*. Journal of Fish Diseases 15, 55-61.

Bailey, T.A. (1983a) Screening fungicides for use in fish culture: evaluation of the agar plug transfer, cellophane transfer, and agar dilution methods. Progressive Fish Culturist 45(1), 24-27.

Bailey, T.A. (1983b) Method for *in vitro* screening of aquatic fungicides. Journal of Fish Diseases 6, 91-100.

Bailey, T.A. (1984) Effects of twenty-five compounds on four species of aquatic fungi (Saprolegniales) pathogenic to fish. Aquaculture 38, 97-104.

Balakhnin, I.A, Dudka, I.A. and Isaeva, N.M. (1990) Testing of fungi on their specific interaction with fish lectins. Mikologiya I Fitopatologiya 24(5), 416-420. (In Russian)

Balasuriya, L.K.S.W. (1994) epizootic ulcerative syndrome in fish in Sri Lanka, country status report. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 39-47. Aquatic Animal Health Research Institute, Bangkok.

Balasuriya, K.S.W., Kulathilake, M. and Subasinghe, R.P. (1990) Preliminary investigations into the experimental transmission of ulcerative disease syndrome in Sri Lanka. Proceedings of the Second Asian Fisheries Forum, Tokyo, Japan, 17-22 April 1990. (ed. by Hirano, R. and Hanyu, I.). Pp. 659-662. Asian Fisheries Society, Manila.

Baqui, M.A. and Chowdhury, M.B.R. (1995) Investigation of bacterial flora in an Indian major carp, *Labeo rohita* cultured in a commercial fish farm of Bangladesh. Abstracts of the Fourth Asian Fisheries Forum, Beijing, China, 16-20 October 1995. P. 37. Asian Fisheries Society, Manila and China Society Fisheries, Beijing.

Barr, D.J.S. (1981) The phylogenetic and taxonomic implications of flagellar rootlet morphology among zoosporic fungi. Biosystems 14(3-4), 359-370.

Barr, D.J.S. (1983a) The zoosporic grouping of plant pathogens - entity or non-entity? In: Buczacki, S.T. (ed.), Zoosporic Plant Pathogens - A Modern Perspective. Pp. 43-83. Academic Press, London.

Barr, D.J.S. (1983b) The genera of phytopathogenic zoosporic fungi. In: Buczacki, S.T. (ed.), Zoosporic Plant Pathogens - A Modern Perspective. Pp. 293-302. Academic Press, London.

Barr, D.J.S., Warwick, S.I. and Desaulniers, N.L. (1996) Isozyme variation, morphology and growth response to temperature in *Pythium ultimum*. Canadian Journal of Botany 74(5), 753-761.

Bartnicki-Garcia, S. (1987) The cell wall: a crucial structure in fungal evolution. In: Rayner, A.D.M., Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 389-404. Cambridge University Press, Cambridge.

Barua, G. (1990) Bangladesh report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 2-4. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Barua, G. (1994) The status of epizootic ulcerative syndrome of fish of Bangladesh. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 13-20. Aquatic Animal Health Research Institute, Bangkok.

Beakes, G.W. (1987) Oomycete phylogeny: ultrastructural perspectives. In: Rayner, A.D.M., Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 405-42. Cambridge University Press, Cambridge.

Beakes, G. and Ford, H. (1983) Esterase isoenzyme variation in the genus *Saprolegnia*, with particular reference to the fish-pathogenic *S. diclina* complex. Journal of General Microbiology 129, 2605-2619.

Beakes, G.W. and Gay, J.L. (1980) Effects of streptomycin on the growth and sporulation of *Saprolegnia* spp. Journal of General Microbiology 119, 361-371.

Beakes, G.W., Wood, S.E. and Burr, A.W. (1994) Features which characterize *Saprolegnia* isolates from salmonid fish lesions - a review. In: Mueller, G.J. (ed.), Salmon Saprolegniasis. Pp. 33-66. Bonneville Power Administration, US Department of Energy, Portland, USA.

Beakes, G.W., Burr, A.W., Wood, S.E. and Hardham, A.R. (1995) The application of spore surface features in defining taxonomic versus ecological groupings in oomycete fungi. Canadian Journal of Botany 73(Suppl. 1), S701-S711.

Beghdadi, A., Richard, C. and Dostaler, D. (1992) L'*Aphanomyces euteiches* des luzernières du Québec: isolement, morphologie et variabilité de la croissance et du pouvoir pathogène. Canadian Journal of Botany 70, 1903-1911. (In French, English abstract)

Berbee, M.L. and Kerwin, J.L. (1993) Ultrastructural and light microscopic localization of carbohydrates and peroxidase catalases in *Lagenidium giganteum* zoospores. Mycologia, 85(5), 734-743.

Bhargava, K.S., Swaup, K. and Singh, C.S. (1971) Fungi parasitic on certain freshwater fishes of Gorakhpur. Indian Biologist 3, 65-69.

Bielenin, A., Jeffers, S.N., Wilcox, W.F. and Jones, A.L. (1988) Separation by protein electrophoresis of six species of *Phytophthora* associated with deciduous fruit crops. Phytopathology 78(11), 1402-1408.

Bimpong, C.E. and Clerk, G.C. (1970) Motility and chemotaxis in zoopsores of *Phytophthora palmivora* (Butl.) Butl. Annals of Botany 34, 617-624.

Bisht, G.S., Bisht, D., Joshi, C. and Khulbe, R.D. (1996) Potential threat to reservoir fishery by fungi in Kumaun Himalaya, India. Current Science 71(9), 720-723.

Bly, J.E. and Clem, L.W. (1992) Temperature and teleost immune functions. Fish & Shellfish Immunology 2, 159-171.

Bly, J.E., Lawson, L.A., Dale, D.J., Szalai, A.J., Durborow, R.M. and Clem, L.W. (1992) Winter saprolegniosis in channel catfish. Diseases of Aquatic Organisms 13, 155-164.

Bly, J.E., Lawson, L.A., Szalai, A.J. and Clem, L.W. (1993) Environmental factors affecting outbreaks of winter saprolegniosis in channel catfish, *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases 16(6), 541-549.

Bly, J.E., Quiniou, M.A., Lawson, L.A. and Clem, L.W. (1996) Therapeutic and prophylactic measures for winter saprolegniosis in channel catfish. Diseases of Aquatic Organisms 24, 25-33.

Bly, J.E., Quiniou, S.M.A., Lawson, L.A. and Clem, L.W. (1997) Inhibition of *Saprolegnia* pathogenic for fish by *Pseudomonas fluorescens*. Journal of Fish Diseases 20, 35-40.

Bondad-Reantaso, M.G., Paclibare, J.O., Lumanlan-Mayo, S.C. and Catap, E.S. (1994) EUS outbreak in the Philippines: a country report. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 61-67. Aquatic Animal Health Research Institute, Bangkok.

Bonner, J. (1997) Fish flesh and foul. The Guardian, Online. 18 June 1997. P.7.

Boonyaratpalin, S. (1989) Bacterial pathogens involved in the epizootic ulcerative of fish in southeast Asia. Journal of Aquatic Animal Health 1, 272-276.

Bootsma, R. (1973) Infections with *Saprolegnia* in pike culture. Aquaculture 2, 385-394.

Brasier, C.M. and Rayner, D.M (1987) Whither terminology below the species level in the fungi? In: Rayner, A.D.M, Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 379-388. Cambridge University Press, Cambridge.

Brasier, C.M., Hamm, P.B. and Hansen, E.M. (1993) Cultural characters, protein patterns and unusual mating behaviour of *Phytophthora gonapodyides* isolates from Britain and North America. Mycological Research 97(11), 1287-1298.

Briard, M., Dutertre, M., Rouxel, F. and Brygoo, Y. (1995) Ribosomal RNA sequence divergence within the Pythiaceae. Mycological Research 99(9), 1119-1127.

Brown, D.F.J. and Brown, L. (1991) Evaluation of the E test, a novel method of quantifying antimicrobial activity. Journal of Antimicrobial Chemotherapy 27, 185-190.

Bruin, G.C.A. and Edgington, L.V. (1983) The chemical control of diseases caused by zoosporic fungi - a many-sided problem. In: Buczacki, S.T. (ed.), Zoosporic Plant Pathogens - A Modern Perspective. Pp. 193-232. Academic Press, London.

Bruno, D.W. and Stamps, D.J. (1987) Saprolegniasis of Atlantic salmon, *Salmo salar* L., fry. Journal of Fish Diseases 10, 513-517.

Bullis, R.A., Noga, E.J. and Levy, M.G. (1990) Immunological relationship of the fish-pathogenic Oomycete *Saprolegnia parasitica* to other Oomycetes and unrelated fungi. Journal of Aquatic Animal Health 2, 223-227.

Bullis, R.A., Noga, E.J. and Levy, M.G. (1996) Production and preliminary characterization of monoclonal antibodies to *Saprolegnia parasitica*. Mycological Research 100(4), 489-494.

Burke, J.B. and Rodgers, L.J. (1981) Identification of pathogenic bacteria associated with the occurence of red spot in sea mullet, *Mugil cephalus* L., in south-eastern Queensland. Journal of Fish Diseases 4, 153-159.

Burkholder, J.M., Noga, E.J., Hobbs, C.H. and Glasgow Jr., H.B. (1992) New phantom dinoflagellate is the caustive agent of major estuarine fish kills. Nature 358, 407-410.

Burr, A.W. (1991). Comparative diplanetic processes of salmonid-pathogenic and saprophytic isolates of the *Saprolegnia parasitica-diclina* complex. PhD thesis, University of Newcastle upon Tyne, 381 pp.

Burr, A.W. and Beakes, G.W. (1994) Characterization of zoospore and cyst surface structure in saprophytic and fish pathogenic *Saprolegnia* species (oomycete fungal protists). Protoplasma 181, 142-163.

Byrt, A., Irving, H.R. and Grant, B.R. (1982a) The effect of organic compounds on the encystment, viability and germination of zoospores of the fungus *Phytophthora cinnamomi*. Journal of General Microbiology 128, 2343-2351.

Byrt, A., Irving, H.R. and Grant, B.R. (1982b) The effect of cations on zoospores of the fungus *Phytophthora cinnamomi*. Journal of General Microbiology 128, 1189-1198.

Cahill, D.M and Hardham, A.R. (1994a) Exploitation of zoospore taxis in the development of a novel dipstick immunoassay for the specific detection of *Phytophthora cinnamomi*. Phytopathology 84, 193-200.

Cahill, D.M and Hardham, A.R. (1994b) A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. Phytopathology 84(11), 1284-1292.

Callinan, R.B. (1994a) Red spot disease - EUS in Australia. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 82-88. Aquatic Animal Health Research Institute, Bangkok.

Callinan, R.B. (1994b) A comparative review of *Aphanomyces* species associated with epizootic ulcerative syndrome, red spot disease and mycotic granulomatosis. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 248-252. Aquatic Animal Health Research Institute, Bangkok.

Callinan, R.B. and Keep, J.A. (1989) Bacteriology and parasitology of red spot disease in sea mullet, *Mugil cephalus* L., from eastern Australia. Journal of Fish Diseases 12, 349-356.

Callinan, R.B., Fraser, G.C. and Virgona, J.L. (1989) Pathology of red spot disease in sea mullet, *Mugil cephalus* L., from eastern Australia. Journal of Fish Diseases 12, 467-479.

Callinan, R.B., Paclibare, J.O., Bondad-Reantaso, M.G., Chin, J.C. and Gogolewski, R.P. (1995a) *Aphanomyces* species associated with epizootic ulcerative syndrome (EUS) in the Philippines and red spot disease (RSD) in Australia: preliminary comparative studies. Diseases of Aquatic Organisms 21, 233-238.

Callinan, R.B., Paclibare, J.O., Reantaso, M.B., Lumanlan-Mayo, S.C., Fraser, G.C. and Sammut, J. (1995b) EUS outbreaks in estuarine fish in Australia and the Philippines: associations with acid sulphate soils, rainfall and *Aphanomyces*. Diseases in Asian Aquaculture II. (ed. by Shariff, M.,

Arthur, J.R. and Subasinghe, R.P.). Pp. 291-298. Fish Health Section, Asian Fisheries Society, Manila.

Callinan, R.B., Sammut, J. and Fraser G.C. (1996a) Epizootic ulcerative syndrome (red spot disease) in estuarine fish - confirmation that exposure to acid sulfate soil runoff and an invasive aquatic fungus, *Aphanomyces* sp., are causative factors. Proceedings of the Second National Conference on Acid Sulfate Soils. Pp. 146-151. Roberts J Smith and Associates and ASSMAC, Australia.

Callinan, R.B., Lumanlan-Mayo, S.C., Rukyani, A. and Mohan, C.V. (1996b) Outcomes of a collaboratives research project on EUS in Australia, Indonesia, the Philippines and India 1992-1995. World Aquaculture 1996 Book of Abstracts, Bangkok, 29 January - 2 February 1996. Pp. 60-61. The World Aquaculture Society.

Callinan, R.B., Chinabut, S., Kanchanakhan, S., Lilley, J.H. and Phillips, M.J. (1997) , Epizootic ulcerative syndrome (EUS) of fishes in Pakistan. A report of the findings of a mission to Pakistan. 9-19 March 1997. Prepared by collaboration between ACIAR, AAHRI, NACA, ODA, NSW-Fisheries and Stirling University. 59 pp.

Cameron, J.N. and Carlile, M.J. (1977) Negative geotaxis of zoospores of the fungus *Phytophthora*. Journal of General Microbiology 98, 599-602.

Carlile, M.J. (1996) The discovery of fungal sex hormones: II antheridiol. Mycologist 10(3), 113-117.

Cavalier-Smith, T. (1987) The origin of Fungi and pseudofungi. In: Rayner, A.D.M., Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 339-353. Cambridge University Press, Cambridge.

Cerenius, L. and Söderhäll, K. (1980) Asexual reproduction in *Aphanomyces astaci*. Abstract of the Second Nordic Symposium on Mycology, University of Uppsala, Sweden, Pp. 4-5.

Cerenius, L. and Söderhäll, K. (1984a) Chemotaxis in *Aphanomyces astaci*, an arthropod-parasitic fungus. Journal of Invertebrate Pathology 43, 278-281.

Cerenius, L. and Söderhäll, K. (1984b) Repeated zoospore emergence from isolated spore cysts of *Aphanomyces astaci*. Experimental Mycology 8, 370-377.

Cerenius, L. and Söderhäll, K. (1985) Repeated zoospore emergence as a possible adaptation to parasitism in *Aphanomyces*. Experimental Mycology 9, 259-263.

Cerenius, L., Olson, L.W., Lange, L. and Söderhäll, K. (1984) The secondary zoospore of *Aphanomyces astaci* and *A. laevis* (Oomycetes, Saprolegniales). Nordic Journal of Botany 4, 697-706.

Cerenius, L., Söderhäll, K., Persson, M. and Ajaxon, R. (1988) The crayfish plague fungus *Aphanomyces astaci*. Diagnosis, isolation and pathobiology. Freshwater Crayfish 7, 131-144.

Chakrabarty, A.N. and Dastidar, S.G. (1991) Repeated isolation of chemoautotrophic nocardioform bacteria from fish epizootic ulcerative syndrome. Indian Journal of Experimental Biology 29, 623-627.

Chang, T.T., Yang, X.Y. and Ko, W.H. (1992) A sensitive method for detecting production of extracellular enzymes by fungi on solid media. Mycologia 84(6), 923-926.

Chen, W. and Hoy, J.W. (1993) Molecular and morphological comparison of *Pythium arrhenomanes* and *P. graminicola*. Mycological Research 97(11), 1371-1378.

Chen, W., Hoy, J.W. and Schneider, W. (1991) Comparisons of soluble proteins and isozymes for seven *Pythium* species and applications of the biochemical data to *Pythium* systematics. Mycological Research 95(5), 548-555.

Chinabut , S. and Lilley, J.H. (1992) The distribution of EUS lesions on infected snakehead fish, *Channa striatus* Bloch. The AAHRI Newsletter 1(2), 1-2. Aquatic Animal Health Research Institute, Bangkok.

Chinabut, S. (1990) The histopathology of EUS in Asia. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). P. 75. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Chinabut, S. (1993) Malachite green - a therapeutic chemical. The AAHRI Newsletter, 2(2), 1. Aquatic Animal Health Research Institute, Bangkok.

Chinabut, S. (1995) Epizootic ulcerative syndrome: the current state of knowledge. In: Shariff, M., Arthur, J.R. and Subasinghe, R.P. (eds), Diseases in Asian Aquaculture II. Pp. 285-290. Fish Health Section, Asian Fisheries Society, Manila.

Chinabut, S., Roberts, R.J., Willoughby, L.G. and Pearson, M.D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. Journal of Fish Diseases 18, 41-47.

Chondar, S.L. and Rao, P.S. (1996) Epizootic ulcerative syndrome disease to fish and its control: a review. World Aquaculture 1996, Book of Abstracts. Bangkok, 29 January - 2 February 1996. P. 77. World Aquaculture Society.

Chulalongkorn University (1983) The Symposium on Fresh Water Fishes Epidemic: 1982-1983. 23-24 June 1983. Chulalongkorn University, Bangkok.

Chulalongkorn University (1985) Proceedings of the Technical Conference on Living Aquatic Resources. 7-8 March 1985. Chulalongkorn University, Bangkok.

Chulalongkorn University (1987) Proceedings of the Second Seminar on Living Aquatic Resources. 17-18 December 1987. Chulalongkorn University, Bangkok.

Clark, G. (1981) Staining Procedures. 4th Ed., Williams and Wilkins, Baltimore.

Clark, S. (1991) Snakeheads. The species and their distribution. Aquarist and Pondkeeper 56(8), 17-22.

Coates, D., Nunn, M.J. and Uwate, K.R. (1989) Epizootic Ulcerative Disease of freshwater fish in Papua New Guinea. Science in New Guinea 15, 1-11.

Coffey, M.D. and Bower, L.A. (1984) *In vitro* variability among isolates of eight *Phytophthora* species in response to phosphorous acid. Phytopathology 74, 738-742.

Coffey, M.D. and Ouimette, D.G. (1989) Phosphonates: antifungal compounds against Oomycetes. In: Boddy, L., Marchant, R. and Read, D.J. (eds). Nitrogen, Phosphorus and Sulphur Utilization by Fungi. Pp. 107-129. Cambridge University Press, Cambridge.

Coker, W.C. (1923) The Saprolegniaceae. 1969 Reprint. The University of North Carolina Press, Verlag Von J. Cramer, Lehre, Germany. 201 pp.

Cooke, D.E.L. and Duncan, J.M. (1997) Phylogenetic analysis of *Phytophthora* secies based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycological Research 101(6), 667-677.

Cooke, D.E.L., Kennedy, D.M, Guy, D.C., Unkles, S.E. and Duncan, J.M. (1996) Relatedness of group I species of *Phytophthora* as assessed by randomly amplified polymorphic DNA (RAPDs) and sequences of ribosomal DNA. Mycological Research 100(3), 297-303.

Cope, M., Webb, M.C., O'Gara, E.T., Philip, B.A. and Hardham, A.R. (1996) Immunocytochemical comparison of peripheral vesicles in zoospores of *Phytophthora* and *Pythium* species. Mycologia 88(4), 523-532.

Costa, H.H. and Wijeyaratne, M.J.S. (1989) Epidemiology of epizootic ulcerative syndrome occurring for the first time among fish in Sri Lanka. Journal of Applied Ichthyology 1, 48-52.

Crawford, A.R., Bassam, B.J., Drenth, A., Maclean, D.J. and Irwin, J.A.G. (1996) Evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis. Mycological Research 100(4), 437-443.

Cross, M.L. and Willoughby, L.G. (1989) Enhanced vulnerability of rainbow trout *(Salmo gairdneri)* to *Saprolegnia* infection, following treatment of the fish with an androgen. Mycological Research 93(3), 379-402.

Cruz-Lacierda, E.R. and Shariff, M. (1995) Experimental transmission of epizootic ulcerative syndrome (EUS) in snakehead *(Ophicephalus striatus).* In: Shariff, M., Arthur, J.R. and Subasinghe, R.P. (eds), Diseases in Asian Aquaculture II. Pp. 327-336. Fish Health Section, Asian Fisheries Society, Manila.

Cutter Jr, V.M. (1941) Observations on certain species of *Aphanomyces.* Mycologia 33, 220-240.

Das, M.K. (1994) Outbreak of the fish disease, epizootic ulcerative syndrome in India - an overview. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp.21-38. Aquatic Animal Health Research Institute, Bangkok.

Das, M.K. and Das, R.K. (1993) A review of the fish disease epizootic ulcerative syndrome in India. Environment and Ecology 11(1), 134-145.

Dastidar, S.G. and Chakrabarty, A.N. (1992) Bacteriological and histopathological studies on epizootic ulcerative syndrome in fish. Journal of Aquaculture in the Tropics 7, 85-90.

Davis, H.S. and Lazar, E.C. (1941) A new fungus disease of trout. Transactions of the American Fisheries Society 70, 264-271.

De, K.C. (1991) Ash cures UDS. Fishing Chimes 11(4), 31, 36.

Deacon, J.W. and Donaldson, S.P. (1993) Molecular recognition in the homing responses of zoosporic fungi, with special reference to *Pythium* and *Phytophthora.* Mycological Research 97(10), 1153-1171.

Dice, L.R. (1945) Measures of the amount of ecologic association between species. Ecology 26(3), 297-302.

Dick, M.W. (1973) Saprolegniales. In: Ainsworth, G.C., Sparrow, F.K. and Sussman, A.S. (eds). The Fungi. An Advanced Treatise. Vol. IVB. Academic Press, New York.

Dick, M.W. (1997) Fungi, flagella and phylogeny. Mycological Research 101(4), 385-394.

Diéguez-Uribeondo, J., Cerenius, L. and Söderhäll, K. (1994) Repeated zoospore emergence in *Saprolegnia parasitica*. Mycological Research 98(7), 810-815.

Diéguez-Uribeondo, J., Huang, T.-S., Cerenius, L. and Söderhäll, K. (1995) Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. Mycological Research 99(5), 574-578.

Diéguez-Uribeondo, J., Cerenius, L. and Söderhäll, K. (1996) Physiological characterization of *Saprolegnia parasitica* isolates from brown trout. Aquaculture 140, 247-257.

Durán, A., Rodríguez Aparicio, L.B., Reglero, A. and Pérez Díaz, J. (1987) Changes in serum enzymes of *Saprolegnia*-infected brown trout, *Salmo trutta* L. Journal of Fish Diseases 10, 505-507.

Durso, L., Lehnen Jr, L.P. and Powell, M.J. (1993) Characteristics of extracellular adhesions produced during *Saprolegnia ferax* secondary zoospore encystment and cystospore germination. Mycologia 85(5), 744-755.

Dykstra, M.J., Noga, E.J., Levine, J.F., Moye, D.W. and Hawkins, J.H. (1986) Characterization of the *Aphanomyces* species involved with ulcerative mycosis (UM) in menhaden. Mycologia 78(4), 664-672.

Dykstra, M.J., Levine, J.F., Noga, E.J., Hawkins, J.H., Gerdes, P., Hargis Jr, W.J., Grier, H.J. and Te Strake, D. (1989) Ulcerative mycosis: a serious menhaden disease of the southeastern coastal fisheries of the United States. Journal of Fish Diseases 12, 175-178.

Egusa, S. (1992) Mycotic granulomatosis. In: Infectious Diseases of Fish. Pp. 392-396. Oxonian Press Pvt. Ltd., New Delhi.

Egusa, S. and Masuda, N. (1971) A new fungal disease of *Plecoglossus altivelis*. Fish Pathology 6, 41-46.

Ellis, A.E. (1987) Inhibition of *Aeromonas salmonicida* extracellular protease by α2 macroglobulin in the serum of rainbow trout. Microbial Pathogenesis 3, 167-177.

Ellis, A.E. and Stapleton, K.J. (1988) Differential susceptibility of salmonid fishes to furunculosis correlates with differential serum enhancement of *Aeromonas salmonicida* extracellular protease activity. Microbial Pathogenesis 4, 299-304.

Elhissy, F.T., Moharram, A.M., Elzayat, S.A. and Massoud, M.S. (1996) Aquatic phycomycetes recovered from Aswan-High Dam Lake (AHDL). Microbiological Research 151(2), 149-156.

El-Sharouny, H.M. and Badran, R.A.M. (1995) Experimental transmission and pathogenicity of some zoosporic fungi to tilapia fish. Mycopathologia 132, 95-103.

Erselius, L.J. and De Vallavieille, C. (1984) Variation in protein profiles of *Phytophthora:* comparison of six species. Transactions of the British Mycological Society 83(3), 463-472.

Estrada-Garcia, M.T., Green, J.R., Booth, J.M., White, J.G. and Callow, J.A. (1989) Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens. Experimental Mycology 13, 348-355.

Faisal, M. and Hargis Jr, W.J. (1992) Augmentation of mitogen-induced lymphocyte proliferation in Atlantic menhaden, *Brevoortia tyrannus,* with ulcer disease syndrome. Fish & Shellfish Immunology 2, 33-42.

FAO (1986) Report of the expert consultation on ulcerative fish diseases in the Asia-Pacific region. (TCP/RAS/4508). Bangkok. 5-9 August 1986. FAO, Regional Office for Asia and the Pacific, Bangkok.

Fegan, M., Manners, J.M., MacLean, D.J., Irwin, J.A.G., Samuels, K.D.Z., Holdom, D.G. and Li, D.P. (1993) Random amplified polymorphic DNA markers reveal a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*. Journal of General Microbiology 139, 2075-2081.

Fitzpatrick, M.S., Schreck, C.B. and Chitwood, R.L. (1995) Evaluation of three candidate fungicides for treatment of adult spring chinook salmon. The Progressive Fish-Culturist 57, 153-155.

Fjalestad, K.T., Gjedrem, T. and Gjerde, B. (1993) Genetic improvement of disease resistance in fish - an overview. Aquaculture 111(1-4), 65-74.

Foster, L.M., Kozak, K.R., Loftus, M.G., Stevens, J.J. and Ross, I.K. (1993) The polymerase chain reaction and its application to filamentous fungi. Mycological Research 97(7), 769-781.

Fowles, B. (1976) Factors affecting growth and reproduction in selected species of *Aphanomyces*. Mycologia 68, 1221-1232.

Fraser, G. and Callinan, R. (1996) A technique for enumeration of propagules of the epizootic ulcerative syndrome fungus in pondwater. World Aquaculture 1996 Book of Abstracts, Bangkok, 29 January - 2 February 1996. P. 131. The World Aquaculture Society.

Fraser, G.C., Callinan, R.B. and Calder, L.M. (1992) *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. Journal of Fish Diseases 15, 173-181.

Frerichs, G.N. (1995) Viruses associated with the epizootic ulcerative syndrome (EUS) of fish in South-East Asia. Veterinary Research 26(5-6), 449-454.

Frerichs, G.N., Millar, S.D. and Roberts, R.J. (1986) Ulcerative rhabdovirus in fish in Southeast Asia. Nature 322(6076), 216.

Frerichs, G.N., Millar, S.D. and Chinabut, S. (1993) Clinical response of snakeheads *(Ophicephalus striatus)* to experimental infection with snakehead fish rhabdovirus and snakehead cell line retrovirus. Aquaculture 116, 297-301.

Goodwin, S.B., Cohen, B.A. and Fry, W.E. (1994) Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. Proceedings of the National Academy of Sciences of the USA 91, 11591-11595.

Goodwin, S.B., Sujkowski, L.S. and Fry, W.E. (1995) Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. Genetics 85(6), 669-676.

Grant, B.R., Irving, H.R. and Radda, M. (1985) The effect of pectin and related compounds on encystment and germination of *Phytophthora palmivora*. Journal of General Microbiology 131, 382-385.

Grant, B.R., Griffith, J.M. and Irving, H.R. (1986) A model to explain ion-induced differentiation in zoospores of of *Phytophthora palmivora*. Experimental Mycology 10, 89-98.

Griffin, M.J. and Coley-Smith, J.R. (1971) Some effects of streptomycin on *Pseudoperonospora humuli*, the downy mildew of hop. Journal of General Microbiology 69, 117-134.

Grocott, R.G. (1955) A stain for fungi in tissue sections and smears. American Journal of Clinical Pathology 25, 975-979.

Guizhen, J. (1990) Peoples Republic of China report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 11-15. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Gunderson, J.H., Elwood, H., Ingold, A., Kindle, K. and Sogin, M.L. (1987) Phylogenetic relationships between chlorophytes, chrysophytes, and oomycetes. Proceedings of the National Academy of Science of the USA 84, 5823-5827.

Haines, A.K. (1983) Fish fauna and ecology. In: Petr, T. (ed.), The Purari tropical environment of high rainfall river basin. Pp. 367-384. Dr W. Junk.

Hajek, A.E., Hodge, K.T., Liebherr, J.K., Day, W.H. and Vandenburg, J.D. (1996) Use of RAPD analysis to trace the origin of the weevil pathogen *Zoophthora phytonomi* in North America. Mycological Research 100(3), 349-355.

Hall, L. and Unestam, T. (1980) The effect of fungicides on survival of the crayfish plague fungus, *Aphanomyces astaci,* Oomycetes, growing on fish scales. Mycopathologia 72(3), 131-134.

Hankin, L. and Anagnostakis, S.L. (1975) The use of solid media for detection of enzyme production by fungi. Mycologia 67, 597-607.

Hansen, E.M. (1987) Speciation in *Phytophthora:* evidence from the *Phytophthora megasperma* complex. In: Rayner, A.D.M., Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 325-337. Cambridge University Press, Cambridge.

Hansen, E.M., Brasier, C.M., Shaw, D.S. and Hamm, P.B. (1986) The taxonomic structure of *Phytophthora megasperma:* evidence for emerging biological species groups. Transactions of the British Mycological Society 87(4), 557-573.

Hantula, J., Dusabenyagasani, M. and Hamelin, R.C. (1996) Random amplified microsatellite (RAMS) - a novel method for characterizing genetic variation within fungi. European Journal of Forest Pathology 26, 159-166.

Hantula, J., Lilja, A. and Parikka, P. (1997) Genetic variation and host specificity of *Phytophthora cactorum* isolated in Europe. Mycological Research 101(5), 565-572.

Hardham, A.R. (1989) Lectin and antibody labelling of surface components of spores of *Phytophthora cinnamomi.* Australian Journal of Plant Physiology 16, 19-32.

Hardham, A.R. (1995) Polarity of vesicle distribution in oomycete zoospores: development of polarity and importance for infection. Canadian Journal of Botany. 73(Suppl.1), S400-S407.

Hardham, A.R. and Suzaki, E. (1986) Encystment of zoospores of the fungus, *Phytophthora cinnamomi,* is induced by specific lectin and monoclonal antibody binding to the cell surface. Protoplasma 133, 165-173.

Hardham, A.R., Suzaki, E. and Perkin, J.L. (1986) Monoclonal antibodies to isolate-, species- and genus -specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi* . Candian Journal of Botany 64, 311-321.

Hardham, A.R., Gubler, F., Duniec, J. and Elliott, J. (1991) A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. Journal of Microscopy 162, 305-318.

Hardham, A.R., Cahill, D.M, Cope, M., Gabor, B.K., Gubler, F. and Hyde, G.J. (1994) Cell surface antigens of *Phytophthora* spores: biological and taxonomic characterization. Protoplasma 181, 213-232.

Hatai, K. (1980a) Studies on pathogenic agents of saprolegniasis in fresh water fishes. Special Report of Nagasaki Prefectural Institute of Fisheries No. 8, Matsugae-cho, Nagasaki, Japan. (In Japanese).

Hatai, K. (1980b) Saprolegniasis in salmonids. Fish Pathology 14(4), 199-206.

Hatai, K. (1989) Fungal pathogens/parasites of aquatic animals. In: B. Austin and D.A. Austin (eds) Methods for the Microbiological Examination of Fish and Shellfish. Pp. 240-272. Ellis Horwood Limited, Chichester.

Hatai, K. (1994) Mycotic granulomatosis in ayu *(Plecoglossus altivelis)* due to *Aphanomyces piscicida.* Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 101-108. Aquatic Animal Health Research Institute, Bangkok.

Hatai, K. and Egusa, S. (1977) Studies on visceral mycosis of salmonids fry - II. Characteristics of fungi isolated from the abdominal cavity of amago salmon fry. Fish Pathology 11, 187-193. (In Japanese)

Hatai, K. and Egusa, S. (1978) Studies on the pathogenic fungus of mycotic granulomatosis - II. Some of the note on the MG-fungus. Fish Pathology 13(2), 85-89. (In Japanese, English abstract)

Hatai, K. and Egusa, S. (1979) Studies on the pathogenic fungus of mycotic granulomatosis - III. Development of the medium for the MG-fungus. Fish Pathology 13(3), 147-152. (In Japanese, English abstract)

Hatai, K. and Hoshiai, G. (1992a) Mass mortality in cultured coho salmon *(Oncorhynchus kisutch)* due to *Saprolegnia parasitica* Coker. Journal of Wildlife Diseases 28(4), 532-536.

Hatai, K. and Hoshiai, G.-I. (1992b) Saprolegniasis in cultured coho salmon *(Oncorhynchus kisutch)*. Fish Pathology 27(4), 233-234.

Hatai, K. and Hoshiai, G.-I. (1993) Characteristics of two *Saprolegnia* species isolated from coho salmon with saprolegniasis. Journal of Aquatic Animal Health 5, 115-118.

Hatai, K. and Willoughby, L.G. (1988) *Saprolegnia parasitica* from rainbow trout inhibited by a bacterium, *Pseudomonas fluorescens*. Bulletin of the European Association of Fish Pathologists 8, 27-29.

Hatai, K., Egusa, S., Takahashi, S. and Ooe, K. (1977a) Study on the pathogenic fungus of mycotic granulomatosis - I. Isolation and pathogenicity of the fungus from cultured ayu infected with the disease. Fish Pathology 11(2), 129-133. (In Japanese, English abstract)

Hatai, K., Egusa, S. and Awakura, T. (1977b) *Saprolegnia shikotsuensis* sp. nov., isolated from koka-nee salmon associated with fish saprolegniasis. Fish Pathology 12, 105-110.

Hatai, K., Egusa, S. and Nomura, T. (1977c) *Saprolegnia australis* Elliott isolated from body surface lesions of rainbow trout fingerlings. Fish Pathology 11, 201-206.

Hatai, K., Willoughby, L.G. and Beakes, G.W. (1990) Some characteristics of *Saprolegnia* obtained from fish hatcheries in Japan. Mycological Research 94(2), 182-190.

Hatai, K., Nakamura, K., Rha, S.A., Yuasa, K. and Wada, S. (1994) *Aphanomyces* infection in dwarf gourami *(Colisa lalia)*. Fish Pathology 29(2), 95-99.

Hearth, J.H. and Padgett, D.E. (1990) Salinity tolerance of an *Aphanomyces* isolate (oomycetes) and its possible relationship to ulcerative mycosis (UM) of Atlantic menhaden. Mycologia 82(3), 364-369.

Heath, M.C. (1987) Evolution of parasitism in the fungi. In: Rayner, A.D.M., Brasier, C.M. and Moore, D. (eds), Evolutionary Biology of the Fungi. Pp. 149-160. Cambridge University Press, Cambridge.

Heath, I.B. and Greenwood, A.D. (1971) Ultrastructural observations on the kinetosomes, and golgi bodies during the asexual life cycle of *Saprolegnia*. Zeitung Fur Zellforschung 112, 371-389.

Heinäaho, M. (1996). Identification of *Saprolegnia* sp. strains in salmon, *Salmo salar* and brown trout, *Salmo trutta*. BSc project thesis, University of Uppsala, Sweden, 20 pp.

Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. Journal of Bacteriology 154(1), 269-277.

Hjelmeland, K., Christie, M. and Raa, J. (1983) Skin mucus protease from rainbow trout, *Salmo gairdneri* Richardson, and its biological significance. Journal of Fish Biology 23, 13-22.

Hoch, H.C. and Mitchell, J.E. (1972a) The ultrastructure of zoospores of *Aphanomyces euteiches* and of their encystment and subsequent germination. Protoplasma 75, 113-138.

Hoch, H.C. and Mitchell, J.E. (1972b) The ultrastructure of *Aphanomyces euteiches* during asexual spore formation. Phytopathology 62, 149-160.

Hodge, K.T., Sawyer, A.J. and Humber, R.A. (1995) RAPD-PCR for identification of *Zoophthora radicans* isolates in biological control of the potato leafhopper. Journal of Invertebrate Pathology 65(1), 1-9.

Hodkinson, M. and Hunter, A. (1970) Immune response of UDN-infected salmon to *Saprolegnia*. Journal of Fish Biology 2, 305-311.

Hodkinson, M. and Hunter, A. (1971) The effects of culture media on *Saprolegnia* antigens. Mycopathologia et Mycologia applicata 43(3-4), 347-353.

Holloway, S.A. and Heath, I.B. (1977) An ultrastructural analysis of the changes in organelle arrangement and structure between the various spore types of *Saprolegnia*. Canadian Journal of Botany 55, 1328-1339.

Holub, E.B., Grau, C.R. and Parke, J.L. (1991) Evaluation of the *forma specialis* concept in *Aphanomyces euteiches*. Mycological Research 95(2), 147-157.

Hoshina, T., Sano, T. and Sunayama, M. (1960) Studies on the saprolegniasis of eel. J. Tokyo Univ. Fish. 47, 59-79.

Hossain, M.S., Alam, M. and Mazid, M.A. (1992) Survey on fish disease epizootic ulcerative syndrome in Chandpur district. Bangladesh Journal of Training and Development 5(1), 55-61.

Hotchkiss, R.D. (1948) A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Archives of Biochemistry 16, 131-141.

Huang, T.-S., Cerenius, L. and Söderhäll, K. (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci,* by random amplification of polymorphic DNA. Aquaculture 126, 1-10.

Hughes, G.C. (1994) Saprolegniasis: then and now: a retrospective. In: Mueller, G.J. (ed.), Salmon Saprolegniasis. Pp. 3-32. Bonneville Power Administration, US Department of Energy, Portland, USA.

ICSF (1992) Enigma of EUS. Consultation on Epizootic Ulcerative Syndrome Vis-A-Vis the Environment and the People. 25-26 May 1992. Trivandrum, Kerala. International Collective in Support of Fishworkers, Madras, India. 40 pp.

Jhingran, A.G. and Das, M.K. (1990) Epizootic ulcerative syndrome in fishes. Bulletin of the Central Inland Capture Fisheries Research Institute No. 65. CIFRI, Barrackpore, India. 14 pp.

Jones, S.W., Donaldson, S.P. and Deacon, J.W. (1991) Behaviour of zoospores and zoospore cysts in relation to root infection by *Pythium aphanidermatum*. New Phytologist 117, 289-301.

Jong, S.-C., Davis, E.E., McManus, C. and Krichevsky, M.I. (1991) Computer coding of the strain features of the saprolegnian fungi. Mycotaxon XLI(2), 407-418.

Jothy, A.A. (1981) Preliminary report on the outbreak of wabak kudison freshwater fish in paddy growing areas in Peninsular Malaysia. Report to the Ministry of Agriculture, Malaysia. December 1981. 17 pp.

Kanchanakhan, S. (1996a) Epizootic ulcerative syndrome (EUS): a new look at the old story. The AAHRI Newsletter, 5(1), 2-3. Aquatic Animal Health Research Institute, Bangkok.

Kanchanakhan, S. (1996b). Field and laboratory studies on rhabdoviruses associated with epizootic ulcerative syndrome (EUS) of fishes. PhD thesis, University of Stirling, Scotland. 278 pp.

Kanouse, B.B. (1932) A physiological and morphological study of *Saprolegnia parasitica*. Mycologia 24, 431-452.

Kaosiri, T. and Zentmyer, G.A. (1980) Protein, esterase and peroxidase patterns in the *Phytophthora palmivora* complex from Cacao. Mycologia 72, 988-1000.

Karim, S.M.R. and Chowdhury, M.B.R. (1995) Bacterial flora in the pond water of two different fish farms in Bangladesh. Abstracts of the Fourth Asian Fisheries Forum. 16-20 October 1995. P. 37. Asian Fisheries Society, Manila and China Society Fisheries, Beijing.

Karunasagar, I., Sugumar, G. and Karunasagar, I. (1995) Virulence characters of *Aeromonas* spp isolated from EUS-affected fish. In: Shariff, M., Arthur, J.R. and Subasinghe, R.P. (eds), Diseases in Asian Aquaculture II. Pp. 307-314. Fish Health Section, Asian Fisheries Society, Manila.

Kasornchandra, J., Engelking, H.M., Lannan, C.N., Rohovec, J.S. and Fryer, J.L. (1992) Characteristics of three rhabdoviruses from snakehead fish *Ophicephalus striatus*. Diseases of Aquatic Organisms 13, 89-94.

Kelly, L.A. and Smith, S. (1996) The nutrient budget of a small eutrophic loch and the effectiveness of straw bales in controlling algal blooms. Freshwater Biology 36(2), 411-419.

Kennedy, D.M and Duncan, J.M. (1995) A papillate *Phytophthora* species with specificity to *Rubus*. Mycological Research 99(1), 57-68.

Khan, M.H. (1997). A study of the activity of the head kidney macrophages from rosy barb, *Puntius schwanenfeldi;* rainbow trout, *Oncorhynchus mykiss* and tilapia *Oreochromis niloticus* against *Aphanomyces invaderis in vivo* and *in vitro.* MSc thesis, University of Stirling, Scotland. (submitted)

Khulbe, R.D. (1983) Pathogenicity of some species of *Pythium* Pringsheim on certain fresh water temperate fishes. Mykosen 26, 273-275.

Khulbe, R.D. (1989) Infection ability of watemolds in some temperate fishes of Himalaya, India. Mycoses 32(2), 84-86.

Khulbe, R.D. and Sati, S.C. (1980) Studies of parastic watermolds of Kumaun Himalaya; host range of *Achlya america* Humphrey on certain temperate fish. Mykosen 24, 177-180.

Khulbe, R.D., Joshi, C. and Bisht, G.S. (1995) Fungal diseases of fish in Nanak Sagar, Nainai Tal, India. Mycopathologia 130, 71-74.

Kitancharoen N., Yuasa, K. and Hatai, K. (1996) Effects of pH and temerature on growth of *Saprolegnia diclina* and *S. parasitica* isolated from various sources. Myoscience 37, 385-390.

Klebs, G. (1899) Zur Physiologie der Fortpflanzung einiger Pilze II. *Saprolegnia mixta* deBary. Jahrbuecher fuer Wissenschaftliche Botanik 33, 71-151. (In German)

Korf, R.P. (1995) Authors, reviewers and editors of articles proposing new names: a few guidelines. Mycotaxon 54, 413-419.

Kraft, J.M. and Boge, W.L. (1994) Development of an antiserum to quantify *Aphanomyces euteiches* in resistant pea lines. Plant Disease, 78(2), 179-183.

Krywienczyk, J. and Dorworth, C.E. (1980) Serological relationships of some fungi of the genus *Pythium.* Canadian Journal of Botany 58, 1412-1417.

Kumar, R.K.C. (1997). Therapeutic application of microalgal and herbal extract in fish disease. MSc thesis, University of Stirling, Scotland. 55 pp. (submitted)

Kumar, D., Dey, R.K. and Sinha, A. (1991) Outbreak of epizootic ulcerative syndrome of fish in India. In: Sinha, V.R.P. and Srivastava, H.C. (eds), Aquaculture Productivity. Pp. 345-356. Oxford and IBH Publishing Company, New Delhi.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lange, L. and Olson, L.W. (1983) The fungal zoospore, its structure and biological significance. In: Buczacki, S.T. (ed.), Zoosporic Plant Pathogens - A Modern Perspective. Pp. 1-42. Academic Press, London.

Larsson, M. (1994) Pathogenicity, morphology and isoenzyme variability among isolates of *Aphanomyces* spp. from weeds and various crop plants. Mycological Research 98(2), 231-240.

Latorre, B.A., Perez, G.F., Wilcox, W.F. and Torres, R. (1995) Comparative protein electrophoretic and isoenzymic patterns of *Phytophthora cryptogea* isolates from Chilean kiwifruit and North American deciduous fruits. Plant Disease 79(7), 703-708.

Lehnen Jr, L.P. and Powell, M.J. (1988) Cytochemical localisation of carbohydrates in zoospores of *Saprolegnia ferax*. Mycologia 80, 423-432.

Lehnen Jr, L.P. and Powell, M.J. (1989) The role of kinetosome-associated organelles in the attachment of encysting secondary zoospores of *Saprolegnia ferax* to substrates. Protoplasma 149, 163-174.

Lehnen Jr, L.P. and Powell, M.J. (1991) Formation of K2-bodies in primary cysts of *Saprolegnia ferax*. Mycologia 83(2), 163-179.

Lehnen Jr, L.P. and Powell, M.J. (1993) Characterization of cell surface carbohydrates on asexual spores of the water mold *Saprolegnia ferax*. Protoplasma 175, 161-172.

Levine, J.F., Hawkins, J.H., Dykstra, M.J., Noga, E.J., Moye, D.W. and Cone, R.S. (1990a) Species distribution of ulcerative lesions on finfish in the Tar-Pamlico River Estuary, North Carolina. Diseases of Aquatic Organisms 8(1), 1-5.

Levine, J.F., Hawkins, J.H., Dykstra, M.J., Noga, E.J., Moye, D.W. and Cone, R.S. (1990b) Epidemiology of ulcerative mycosis in Atlantic menhaden in the Tar-Pamlico River Estuary, North Carolina. Journal of Aquatic Animal Health 2(3), 162-171.

Lewis, D.H. (1973) Concepts in fungal nutrition and the origin of biotrophy. Biological Reviews 48, 261-278.

Li, K.H., Wise, D.J. and Robinson, E.H. (1996) Chemical prevention and treatment of winter saprolegniosis (winter kill) in channel catfish *Ictalurus punctatus*. Journal of the World Aquaculture Society 27(1), 1-6.

Lian, C.X. (1990) Peoples Republic of China report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 8-10. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Lilley, J.H. (1992) Assaying pond water for spores of saprolegniaceous fungi. Proceedings of the Seminar on Fisheries. National Inland Fisheries Research Institute, 16-18 September 1992. Pp. 79-82. Department of Fisheries, Kasetsart University, Bangkok.

Lilley, J.H. and Frerichs, G.N. (1994) Comparison of rhabdoviruses associated with epizootic ulcerative syndrome (EUS) with respect to their structural proteins, cytopathology and serology. Journal of Fish Diseases 17, 513-522.

Lilley, J.H., Phillips, M.J. and Tonguthai, K. (1992) A review of epizootic ulcerative syndrome (EUS) in Asia. Aquatic Animal Health Research Institute and Network of Aquaculture Centres in Asia-Pacific, Bangkok. 73 pp.

Limsuwan, C. and Chinabut, S. (1983) Histological changes of some freshwater fishes during 1982-83 disease outbreak. In: Proceedings of the Symposium on Freshwater Fish Epidemic 1982-3, Bangkok, 23-24 June 1983. P. 255 Chulalongkorn University, Bangkok.

Lio-Po, G.D., Albright, L.J. and Alapide-Tendencia, E.V. (1992) *Aeromonas hydrophila* in the epizootic ulcerative syndrome (EUS) of snakehead, *Ophicephalus striatus*, and catfish, *Clarias batrachus*: quantitative estimation in natural infection and experimental induction of dermo-muscular necrotic lesion. In: Shariff, M., Subasinghe, R.P. and Arthur, J.R. (eds). Diseases in Asian Aquaculture I, Pp. 461-474. Fish Health Section, Asian Fisheries Society, Manila.

Llobrera, A. and Gacutan, R.Q. (1987) *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines. Aquaculture 67, 273-278.

Lumanlan-Mayo, S.C., Catap, E.S., Paclibare, J.O., Callinan, R.B. and Fraser, G.C. (1996) Epizootic ulcerative syndrome (EUS) in rice-fish culture systems. World Aquaculture 1996 Book of

Abstracts, Bangkok, 29 January - 2 February 1996. Pp. 240-241. The World Aquaculture Society.

Magarinos, B., Pazos, F., Santos, Y., Romalde, J.L. and Toranzo, A.E. (1995) Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish. Diseases of Aquatic Organisms, 21(2), 103-108.

Magee, J.T. (1993) Whole organism fingerprinting. In: Goodfellow M. and O'Donnell A.G. (eds) Handbook of New Bacterial Systematics. Pp. 383-427. Academic Press, London.

Magee, J.T., Randle, E.A., Gray, S.J. and Jackson, S.K. (1993) Pyrolysis mass spectrometry characterisation and numerical taxonomy of *Aeromonas* spp. Antonie van Leeuwenhoek 64, 315-323.

Majer, D., Mithen, R., Lewis, B.G., Vos, P. and Oliver, R.P. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100(9), 1107-1111.

Manchester, L.N., Toole, A. and Goodacre, R. (1995) Characterization of *Carnobacterium* species by pyrolysis mass spectrometry. Journal of Applied Bacteriology 78(1), 88-96.

Manihar, S.R., Varma, K.L. and Das, H.R. (1990) Studies on hemagglutinins (lectins) from plasma of murrel fish, family Channidae. Indian Journal of Biochemistry and Biophysics 27, 464-470.

Manihar, S.R., Das, R.H. and Das, H.R. (1991) T-antigen-binding lectin from *Channa leucopunctatus* (murrel) plasma. Carbohydrate Research 213, 251-261.

Marin de Mateo, M., Adams, A., Richards, R.H., Castagnaro, M. and Hedrick, R.P. (1993) Monoclonal antibody and lectin probes recognize developmental and sporogonic stages of PKX, the causative agent of proliferative kidney disease in European and North American salmonid fish. Diseases of Aquatic Organisms 15, 23-29.

Marking, L.L., Rach, J.J. and Schreier, T.M. (1994a) Evaluation of antifungal agents for fish culture. The Progressive Fish-Culturist 56(4), 225-231.

Marking, L.L., Rach, J.J. and Schreier, T.M. (1994b) Search for antifungal agents in fish culture. In: Mueller, G.J. (ed.) Salmon Saprolegniasis. Pp. 131-148. Bonneville Power Administration, US Department of Energy, Portland, USA.

Marren, P. (1986) The lethal harvest of crayfish plague. New Scientist 30, 46-50.

Matthews, V.D. (1935) Notes on some Oomycetes from the viscinity of Mountain Lake, Virginia. Journal of the Elisha Mitchell Scientific Society 51, 306-311.

McGarey, D.J., Beatty, T.K., Alberts, V.A., Te Strake D. and Lim, D.V. (1990) Investigations of potential microbial pathogens associated with ulcerative disease syndrome (UDS) of Florida fish. In: Pathology in Marine Science. Pp. 65-75. Academic Press, Inc., San Diego.

McKenzie, R.A. and Hall, W.T.K. (1976) Dermal ulceration of mullet *(Mugil cephalus)*. Australian Veterinary Journal 52, 230-231.

McLaughlin, D.J., Barres, M.E. and Szabo, L.J. (1995) Molecules and morphology in basidiomycete phylogeny. Canadian Journal of Botany 73(Suppl.1), S684-S692.

Metzenberg, R.L. (1991) Benefactor's lecture: The impact of molecular biology on mycology. Mycological Research 95(1), 9-13.

Min, H.-K., Hatai, K, and Bai, S. (1994) Some inhibitory effects of chitosan on fish-pathogenic Oomycetes, *Saprolegnia parasitica*. Fish Pathology 29(2), 73-77.

Mitchell, J.I., Roberts, P.J. and Moss, S.T. (1995) Sequence or structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. Mycologist 9(2), 67-75.

Mitra, S.D. and Varshney, P.K. (1990) Homeo drug for curing ulcerative disease syndrome. Fishing Chimes 10(3), 13-14.

Miyazaki, T. (1994) Comparison among mycotic granulomatosis, saprolegniasis and anaaki-byo in fishes: a Japanese experience. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 253-270. Aquatic Animal Health Research Institute, Bangkok.

Miyazaki, T. and Egusa, S. (1972) Studies on mycotic granulomatosis in fresh water fishes - I. Mycotic granulomatosis in goldfish. Fish Pathology 7, 15-25. (In Japanese)

Miyazaki, T. and Egusa, S. (1973a) Studies on mycotic granulomatosis in fresh water fishes - II. Mycotic granulomatosis in ayu. Fish Pathology 7, 125-133. (In Japanese)

Miyazaki, T. and Egusa, S. (1973b) Studies on mycotic granulomatosis in fresh water fishes - III. Mycotic granulomatosis in blue-gill. Fish Pathology 8, 41-43. (In Japanese)

Miyazaki, T. and Egusa, S. (1973c) Studies on mycotic granulomatosis in fresh water fishes - IV. Mycotic granulomatosis in wild fishes. Fish Pathology 8, 44-47. (In Japanese)

Miyazaki, T. and Jo, Y. (1985) Studies on ichthyophonus disease of ayu. Fish Pathology 20(1), 45-48.

Mohan, C.V. and Shankar, K.M. (1994) Epidemiological analysis of epizootic ulcerative syndrome of fresh and brackishwater fishes of Karnataka, India. Current Science 66(9), 656-658.

Mohan, C.V. and Shankar, K.M. (1995) Role of fungus in epizootic ulcerative syndrome of fresh- and brackishwater fishes of India: a histopathological assessment. In: Shariff, M., Arthur, J.R. and Subasinghe, R.P. (eds), Diseases in Asian Aquaculture II. Pp. 299-305. Fish Health Section, Asian Fisheries Society, Manila.

Molina, F.I., Jong, S.-C. and Ma, G. (1995) Molecular characterization and identification of *Saprolegnia* by restriction analysis of genes coding for ribosomal RNA. Antonie van Leeuwenhoek 68, 65-74.

Munday, B.L. (1985) Ulcer disease in cod *(Pseudophycis bachus)* from the Tamar River. Tasmanian Fisheries Research 27, 15-18.

Musgrave, A., Ero, L., Scheffer, R. and Oehlers, E. (1977) Chemotropism of *Achlya bisexualis* germ hyphae to casein hydrolysate and amino acids. Journal of General Microbiology 101, 65-70.

Myers, G.S. and Shapovalov. (1931) On the identity of *Ophicephalus* and *Channa,* two genera of labyrinth fishes. Peking Natural History Bulletin 6(2), 33-37.

Nakamura, K., Yuasa, K., Sinmuk, S., Hatai, K. and Hara, N. (1995) The ubiquinone system in Oomycetes. Myoscience 36, 121-123.

National Workshop on Ulcerative Disease Syndrome in Fish (1990) Proceedings of the National Workshop on Ulcerative Disease Syndrome in Fish, Calcutta, India, 6-7 March 1990.

Neelam, B., Thompson, K.D., Price, N.C., Tatner, M.F., Adams, A., Ellis, A.E. and Stevens, L. (1995) Development of monoclonal antibodies to iron-regulated outer membrane proteins and lipopolysaccharide of *Aeromonas salmonicida*. Diseases of Aquatic Organisms 21, 201-208.

Nei, M. and Li, W.-H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Science of the USA 76(10), 5269-5273.

Neish, G.A. (1977) Observations on saprolegniasis of adult sockeye salmon, *Onchorynchus nerka* (Walbaum). Journal of Fish Biology 10, 513-522.

Neish, G.A. and Hughes, G.C. (1980) Fungal diseases of fish. In: Snieszko S.F. and Axelrod H.R. (eds). Diseases of Fishes. Book Six TFH Publications, NJ, USA. 159 pp.

Niemann, G.J., Van der Bij, A., Brandt-der Boer, B., Boon, J.J. and Baayen, R.P. (1991) Differential response of four carnation cultivars to races 1 and 2 of *Fusarium oxysporum* f. sp. *dianthi* and to *Phialophora cinerescens*. Physiological and Molecular Plant Pathology 38, 117-136.

Ninawe, A.S. (1993) Ulcerative syndrome widespread in India. Fish Farmer, September-October 1993, Pp. 20-21.

Ninni, A.P. (1865) Sulla mortalita dei gamberi *(Astacus fluviatilis* L.) nel veneto e piu particolarmente nella provincia trevigiana. Atti Instituto Veneto, Series III 10, 1203-9. (In Italian)

Noga, E.J. (1993a) Fungal diseases of marine and estuarine fishes. In: Couch, J.A. and Fournie, J.W. (eds), Pathobiology of Marine and Estuarine Organisms. Pp. 85-110. CRC Press, Inc, Baton Raton, Fl, USA.

Noga, E.J. (1993b) Water mold infections of freshwater fish: recent advances. Annual Review of Fish Diseases 3, 291-304.

Noga, E.J. (1994) Epidemic ulcerative diseases recently affecting estuarine fishes of the western Atlantic ocean. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 89-100. Aquatic Animal Health Research Institute, Bangkok.

Noga, E.J. and Dykstra, M.J. (1986) Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). Journal of Fish Diseases 9, 47-53.

Noga, E.J., Levine, J.F., Dykstra, M.J. and Hawkins, J.H. (1988) Pathology of ulcerative mycosis in Atlantic menhaden *Brevoortia tyrannus*. Diseases of Aquatic Organisms 4, 189-197.

Noga, E.J., Dykstra, M.J. and Wright, J.F. (1989) Chronic inflammatory cells with epithelial cell characteristics in teleost fishes. Veterinary Pathology 26, 429-437.

Noga, E.J., Wright, J.F., Levine, J.F., Dykstra, M.J. and Hawkins, J.H. (1991) Dermatological diseases affecting fishes of the Tar-Pamlico Estuary, North Carolina. Diseases of Aquatic Organisms 10(2), 87-92.

Noga, E.J., Khoo, L., Stevens, J.B., Fan, Z. and Burkholder, J.M. (1996) Novel toxic dinoflagellate causes epidemic disease in estuarine fish. Marine Pollution Bulletin, 32(2), 219-224.

Nolard-Tintigner, N. (1973) Etude experimentale sur l'epidemiologie et la pathogenie de la Saprolégniose chez *Lebistes reticulatus* Peters et *Xiphophorus helleri* Heckel. Acta Zoologica et Pathologica Antverpiensia 57, 1-127. (In French)

Ogbonna, C.I.C. and Alabi, R.O. (1991) Studies on species of fungi associated with mycotic infections of fish in a Nigerian freshwater fish pond. Hydrobiologia 220, 131-135.

Oláh, J. and Farkas, J. (1978) Effect of temperature, pH, antibiotics, formalin and malachite green on the growth and survival of *Saprolegnia* and *Achlya* parasitic on fish. Aquaculture 13, 273-288.

Olson, L.W., Cerenius, L., Lange, L. and Söderhäll, K. (1984) The primary and secondary spore cysts of *Aphanomyces* (Oomycetes, Saprolegniales). Nordic Journal of Botany 4(5), 681-696.

Oudemans, P. and Coffey, M.D. (1991) Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. Mycological Research 95(1), 19-30.

Paclibare, J.O., Catap, E.S. and Callinan, R.B. (1994) Fungal isolation from EUS-affected fish in the Philippines. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 238-243. Aquatic Animal Health Research Institute, Bangkok.

Pal, J. and Pradhan, K. (1990) Bacterial involvement in ulcerative condition of air-breathing fish from India. Journal of Fish Biology 36, 833-839.

Paternoster, M.P. and Burns, R.G. (1996) A novel medium for the oosporogenesis of *Aphanomyces cochlioides*. Mycological Research, 100(8), 936-938.

Paterson, R.R.M. and Bridge, P.D. (1994) Biochemical techniques for filamentous fungi. International Mycological Institute, Technical Handbooks No. 1. 125 pp.

Pearce, M. (1990) Epizootic ulcerative syndrome technical report, December 1987 - September 1989. Fisheries Report No.22. Northern Territory Department of Primary Industry and Fisheries, Northern Territory, Australia. 82 pp.

Peduzzi, R. and Bizzozero, S. (1977) Immunochemical investigation of four *Saprolegnia* species with parasitic activity in fish: serological and kinetic characterization of a chymotrypsin-like activity. Microbial Ecology 3, 107-118.

Peduzzi, R., Nolard-Tintigner, N. and Bizzozero, S. (1976) Investigations on saprolegniasis. II. Study on the process of penetrations showing evidence of a proteolytic enzyme and histopathological aspects. Rivista Italiana Piscicolt. Ittiopatol. 11, 109-117. (In French).

Persson, M. and Söderhäll, K. (1986) $CaCl_2$ -induced germination and peptidase secretion in *Aphanomyces astaci*. Experimental Mycology 10, 205-213.

Petersen, A., Jegstrup, I. and Olson, L.W. (1994) Screening for bacterial antagonists against *Saprolegnia parasitica* with BASF Pluronic Polyol F127. In: Mueller, G.J. (ed.), Salmon Saprolegniasis. Pp. 149-160. Bonneville Power Administration, US Department of Energy, Portland, USA.

Petersen, A.B., Olson, L.W. and Rosendahl, S. (1996) Use of polyclonal antibodies to detect oospores of *Aphanomyces*. Mycological Research 100(4), 495-499.

Phillips, M.J. (1989) A report on the NACA workshop on the regional research programme on ulcerative syndrome in fish and the environment, 20-24 March 1989. Network of Aquaculture Centres in Asia-Pacific, Bangkok. 59 pp.

Phillips, M.J. and Keddie, H.G. (1990) Regional research programme on relationships between epizootic ulcerative syndrome in fish and the environment. A Report on the Second Technical Workshop, 13-26 August 1990. Network of Aquaculture Centres in Asia-Pacific, Bangkok. 133 pp.

Pichyangkura, S. and Bodhalamik, V. (1983) The study of *Achlya* sp of fishes disease in *Ophicephalus striatus*. The Symposium on Fresh Water Fishes Epidemic: 1982-1983. 23-24 June 1983. Pp. 197-205. Chulalongkorn University, Bangkok. (In Thai, English abstract).

Pichyangkura, S. and Tangtrongpiros, J. (1985) The relationship between microscopic exam of *Achlya* sp. infection and characteristic of lesions *Ophicephalus striatus*. Proceedings of the Living Aquatic Resources. 7-8 March 1985. Pp. 19-23. Chulalongkorn University, Bangkok. (In Thai, English abstract).

Pickering, A.D. and Richards, R.H. (1980) Factors affecting the structure, function and biota of the salmonid epidermis. Proceedings of the Royal Society of Edinburgh 79B, 93-104.

Pickering, A.D. and Willoughby, L.G. (1977) Epidermal lesions and fungal infection on the perch, *Perca fluviatilis* L., in Windemere. Journal of Fish Biology 11, 349-354.

Pickering, A.D., Willoughby, L.G. and McGrory, C.B. (1979) Fine structures of secondary zoospore cyst cases of *Saprolegnia* isolates from infected fish. Transactions of the British Mycological Society 72, 427-436.

Powell, M.J. and Blackwell, W.H. (1995) Searching for homologous ultrastructural characters in zoosporic fungi. Canadian Journal of Botany 73(Suppl.1), S693-S700.

Powell, M.J., Lehnen Jr, L.P. and Bortnick, R.N. (1985) Microbody-like organelles as taxonomic markers among Oomycetes. Biosystems 18, 321-334.

Puckeridge, J.T., Walker, K.F., Langdon, J.S., Daley, C. and Beakes, G.W. (1989) Mycotic dermatitis in a freshwater gizzard shad, the bony bream, *Nematalosa erebi* (Gunther), in the River Murray, South Australia. Journal of Fish Diseases 12, 205-221.

Qureshi, T.A., Chouhan, R., Prasad, Y. and Mastan, S.A. (1995) Mycological studies on EUS affected catfish, *Mystus cavasius*. Abstracts of the Fourth Asian Fisheries Forum. 16-20 October 1995. P. 38. Asian Fisheries Society, Manila and China Society Fisheries. Beijing.

Raman, R.P. (1992) EUS strikes in the brackishwaters of Chilka Lagoon in India. Fish Health Section Newsletter, 3(2), 3-4. Asian Fisheries Society, Manila.

Rand, T.G. and Munden, D. (1992) Enzyme involvement in the invasion of brook char, *Salvelinus fontinalis* (Mitchill), eggs by *Saprolegnia diclina* (Oomycotina: Saprolegniaceae). Journal of Fish Diseases 15, 91-94.

Rand, T.G. and Munden, D. (1993a) Involvement of zoospores of *Saprolegnia diclina* (Oomycotina: Saprolegniaceae) in the attachment to and invasion of eggs of brook trout under experimental conditions. Journal of Aquatic Animal Health 5, 233-239.

Rand, T.G. and Munden, D. (1993b) Chemotaxis of zoospores of two fish-egg-pathogenic strains of *Saprolegnia diclina* (Oomycotina: Saprolegniaceae) toward salmonid egg chorion extracts and selected amino acids and sugars. Journal of Aquatic Animal Health 5, 240-245.

Rantamäki, J., Cerenius, L. and Söderhäll, K. (1992) Prevention of transmission of the crayfish plague fungus *(Aphanomyces astaci)* to the freshwater crayfish *Astacus astacus* by treatment with $MgCl_2$. Aquaculture 104, 11-18.

Reantaso, M.B. (1991) EUS in brackishwaters of the Philippines. Fish Health Section Newsletter, 2(1), 8-9. Asian Fisheries Society, Manila.

Rennerfelt, E. (1936) Untersuchgen uber die Entwicklung und Biologie des Krebspestpilze, *Aphanomyces astaci* Schikora. Mitt. Anst. f. Binnerfischerei bei Drottningholm, Stockholm No. 10, 21 pp. (In German)

Rha, S.-A., Sinmuk, S., Wada, S., Yuasa, K., Nakamura, K., Hatai, K. and Ishii, H. (1996) Pathogenicity to ayu *(Pecoglossus altivelis)* of *Aphanomyces* sp. isolated from dwarf gourami *(Colisa lalia)*. The Bulletin of thé Nippon Veterinary and Animal Science University 45, 9-15.

Richards, R.H. and Pickering, A.D. (1979) Changes in serum parameters of *Saprolegnia*-infected brown trout, *Salmo salar* L. Journal of Fish Diseases 2, 197-206.

Roberts, R.E. (1963) A study of the distribution of certain members of the saprolegniales. Transactions of the British Mycological Society 46(2), 213-224.

Roberts R.J. (1989) Fish Pathology 2nd Ed. Baillière Tindall, London.

Roberts, R.J. and Bullock, A.M. (1980) The skin surface ecosystem of teleost fishes. Proceedings of the Royal Society of Edinburgh 79B, 87-91.

Roberts, R.J., Shearer, W.M., Munro, A.L.S. and Elson, K.G.R. (1970) Studies on the ulcerative dermal necrosis of salmonids II. The sequential pathology of the lesions. Journal of Fish Biology 2, 373-378.

Roberts, R.J., Shearer, W.M., Munro, A.L.S. and Elson, K.G.R. (1974) Studies on the ulcerative dermal necrosis of salmonids II. The sequential pathology of the lesions. In: Diseases of Fish. Pp. 150-164. MSS Information Corporation.

Roberts, R.J., Macintosh, D.J., Tonguthai, K., Boonyaratpalin, S., Tayaputch, N., Phillips, M.J. and Millar, S.D. (1986) Field and laboratory investigations into ulcerative fish diseases in the Asia-Pacific region. Technical Report of FAO Project TCP/RAS/4508. Bangkok. 214 pp.

Roberts, R.J., Wootten, R., MacRae, I., Millar, S. and Struthers, W. (1989) Ulcerative disease survey, Bangladesh. Final report to the Government of Bangladesh and the Overseas Development Administration. Institute of Aquaculture, Stirling, Scotland. 105 pp.

Roberts, R.J., Willoughby, L.G. and Chinabut, S. (1993) Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. Journal of Fish Diseases 16, 169-183.

Roberts, R.J., Campbell, B. and MacRae, I.H. (1994a) Proceedings of the Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994, The Aquatic Animal Health Research Institute, Bangkok. 282 pp.

Roberts, R.J., Frerichs, G.N., Tonguthai, K. and Chinabut, S. (1994b) Epizootic Ulcerative Syndrome of Farmed and Wild Fishes. In: Muir, J.F. and Roberts, R.J. (eds), Recent Advances in Aquaculture V. Pp. 207-239. Blackwell Science, Oxford.

Rodgers, M. (1988) The biology of the crayfish plague (*Aphanomyces astaci*) in Great Britain. PhD thesis, City of London Polytechnic.

Rukyani, A. (1994) Status of epizootic ulcerative disease in Indonesia. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 68-75. Aquatic Animal Health Research Institute, Bangkok.

Sadowski, L.A. and Powell, M.J. (1990) Cytochemical detection of polysaccharides in zoospores of *Aphanomyces euteiches.* Canadian Journal of Botany 68, 1379-1388.

Saitanu, K., Wongsawang, S., Sunyasootcharee, B. and Sahaphong, S. (1986) Snakehead fish virus isolation and pathogenicity studies. Proceedings of the First Asian Fisheries Forum (ed. by Maclean, J.L., Dizon, L.B. and Hosillos, L.V.). Pp.327-330. Asian Fisheries Society, Manila.

Saitou, N. and Nei, M. (1987) The Neighbor-joining Method - a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4), 406-425.

Sammut, J., Callinan, R.B. and Cribb, B. (1996) Pathological changes in skin and gills of sand whiting *Sillago ciliata* . Microcosmopolitan, University of Sydney, 5-9 February 1996. (ed. by Cox G.). Pp. 163-164. Australian Society for Electron Microscopy.

Sanaullah, M., Hjeltnes, B. and Pittman, K. (1996) *Myxobolus* spp. (Myxozoa: Myxosporea) as primary causative agents of epizootic ulcerative syndrome (EUS) in fishes from Beel Mahmoodpur, Faridpur, Bangladesh. Proceedings of the Second World Fisheries Congress, Vol 1, 28 July - 2 August 1996. Pp. 34-35. Brisbane, Australia.

Sanjeevaghosh, D. (1991) EUS ravages Kerala inland fisheries. Fishing Chimes 11(9), 47-49.

Sarkar, K.K. (1995) Banana commands ulceration. Abstracts of the Fourth Asian Fisheries Forum, Beijing, China, 16-20 October 1995. P. 45. Asian Fisheries Society, Manila and China Society Fisheries, Beijing.

Sati, S.C., Mer, G.S. and Khulbe, R.D. (1982) Studies on parastic watermolds: some new host records for *Saprolegnia parasitica* Coker. Mykosen 25, 638-640.

Schikora, F. (1903) Ubr die Krebspest und ihren Erreger. Fisherei Zeitung 6, 353-355. (In German)

Schikora, F. (1906) Die Krebspest. Fisherei Zeitung 9, 529-532, 561-566, 581-583. (In German)

Schoulties, C.I. and Yang, C.Y. (1971) Electronic particle counting of zoospores of *Aphanomyces euteiches*. Mycologia 63, 169-171.

Schreurs, W.J.A., Harold, R.L. and Harold, F.M. (1989) Chemotropism and branching as alternative resoponses of *Achlya bisexualis* to amino acids. Journal of General Microbiology 135, 2519-2528.

Schuurkamp, G.J. and Hortle, K.G. (1987) Fish ulcer disease from the Ok Tedi area of Western Province, Papua New Guinea. Science in New Guinea 13(1), 15-21.

Scott, W.W. (1961) A monograph of the genus *Aphanomyces*. Virginia Agricultural Experiment Station Technical Bulletin 151. Blacksburg, Virginia, USA. 95 pp.

Scott, W.W. (1964) Fungi associated with fish diseases. Developments in Industrial Microbiology 5, 109-123.

Scott, W.W. and O'Bier, A.H. (1962) Aquatic fungi associated with diseased fish and fish eggs. The Progressive Fish Culturist, January 1962. Pp. 3-14.

Scott, W.W. and Warren Jr, C.O. (1964) Studies of the host range and chemical control of fungi associated with diseased tropical fish. Virginia Agricultural Experiment Station Technical Bulletin No. 171, 24 pp. Blacksburg, Virginia, USA.

Secombes, C.J. (1985) The *in vitro* formation of teleost multinucleate giant cells. Journal of Fish Diseases 8, 461-464.

Sequerra, J., Marmeisse, R., Valla, G., Normand, P., Capellano, A. and Moiroud, A. (1997) Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositatum* inferred from

RFLP analysis of the ribosomal intergenic spacer and Random Amplified Polymorphic DNA. Mycological Research 101(4), 465-472.

Seymour, R.L. (1970) The genus *Saprolegnia*. Verlag Von J. Cramer, Germany, 124 pp.

Shanor, L. and Saslow, H.B. (1944) *Aphanomyces* as a fish parasite. Mycologia 36, 413-415.

Shariff, M. and Law, A.T. (1980) An incidence of fish mortality in Bekok River, Johore, Malaysia. Proceedings of the International Symposium on Conservation Input from Life Sciences. 27-30 October 1980. Pp. 153-162. Universiti Kebangsaan, Bangi, Selangor, Malaysia.

Shariff, M. and Saidin, T.H. (1994) Status of epizootic ulcerative syndrome in Malaysia since 1986. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. (ed. by Roberts, R.J., Campbell, B. and MacRae, I.H.). P. 48. AAHRI, Bangkok.

Sharifpour, I. (1997). Histology of the inflammatory response of the carp (*Cyprinus carpio* L.) to various stimuli. PhD thesis, University of Stirling, Scotland. 373 pp.

Shephard, K.L. (1994) Functions for fish mucus. Reviews in Fish Biology and Fisheries 4(4), 401-429.

Shrestha, G.B. (1994) Status of epizootic ulcerative syndrome (EUS) and its effects on aquaculture in Nepal. Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994 (ed by Roberts, R.J., Campbell, B. and MacRae, I.H.). Pp. 49-57. Aquatic Animal Health Research Institute, Bangkok.

Shute, L.A., Berkeley, R.C.W., Norris, J.R. and Gutteridge, C.S. (1985) Pyrolysis mass spectrometry in bacterial systematics. In: Goodfellow, M. and Minnikin, D.E. (eds), Chemical Methods in Bacterial Systematics Vol. 20, Pp. 95-114.

Singh, V.O. and Bartnicki-Garcia, S. (1975) Lysis of zoospores of *Phytophthora palmivora* induced by concanavalin A. Experimentia 31, 243-244.

Singhal, R.N., Jeet, S. and Davies, R.W. (1987) Experimental transmission of *Saprolegnia* and *Achlya* to fish. Aquaculture 64, 1-7.

Sinmuk, S., Suda, H. and Hatai, K. (1996) *Aphanomyces* infection in juvenile soft-shelled turtle, *Pelodiscus sinensis*, imported from Singapore. Myoscience 37, 249-254.

Skliris, G. (1995). EUS: a vaccine pilot study. MSc thesis, University of Stirling, Scotland, 59 pp.

Smith, D. and Onions, A.H.S. (1994) The preservation and maintenance of living fungi. International Mycological Institute, Technical Handbooks No. 2. 122 pp.

Smith, S.N., Chohan, R., Howitt, S.G. and Armstrong, R.A. (1994) Proteolytic activity amongst selected *Saprolegnia* species. Mycological Research 98(4), 389-395.

Sneath, P.H. and Sokal, R.R. (1973) Numerical Taxonomy W.H. Freeman Co., San Francisco.

Soe, U.M. (1990) Myanmar report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 35-38. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Sohnle, P.G. and Chusid, M.J. (1983) Defense against infection with filamentous fungi in rainbow trout. Comparative Biochemistry and Physiology 74A(1), 71-76.

Southgate, P. (1983). Studies on the crayfish plague fungus, *Aphanomyces astaci*. MSc thesis, University of Stirling, Scotland. 109 pp.

Spanggaard, B., Skouboe, P., Rossen, L. and Taylor, J.W. (1996) Phylogenetic relationships of the intercellular fish pathogen *Ichthyophonus hoferi* and fungi, chanoflagellates and the rosette agent. Marine Biology 126, 109-115.

SPSS (1988) SPSS-X Users Guide. 3rd ed., SPSS Inc, Chicago, IL, USA.

Srivastava, R.C. (1979) Aphanomycosis - a new threat to fish population. Mykosen 22(1), 25-29.

Srivastava, R.C. (1980a) Fungal parasites of certain freshwater fishes of India. Aquaculture 21, 387-392.

Srivastava, R.C. (1980b) Studies in fish-mycopathology - a review. Part III. Mykosen 23(8), 462-469.

Srivastava, G.C. and Srivastava, R.C. (1977a) Host range of *Achlya prolifera* (Nees) de Bary on certain fresh water teleosts. Mycopathologia 61(1), 61-62.

Srivastava, G.C. and Srivastava, R.C. (1977b) *Dictyuchus anomalous* (Nagai), a new pathogen of fresh water teleosts. Current Science 46(4), 118.

Srivastava, R.C. and Srivastava, G.C. (1978) Fungi associated with the diseases of freshwater fishes. Mycopathologia 63(2), 121-126.

Stretton, R.J., Campbell, M. and Burns, D.T. (1976) Pyrolysis gas chromatography as an aid to the identification of *Aspergillus* species. Journal of Chromatography 129, 321-328.

Stuart, M.R. and Fuller, H.T. (1968) Mycological aspects of diseased Atlantic salmon. Nature 217, 90-92.

Subasinghe, R.P. (1993) Effects of controlled infections of *Trichodina* sp on transmission of epizootic ulcerative syndrome (EUS) to naive snakehead, *Ophicephalus striatus* Bloch. Journal of Fish Diseases 16, 161-164.

Subasinghe, R.P. and Jayasinghe, L. (1990) Effects of parasite induced stress on transmission of epizootic ulcerative syndrome (EUS) to snakehead *(Ophicephalus striatus)*. Symposium on Diseases in Asian Aquaculture, Bali, Indonesia, 26-29 November 1990. Asian Fisheries Society, Manila.

Suthi, G. (1991) Pathogenicity of motile aeromonads for *Puntius schwanfeldi* and *Oreochromis niloticus* with particular reference to the ulcerative disease syndrome (EUS). MSc thesis. University of Stirling, Scotland. 71 pp.

Svensson, E. and Unestam, T. (1975) Differential induction of zoospore encystment and germination in *Aphanomyces astaci*, Oomycetes. Physiologia Plantarum 35, 210-216.

Swahn, J.-Ö. (1994) Maypoles, Crayfish and Lucia - Swedish Holidays and Traditions. The Swedish Institute, Stockholm. 49 pp.

Szalai, A.J., Norcum, T., Bly, J.E. and Clem, L.W. (1992) Isolation of an acute-phase phosphorycholine-reactive pentraxin from channel catfish *(Ictalurus punctatus* Rafinesque). Comparative Biochemistry and Physiology 102B, 535-543.

Szalai, A.J., Bly, J.E. and Clem, L.W. (1994) Changes in serum concentrations of channel catfish *(Ictalurus punctatus* Rafinesque) phosphorycholine-reactive protein (PRP) in response to inflammatory agents, low temperature-shock and infection by the fungus *Saprolegnia* sp. Fish & Shellfish Immunology 4, 323-336.

Söderhäll, K. (1978). Interactions between a parasitic fungus, *Aphanomyces astaci*, Oomycetes, and its crayfish host. II Studies on the fungal enzymes and on the activation of crayfish prophenoloxidase by fungal components. PhD thesis, Uppsala University, Sweden. 22 pp.

Söderhäll, K. and Cerenius, L. (1987) Controlled growth and development in filamentous Oomycetes with emphasis on *Aphanomyces* spp. In: Fuller, M.S. (ed.), Zoosporic Fungi in Teaching and Research. No. 3, Pp. 264-267. Palfrey Contributions in Botany.

Söderhäll, K. and Unestam, T. (1975) Properties of extracellular enzymes from *Aphanomyces astaci* and their relevance in the penetration process of crayfish cuticle. Physiologia Plantarum 35, 140-146.

Söderhäll, K., Svensson, E. and Unestam, T. (1977) An inexpensive and effective method for elimination of the crayfish plague: barriers and biological control. Freshwater Crayfish 3, 333-342.

Söderhäll, K., Svensson, E. and Unestam, T. (1978) Chitinase and protease activities in germinating zoospore cysts of a parasitic fungus, *Aphanomyces astaci*, Oomycetes. Mycopathologia 64(1), 9-11.

Söderström, B. and Frisvad, J.C. (1984) Separation of closely related asymmetric penicillia by pyrolysis gas chromatography and mycotoxin production. Mycologia 76(3), 408-419.

Tehler, A. (1995) Morphological data, molecular data, and total evidence in phylogenetic analysis. Canadian Journal of Botany 73(Suppl.1), S667-S676.

Tibayrenc, M. (1996) Towards a unified evolutionary genetics of microorganisms. Annual Review of Microbiology 50, 401-429.

Tiffney, W.N. (1939a) The host range of *Saprolegnia parasitica*. Mycologia 31, 310-321.

Tiffney, W.N. (1939b) The identity of certain species of saprolegniaceae parasitic to fish. Journal of the Elisha Mitchell Scientific Society 55, 134-153.

Tika Ram, S.M., Chhabra, M.B., Batra, U.K. and Mahipal, S.K. (1994) The association of the pathogenic myxosporida as the cause of ulcerative disease syndrome in fish. Indian Veterinary Journal 71, 293-294.

Tonguthai, K. (1985) A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok. 39 pp.

Torres, J.L., Shariff, M. and Law, A.T. (1990) Identification and virulence screening of *Aeromonas* spp isolated from healthy and epizootic ulcerative syndrome (EUS)-infected fish. Proceedings of the Second Asian Fisheries Forum, Tokyo, Japan, 17-22 April 1989. (ed. by Hirano, R. and Hanyu, I.). Pp. 663-667. Asian Fisheries Society, Manila.

Ulcerative Fish Disease Committee (1983) Practical Report of the Ulcerative Fish Disease Committee 1982-1983, Bangkok.

Unestam, T. (1965) Studies on the crayfish plague fungus *Aphanomyces astaci*. I. Some factors affecting growth *in vitro*. Physiologia Plantarum 18, 483-505.

Unestam, T. (1966) Chitinolytic, cellulolytic and pectinolytic activity *in vitro* of some parasitic and saprophytic Oomycetes. Physiologia Plantarum 19, 15-30.

Unestam, T. (1969a) On the adaptation of *Aphanomyces astaci* as a parasite. Physiologia Plantarum 22, 221-235.

Unestam, T. (1969b) On the physiology of zoospore production in *Aphanomyces astaci.* Physiologia Plantarum 22, 236-245.

Unestam, T. and Gleason, F.H. (1968) Comparative physiology of respiration in aquatic fungi II. The Saprolegniales, especially *Aphanomyces astaci.* Physiologia Plantarum 21, 573-588.

Unestam, T. and Svensson, E. (1971) Physiological variation in strains of *Aphanomyces astaci.* Physiologia Plantarum 25, 414-416.

Unestam, T. and Weiss, D.W. (1970) The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci:* responses to infection by a susceptible and a resistant species. Journal of General Microbiology 60, 77-90.

Untereiner, W.A., Straus, N.A. and Malloch, D. (1995) A molecular-morphotaxonomic approach to the systematics of the Herpotrichiellaceae and allied black yeasts. Mycological Research 99(8), 897-913.

Valairatana, W. and Willoughby, L.G. (1993) Spore assay of *Achlya* (Fungi, Phycomycetes) from a fish pond; apparently demonstrating strains of the genus with widely differing growth rates. The AAHRI Newsletter, 1-2. Aquatic Animal Health Research Institute, Bangkok.

Valairatana, W. and Willoughby, L.G. (1994) The aquatic fungi *Aphanomyces* and *Pythium,* as wound pathogens on a soft shell turtle *(Trionyx cartilogineus).* The AAHRI Newsletter, 3(1), 2. Aquatic Animal Health Research Institute, Bangkok.

Virgona, J.L. (1992) Environmental factors influencing the prevalence of a cutaneous ulcerative disease (red spot) in the sea mullet *Mugil cephalus* L., in the Clarence River, New South Wales, Austalia. Journal of Fish Diseases 15, 363-378.

Vishniac, H.S. and Nigrelli, R.F. (1957) The ability of the saprolegniaceae to parasitise platyfish. Zoologica: New York Zoological Society, 42, 131-133.

Wada, S., Hatai, K. and Ishii, H. (1993) Mycotic gastritis of juvenile ayu *(Plecoglossus altivelis)* caused by *Saprolegnia diclina* Type-1. Journal of Wildlife Diseases, 29(4), 587-590.

Wada, S, Yuasa, K, Rha, S., Nakamura, K. and Hatai, K. (1994) Histopathology of *Aphanomyces* infection in dwarf gourami *(Colisa lalia).* Fish Pathology 29(4), 229-237.

Wada, S., Rha, S.-A., Kondoh, T., Suda, H., Hatai, K. and Ishii, H. (1996) Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida.* Fish Pathology 31(2), 71-80.

Wang, H.S. and LeJohn, H.B. (1974a) Analogy and homology of the dehydrogenases of Oomycetes. III. Isozyme patterns of malic and isocitric dehydrogenases. Canadian Journal of Microbiology 20, 581-586.

Wang, H.S. and LeJohn, H.B. (1974b) Analogy and homology of the dehydrogenases of Oomycetes. II. Regulation by GTP of D(-)lactic dehydrogenases and isozyme patterns. Canadian Journal of Microbiology 20, 575-580.

Wang, H.S. and LeJohn, H.B. (1974c) Analogy and homology of the dehydrogenases of Oomycetes. I. Regulation of glutamic dehydrogenases and isozyme patterns. Canadian Journal of Microbiology 20, 567-574.

Waterhouse, G.M. (1942) Water moulds of Hogsmill River. Transactions of the British Mycological Society 25, 315-325.

Weijman, A.C.M., van Eijk, G.W., Roeijmans, H.J., Windig, W., Haverkamp, J. and Turkensteen, L.J. (1984) Mass spectrometric techniques as aids in the diagnosis of gangrene in potatoes caused by *Phoma exigua* var. *foveata*. Netherlands Journal of Plant Pathology 90(3), 107-115.

White, J.G., Lyons, N.F., Wakeham, A.J., Mead, A. and Green, J.R. (1994a) Serological profiling of the fungal genus *Pythium*. Physiological and Molecular Plant Pathology 44(5), 349-361.

White, G.C., Sisson, P.R., Freeman, R. and Cookson, B.D. (1994b) Rapid characterisation of *Candida albicans* by pyrolysis mass spectrometry. Journal of Medical Microbiology 41, 98-105.

Widagdo, D. (1990) Indonesia report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 18-22. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Willoughby, L.G. (1968) Atlantic salmon disease fungus. Nature 217, 872-873.

Willoughby, L.G. (1970) Mycological aspects of a disease of young perch in Windermere. Journal of General Microbiology 65, 325-337.

Willoughby, L.G. (1977) An abbreviated life cycle in the salmonid fish *Saprolegnia*. Transactions of the British Mycological Society 69(1), 133-166.

Willoughby, L.G. (1978) Saprolegnias of salmonid fish in Windemere: a critical analysis. Journal of Fish Diseases 1, 51-67

Willoughby, L.G. (1986) An ecological study of water as the medium for growth and reproduction of the Saprolegnia from salmonid fish. Transactions of the British Mycological Society 87(4), 493-502.

Willoughby, L.G. (1989) Continued defence of salmonid fish against *Saprolegnia* fungus, after its establishment. Journal of Fish Diseases 12, 63-67.

Willoughby, L.G. (1993) A new destructive disease (epizootic ulcerative syndrome) of freshwater fishes in southeast Asia. Freshwater Forum 3(2), 110-121.

Willoughby, L.G. (1994) Fungi and fish diseases. Pisces Press, Stirling. 57 pp.

Willoughby, L.G. (1997a) *Saprolegnia polymorpha* sp. nov., a fungal parasite on Koi carp, in the UK. Nova Hedwigia (In press)

Willoughby, L.G. (1997b) *Achlya diffusa* (Fungi, Oomycota) from fish ponds in Thailand. Nova Hedwigia 64(3-4), (in press)

Willoughby, L.G. and Chinabut, S. (1996) Self-staling in *Aphanomyces invaderis*, the fungal pathogen of freshwater, tropical fish affected by epizootic ulcerative syndrome (EUS). The AAHRI Newsletter, 5(2), 2-3. Aquatic Animal Health Research Institute, Bangkok.

Willoughby, L.G. and Copland, J.W. (1984) Temperature - growth relationships of *Saprolegnia* pathogenic to fish, especially eels cultivated in warm water. Nova Hedwigia 39, 35-55.

Willoughby, L.G. and Lilley, J. (1992) The ecology of aquatic fungi in Thailand, and the fish disease relationship. The AAHRI Newsletter, 1(1), 5-6. Aquatic Animal Health Research Institute, Bangkok.

Willoughby, L.G. and Pickering, A.D. (1977) Viable Saprolegniaceae spores on the epidermis of the salmonid fish *Salmo trutta* and *Salvelinus alpinus*. Transactions of the British Mycological Society 68(1), 91-95.

Willoughby, L.G. and Roberts, R.J. (1992a) Towards strategic use of fungicides against *Saprolegnia parasitica* in salmonid fish hatcheries. Journal of Fish Diseases 15, 1-13.

Willoughby, L.G. and Roberts, R.J. (1992b) The ecology of *Olpidiopsis incrassata, Rozellopsis septigena* and *Woronina polycystis,* biflagellate fungal parasites of *Saprolegnia* fungus in fresh water. Nova Hedwigia 55(1-2), 1-9.

Willoughby, L.G. and Roberts, R.J. (1994a) Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. Journal of Fish Diseases 17, 541-543.

Willoughby, L.G. and Roberts, R.J. (1994b) Loss and recovery of zoospore motility in an isolate of *Aphanomyces* from a diseased fish. Mycological Research 98(12), 1463-1464.

Willoughby, L.G. and Roberts, R.J. (1994c) Zoospore motility, its loss and recovery, in an isolate of *Aphanomyces* from a diseased fish in Thailand. In: Mueller, G.J. (ed.), Salmon Saprolegniasis. Pp. 99-108. Bonneville Power Administration, US Department of Energy, Portland, USA.

Willoughby, L.G., McGrory, C.B. and Pickering, A.D. (1983) Zoospore germination of *Saprolegnia* pathogenic to fish. Transactions of the British Mycological Society 80(3), 421-435.

Willoughby, L.G., Roberts, R.J. and Chinabut, S. (1995) *Aphanomyces invaderis* sp. nov., the fungal pathogen of freshwater tropical fishes affected by epizootic ulcerative syndrome (EUS). Journal of Fish Diseases 18, 273-275.

Wilson, J.G.M. (1976) Immunological aspects of fungal disease in fish. In: Jones, E.B.G. (ed.), Recent Advances in Aquatic Mycology. Pp. 573-601. Elek Science, London.

Wilson, K.D. and Lo, K.S. (1992) Fish disease in Hong Kong and the potential role of the veterinarian. Proceedings of the 23rd Annual Conference of the International Association for Aquatic Animal Medicine (IAAAM), Hong Kong, 18-22 May 1992.

Windig, W. and Haverkamp, J. (1982) Pyrolysis mass spectrometry, I. *Rhodosporidium*. Studies in Mycology 22, 56-59.

Winstanley, T.G., Magee, J.T., Limb, D.I., Hindmarch, J.M., Spencer, R.C., Whiley, R.A., Beighton, D. and Hardie, J.M. (1992) A numerical taxonomic study of the *"Streptococcus milleri"* group based upon conventional phenotypic tests and pyrolysis mass spectrometry. Journal of Medical Microbiology 36(3), 149-155.

Wolke, R.E. (1975) Pathology of bacterial and fungal diseases affecting fish. In: Ribelin, W.E. and Migaki, G. (eds), The Pathology of Fishes. Pp. 33-116. University of Wisconsin Press, Winsconsin.

Wood, S.E. (1988) The monitoring and identification of *Saprolegnia parasitica* and its infection of salmonid fish. PhD thesis. The University of Newcatle upon Tyne. 175 pp.

Wood, S.E., Willoughby, L.G. and Beakes, G.W. (1986) Preliminary evidence for inhibition of *Saprolegnia* fungus in the mucus of brown trout, *Salmo trutta* L., following experimental challenge. Journal of Fish Diseases 9, 557-560.

Wood, S.E., Willoughby, L.G. and Beakes, G.W. (1988) Experimental studies on uptake and interaction of spores of the *Saprolegnia diclina-parasitica* complex with external mucus of brown trout *(Salmo trutta)*. Transactions of the British Mycological Society 90(1), 63-73.

Xuan, T.T. (1990) Vietnam report. Regional Research Programme on Relationships Between Epizootic Ulcerative Syndrome in Fish and the Environment. A Report on the Second Technical Workshop, 13-26 August 1990. (ed. by Phillips, M.J. and Keddie, H.G.). Pp. 62-63. Network of Aquaculture Centres in Asia-Pacific, Bangkok.

Yadav, M., Indira, G. and Ansary, A. (1992) Cytotoxin elaboration by *Aeromonas hydrophila* isolated from fish with epizootic ulcerative syndrome. Journal of Fish Diseases 15(2), 183-189.

Yeh, Y. (1989). Inter- and intra-specific restriction fragment length polymorphisms in the genus *Aphanomyces*. PhD thesis, Texas A&M University.

Yuasa, K. and Hatai, K. (1994a) Physiological and biochemical characteristics of some water moulds from fishes in Japan. Proceedings of the Third Asian Fisheries Forum. (ed. by Chou, L.M, Munro, A.D, Lam, T.J., Chen, T.W., Cheong, L.K.K., Ding, J.K., Hooi, K.K., Khoo, H.W., Phang, V.P.E., Shim, K.F. and Tan, C.H.). Pp. 298-301. Asian Fisheries Society, Manila.

Yuasa, K. and Hatai, K. (1994b) An attempt of classification by biological characteristics of some pathogenic water moulds from freshwater fishes. Nippon Kingakukai Kaiho 35, 104-110. (In Japanese, English abstract)

Yuasa, K. and Hatai, K. (1995a) Drug sensitivity of some pathogenic water moulds isolated from freshwater fishes. Journal of Antibacterial and Antifungal Agents 23(4), 213-219. (In Japanese, English abstract)

Yuasa, K. and Hatai, K. (1995b) Relationship between pathogenicity of *Saprolegnia* spp isolates to rainbow trout and their biological characteristics. Fish Pathology 30(2), 101-106.

Yuasa, K. and Hatai, K. (1996a) Some biochemical characteristics of the genera *Saprolegnia, Achlya* and *Aphanomyces* isolated from fishes with fungal infection. Myoscience 37, 477-479.

Yuasa, K. and Hatai, K. (1996b) Investigation of effective chemicals for treatment of saprolegniasis caused by *Saprolegnia parasitica*. Journal of Antibacterial and Antifungal Agents 24(1), 27-31. (In Japanese, English abstract)

Zhang, K.M., Zheng, F.C., Li, Y.D., Ann, P.J. and Ko, W.H. (1994) Isolates of *Phytophthora colocasiae* from Hainan island in China: evidence suggesting an Asian origin of this species. Mycologia 86(1), 108-112.

Zheng, X.B. and Ko, W.H. (1996) Continuing variation in successive asexual generations of *Phytophthora cinnamomi* following sexual reproduction. Canadian Journal of Botany 74, 1181-1185.

Zoological Society of Assam (1988) Proceedings of the Symposium on Recent Outbreak of Fish Diseases in North Eastern India. 30 December 1988. Organised by Zoological Society of Assam, Guwahati, Assam, India. 23 pp.

APPENDIX ONE

List of fungal isolates used in comparative studies

(a) EUS, RSD and MG pathogenic *Aphanomyces* isolates

	Isolate	Pathog-enicity	Growth data	Chemical suscep-tibility	EM	Immuno cyto-chemistry	SDS PAGE	Western blots	PyMS	RAPD
Chapter		**3**	**4**	**5**	**7**	**7**	**8**	**8**	**10**	**11**
EUS - Thailand	TA1	X	X	X			X		X	X
	RF6	X	X	X			X	X	X	X
	RF8	X	X	X			X		X	X
	S1PA	X	X	X			X		X	
	S3PA	X	X	X					X	X
	G2PA	X	X	X			X	X	X	X
	PA1	X	X	X		X			X	X
	PA3	X	X	X						
	PA4	X	X	X			X			X
	PA5	X	X	X			X			
	PA7	X	X	X			X	X	X	
	PA8	X	X		X					
	PA10	X	X	X			X			
	96PA								X	X
EUS - Bangladesh	BR	X	X	X			X		X	X
	BH	X	X	X			X	X	X	X
	BS	X	X	X			X		X	
EUS - Indonesia	36/1P	X	X	X			X	X	X	X
EUS - Philippines	30P	X	X	X						X
	33P	X	X	X	X	X	X		X	X
	34P	X	X	X					X	X
	10D	X	X	X			X	X	X	X
RSD - Australia	3P	X	X	X			X		X	
	4P	X	X	X			X	X	X	X
	10P	X	X	X			X		X	X
	24P	X	X	X		X	X	X	X	X
MG - Japan	NJM9030	X	X	X			X	X	X	X
	NJM9201									X

(b) Non-Asian pathogens; saprophytic *Aphanomyces, Achlya and Saprolegnia* spp; and *Phytophthora*

	Isolate	Pathog-enicity	Growth data	Chemical suscep-tibility	EM	Immuno cyto-chemistry	SDS PAGE	Western blots	PyMS	RAPD
Chapter		**3**	**4**	**5**	**7**	**7**	**8**	**8**	**10**	**11**
UM-*Aphanomyces*	84-1240	X	X	X	X		X	X	X	X
	84-1249								X	
	84-1282								X	X
Aphanomyces	J1									X
astaci	PL									X
	KV									X
	PC									X
	FDL457	X	X	X			X		X	X
	FDL458	X	X	X			X	X	X	X
Saprophytic	TF5	X	X	X			X	X	X	
Aphanomyces	TF33	X	X	X			X	X	X	
spp	TF41	X	X	X			X	X	X	X
	TF54	X	X	X						X
	T1SA	X	X	X			X	X		
	A2SA	X	X	X					X	
	F3SA	X	X	X	X	X	X	X	X	X
	SSA	X	X	X			X	X	X	
	WSA	X	X	X	X	X	X	X	X	X
	SA11	X	X	X			X		X	X
Aphanomyces	ASEAN1	X	X	X	X		X	X	X	
laevis	ASEAN3	X	X	X					X	X
Achlya sp	S2AC	X	X	X			X		X	
	AC2	X	X	X			X			
	AC5	X	X							
	AC10	X	X	X						
Achlya diffusa	W2BAC	X	X	X			X		X	
Saprolegnia sp	TF20S	X	X						X	
	TF23	X	X	X						
	TF24	X	X							
	TF25	X	X	X						
	TF26	X	X							
	TF29	X	X	X			X		X	
S. diclina	TF27	X	X	X						
	TF31	X	X	X			X			
	E3	X	X	X					X	
S. australis	*S. aust*	X	X	X			X		X	
S. ferax	P32	X	X	X			X		X	
S. parasitica	TP41	X	X	X		X	X		X	
Phytophthora cinnamomi						X				

APPENDIX TWO

(a) Formulae for media

GP (glucose-peptone) broth

3 g/l	glucose (BDH)
1 g/l	peptone (bacteriological, Oxoid)
0.128 g/l	$MgSO_4.7H_2O$ (Hopkins & Williams, Chadwell Heath, Essex)
0.014 g/l	KH_2PO_4 (BDH)
29.3 mg/l	$CaCl_2.2H_2O$ (Sigma)
2.4 mg/l	$FeCl_3.6H_2O$ (Sigma)
1.8 mg/l	$MnCl_2.4H_2O$ (BDH)
3.9 mg/l	$CuSO_4.5H_2O$ (BDH)
0.4 mg/l	$ZnSO_4.7H_2O$ (BDH)

GPY (glucose-peptone-yeast) broth

as GP broth with:

0.5 g/l	yeast (BDH)

GP agar

as GP broth with:

12 g/l	technical agar (Oxoid No. 3)

GPY agar

as GPY broth with:

12 g/l	technical agar (Oxoid No. 3)

GP-PenOx broth

prepare GP broth and after autoclaving and cooling to 50°C add:

100 units/ml	penicillin-K (Sigma)
10 μg/ml	oxolinic acid (Sigma)

GP-PenStrep agar

prepare GP agar and after autoclaving and cooling to 50°C add:

100 units/ml	penicillin-K (Sigma)
10 μg/ml	streptomycin sulphate (Sigma)

concentrated assay broth (50 ml)

4.5 g	glucose (BDH)
1.5 g	peptone (bacteriological, Oxoid)
0.19 g	$MgSO_4.7H_2O$ (Hopkins & Williams, Chadwell Heath, Essex)
0.02 g	KH_2PO_4 (BDH)
44 mg	$CaCl_2.2H_2O$ (Sigma)
3.6 mg	$FeCl_3.6H_2O$ (Sigma)
2.7 mg	$MnCl_2.4H_2O$ (BDH)
5.9 mg	$CuSO_4.5H_2O$ (BDH)
0.6 mg	$ZnSO_4.7H_2O$ (BDH)

Autoclave glucose separately to prevent carmelisation. After autoclaving and cooling to 50°C add:

15 mg (15000 units)	penicillin-K (Sigma)
15 mg	oxolinic acid (Sigma)

Add 1ml of concentrated assay broth to Petri dish with 29ml of test solution.

PG-1 broth

3 g/l	glucose (BDH)
6 g/l	peptone (bacteriological, Oxoid)
0.17 g/l	$MgCl_2.6H_2O$ (BDH)
0.15 g/l	$CaCl_2.2H_2O$ (BDH)
0.37 g/l	KCl (BDH)
0.02 g/l	$FeCl_3.6H_2O$ (Sigma)
0.044 g/l	Na_2 EDTA (Sigma)

Buffer with 13 mM sodium phosphate (BDH). Adjust pH to 6.3. Autoclave the glucose and sodium phosphate buffer separately from the other ingredients.

sodium phosphate buffer

make up stocks of:
31.2 g/l solution A - $NaH_2PO_4.2H_2O$ (BDH) - store at 4°C
71.7 g/l solution B - $Na_2HPO_4.12H_2O$ (BDH) - store at room temperature
407.5 ml solution A, 92.5 ml solution B and 500 ml distilled water are combined to make 1000 ml phosphate buffer (100 mM). 130 ml of this buffer is used in 1000 ml PG-1.

PG-1 agar

as PG-1 broth with:

12 g/l	technical agar (Oxoid No. 3)

V8 broth

5%	V8 juice (Campbell Grocery Products Ltd)
0.2%	$CaCO_3$ (BDH)

Adjust pH to 6.1

APW (autoclaved pond water)

Pond or lake water known to support fungal growth is filtered through Whatman 541 filter paper. One part pond water is combined to two parts distilled water and autoclaved.

(b) Water quality of lake/pond water used in APW

Stirling/Newcastle - Airthrey Loch water
(monthly sampling between 20/1/94 - 20/1/95 at surface, 2m and 4m depth, from Kelly and Smith, 1996)

	mean	range
Loch area	6.9 ha	
Loch mean depth	1.85	up to 4.5 m
Macrophyte cover	-	up to 25%
Temperature	9.3	2.0 - 20.0 °C
Dissolved oxygen	10.7	8.1 - 13.5 mg/l
pH	-	6.85 (Feb) - 9.54 (May)
Secchi disc	1.4	0.8 - 2.3 m
Total phosphorus	0.61	34.1 (May) - 83.1 (July) µg/l
Dissolved reactive phosphorus	2.2	not detectable - 9.8 (Jan) µg/l
Total silica	0.90	0.07 - 3.00 (Jan) mg/l
Molybdate reactive silicate	0.53	0.05 - 1.99 (Jan) mg/l
Chlorophyll *a*	34.0	2.7 - 102.9 (Jan) µg/l

(monthly sampling between 1/2/95 - 31/7/95, from L.A. Kelly (pers. comm.)

	mean	range
Alkalinity	1.0	0.9-1.16 meq/l
Total hardness ($CaCO_3$)	49	42-55 mg/l
Conductivity	232	216-250 uS/cm

Bangkok - National Inland Fisheries Instiute (NIFI) pond water
(analysis of surface water done at NIFI at 11:00am 3/3/97)

Temperature	29 °C
Alkalinity	161 mg/l
Hardness	413 mg/l
Dissolved oxygen	3.8 mg/l
pH	7.55
NO_2	0.58 mg/l
NO_3	1.30 mg/l
ammonia	0.06 mg/l
ortho PO_4	1.00 mg/l
total PO_4	1.24 mg/l

high ammonia and high temp and pH mean that proportionof unionised ammionia would be high

Hälsjön Lake near Uppsala

	Oct 1973	Nov 1974	Autumn 1978	Autumn 1979
pH	8.6	7.3		
NH_4 -N	0.022	0.085	0.030	0.088 mg/l
NO_2-N	0.004	0.004	0.016	0.023 mg/l
NO_3-N	0.202	0.195	0.346	0.259 mg/l
organic N	0.40	0.43	0.813	0.745 mg/l
total N	0.63	0.71	1.48	1.12 mg/l
PO_4-P	0.009	0.005	0.046	0.065 mg/l
general P	0.033	0.017	0.079	0.033 mg/l
total P	0.042	0.019	0.125	0.98 mg/l
COD	38	24 mg/l		
conductivity	164	163		

APPENDIX THREE

Histology staining procedures

H&E (haemotoxylin and eosin) (Clark, 1981)

i) Dewax and bring sections to distilled water	
ii) Stain in alum haemotoxylin	3 min
iii) Differentiate in acid alcohol	
iv) Blue in tap water	
v) Wash in running tap water	
vi) Counterstain with 1% eosin	1-3 min

Grocott silver stain (Grocott, 1955)

Using this technique, care must be taken that all glassware is washed well in distilled water and slides are handled only with plastic forceps otherwise extraneous silver staining will occur.

i) Dewax and bring sections to distilled water	
ii) 5% chromic acid	60 min
iii) Wash in tap water	
iv)1% sodium bisulphite	1 min
v) Running tap water	5 min
vi) Rinse 3 times in distilled water	
vii) Stain in the silver solution in the dark at 50°C	30-60 min

Stock methenamine solution: 3g methenamine (hexamine) dissolved in 95 ml distilled water bringing the volume to 100ml. Add 5 ml of a 5% aqueous solution of silver nitrate. Any white precipitation is redissolved with shaking. To 25 ml of this stock is added 25 ml distilled water and 2 ml of a 5% solution of borax just before using.

viii) Rinse 3 times in distilled water	
ix) Tone in 0.1% gold chloride	3-5 min
x) Rinse in distilled water	
xi) 2% sodium thiosulphate	2 min
xii) Running tap water	

A counterstain may be applied. Fungus should be a deep black, the inner parts of the hyphaemay be a red-rose colour. Background colour depends on any counterstain used.

PAS (periodic acid - Schiffs) (Hotchkiss, 1948)

i) Dewax and bring sections to distilled water	
ii) 1% periodic acid	10 min
iii) Running tap water	5 min
iv) Schiffs reagent	10 min
v) 3 rinses in 0.5% sodium metabisulphite	3 x 2 min
vi) Running water	5 min