FINAL REPORT

Molecular Diagnostic Tests to Detect Epizootic Ulcerative Syndrome (*Aphanomyces invadans*), and Crayfish Plague (*Aphanomyces astaci*)

Ms. Nicky Buller

June 2004

FRDC Project No. 2001/621

N. B. Buller

Aquatic Animal Health Subprogram: molecular diagnostic tests to detect epizootic ulcerative syndrome (*Aphanomyces invadans*) and crayfish plague (*Aphanomyces astaci*)

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Australian Government
Department of Agriculture, Fisheries and Forestry

CEFAS
MURDOCH UNIVERSITY FISHERIES

Australian Government
Fisheries Research and Development Corporation

Project Investigators

PROJECT TITLE

Molecular Diagnostic Tests to Detect Epizootic Ulcerative Syndrome (*Aphanomyces invadans*), and Crayfish Plague (*Aphanomyces astaci*)

PROJECT NUMBER FRDC 2001/621

RESEARCH ORGANISATION

Name: Murdoch University

PRINCIPAL INVESTIGATOR

Name: Nicky Buller

Position: Senior Microbiologist
Department of Agriculture Western Australia

Postal Address: Animal Health Laboratories
Department of Agriculture Western Australia
3 Baron-Hay Court
South Perth, WA 6151

Phone: 08-93683425
Facsimile: 08-94741881

CO-INVESTIGATORS

Names: Dr Stan Fenwick (Murdoch University)
Dr Phil Nicholls (Murdoch University)
Dr David Alderman (CEFAS, UK)
Dr Brian Jones (Department of Fisheries WA)
Heather McLetchie (Research Officer)

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1. NON TECHNICAL SUMMARY

2001/621 Molecular Diagnostic Tests to Detect Epizootic Ulcerative Syndrome (*Aphanomyces invadans*), and Crayfish Plague (*Aphanomyces astaci*)

PRINCIPAL INVESTIGATOR: Ms Nicky Buller
ADDRESS: Animal Health Laboratories
Department of Agriculture Western Australia
3 Baron-Hay Court
South Perth Western Australia 6151

Telephone: 08 9368 3425 Fax: 08 9474 1881
E-mail: nbuller@agric.wa.gov.au

OBJECTIVES:

1 Develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples.

2 Develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples.

3 Develop a rapid molecular diagnostic test for the detection of *Aphanomyces invadans,* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

4 Develop a rapid molecular diagnostic test for the detection of *Aphanomyces astaci,* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

5 Transfer of this technology in the form of a kit for initial distribution and evaluation to selected laboratories.

6 Write up Australian Standard Diagnostic Techniques based on the above tests and in the format supplied by AFFA.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

A PCR has been developed that is specific for the detection and identification of *Aphanomyces invadans* (epizootic ulcerative syndrome) from fungal cultures and fish tissue. DNA extraction methods were optimised for detection of oomycete fungus from broth culture and for direct detection in fish tissue.

A number of primers for the detection of *A. astaci* (crayfish plague) have been designed and these will be developed into a specific PCR in a later project (FRDC 2004/91)

Crayfish Plague and Epizootic Ulcerative Syndrome are two fungal diseases that affect freshwater crayfish and freshwater finfish, respectively. Crayfish Plague, which is caused by *Aphanomyces astaci*, is exotic to Australia, but is capable of causing massive stock losses of up to 100% should the disease occur in Australia. It is present in North America where the native freshwater crayfish are largely resistant to the disease or act as carriers of the disease. Crayfish plague was transferred to Europe in the 1870's through infected stock and has wiped out many of the native freshwater crayfish in a number of countries. Epizootic Ulcerative Syndrome (EUS), which is due to infection by *Aphanomyces invadans*, is endemic in many fish in the river systems throughout Australia. The disease causes economic loss to the freshwater finfish aquaculture industry and affects local native stocks. Both diseases are almost impossible to eradicate.

Both diseases may be difficult to diagnose, especially if laboratory personnel are not sufficiently skilled in recognising or differentiating the diseases. The culture and identification of both fungi can be difficult and prolonged. Isolation and identification may be hampered by overgrowth from other fungi that contaminate the lesion or the isolation medium. Culture is tedious and may take up to 15 days before fungal growth is seen. It can then be difficult to identify the fungus and often necessitates the culture being sent to a specialist laboratory for confirmation of identification. Examination of histological samples requires expertise and although the presence of wider than normal hyphae is suggestive of Aphanomyces, it is not definitive.

Early detection and diagnosis of the disease is crucial to ensure rapid disease response and containment, particularly for Crayfish plague, which is exotic to Australia and the Asian region. Therefore, there was a need to improve diagnostic capabilities of Australian laboratories and this objective formed part of the federal budget initiative to improve disease diagnosis in aquaculture.

The research undertaken in this project sought to provide two diagnostic tests for each disease based on molecular biological techniques that isolated and identified the fungi based on their DNA. The two techniques were the Polymerase Chain Reaction (PCR) and Fluorescent *In-Situ* Hybridization (FISH). Both techniques identify an organism by detection of a section of DNA that is unique to that organism. The research involves searching for a

section of DNA that is unique to that organism and then proving this by testing all fungi that are genetically similar or that may be found in the same ecological location. For the detection and identification of *A. invadans* the tests were applied to fungal culture material, fresh tissue, and paraffin-embedded tissue. For *A. astaci* the tests were applied to paraffin-embedded material and formalin-fixed tissue.

Optimal extraction of DNA from samples is of prime importance for achieving an accurate and sensitive result. Two commercial methods were suitable; the DNAzol reagent (Invitrogen™, Life Technologies) and the DNeasy mini plant tissue kit (Promega).

A PCR that was specific to *A. invadans* was achieved. The PCR can be used on culture material and fresh fish tissue using primers AIF14 + AIR10. The test is specific and does not show cross-reaction with closely related oomycete fungi such as *Saprolegnia* species. It is also specific against fungi that are likely to occur as plate contaminants on laboratory media, such as *Aspergillus* species and *Penicillium* species. Variable results have been obtained for use on paraffin-embedded tissue, both seeded material and diagnostic samples. This unreliable detection of DNA in paraffin-embedded material may be due to the effect of the formalin fixative on the DNA, the DNA extraction method or the large size (554 base pairs) of the amplicon (amplified DNA segment). Investigations are continuing for this type of sample.

The FISH method for *A. invadans* using fluorophores based on the primers that were specific for the PCR were unable to produce a test that was specific, as cross-reaction occurred with *Saprolegnia* species. The technique was only applied to fungal culture.

A number of primers were designed for the specific detection of *A. astaci*. However, no product was amplified from formalin-fixed tissue even though a very weak amplicon of 750 base pairs was amplified using universal primers to the large subunit gene (28S rDNA). This indicated that fungal material was present in the sample and that the DNA extraction method was suitable. The primers were designed to the virulence genes that have been sequenced for *A. astaci*. These were the subtilisin-like proteinase precursor gene, the trypsin proteinase precursor gene and the chi 1 gene for a putative chitinase. One reason for non-amplification may be due to low copy number of the genes in *A. astaci*, although this was addressed by using a nested PCR to increase the sensitivity of the test. Another reason may be due to damaged DNA caused by the formalin fixative.

A future project, FRDC 2004-091 will address the problems resulting from the use of formalin-fixed tissue for *A. astaci* detection. The problems caused by lack of fresh DNA for *A. astaci* will be overcome through the Australian Animal Health Reference Laboratory, which has imported cultures of *A. astaci* so as to provide fresh DNA for this project. The project will also continue to develop the *A. invadans* PCR for use on paraffin-embedded material and continue with the FISH method for both fungi. These methods will then be validated by laboratories in Australia, New Zealand, Thailand and United States of America.

KEYWORDS: **PCR, fluorescent in-situ hybridization, FISH, Epizootic Ulcerative Syndrome (EUS), Crayfish plague, *Aphanomyces astaci*, *Aphanomyces invadans***.

2. ACKNOWLEDGMENTS

The following individuals and organisations are gratefully acknowledged for supplying fungal cultures, fish samples, technical assistance or advice during the project.

Dr Vicki Blaser, National Fish Health Research Laboratory, Leetown Science Centre, USA for the gift of cultures of *Aphanomyces invadans* isolated from diseased menhaden in the USA.

Dr Chris Burke, University of Tasmania and Dr Barry Munday for supplying a culture of *Aphanomyces invadans*.

Dr Richard Callinan and Dr Matt Landos, Department of Fisheries New South Wales, Australia for gift of cultures of *Aphanomyces invadans*.

Julia Carson, Technical Officer, Animal Health Laboratories, Department of Agriculture Western Australia, for preparation of culture media.

Dr Supranee Chinabut and Thitiporn Petchinda, Aquatic Animal Health Research Insitute, Thailand, for the gift of cultures of *Aphanomyces invadans*.

Dr Jim Lilley who gave his time to visit the Animal Health Laboratory at Department of Agriculture Western Australia to explain isolation techniques for Aphanomyces and to discuss his research in this area. Dr Lilley also provided the group with reference papers and a draft of a paper that was to be submitted to a journal.

Dr Annette Thomas, Department of Primary industries, Queensland, Australia, for supplying stored cultures of *A. invadans*.

Mr Ian Vaughan, fisherman, Western Australia, for his assistance in collecting infected fish from the Swan River system and for his enthusiasm for the project.

Dr Richard Whittington, University of Sydney, for supplying references and his laboratory method for the preparation of DNA extraction from paraffin-embedded tissue.

Colleagues at Animal Health Laboratories, Department of Agriculture Western Australia, for their support and assistance during the project.

Colleagues at Fish Health Unit, Department of Fisheries Western Australia, for their support and assistance during the project.

3. BACKGROUND

Epizootic Ulcerative Syndrome (EUS) and Crayfish Plague are two fungal diseases that affect freshwater fish and freshwater crayfish, respectively. EUS is caused by *Aphanomyces invadans* and Crayfish Plague is due to *A. astaci*. Members of the *Aphanomyces* genus belong to a group of water moulds (Oomycetes) from the family *Saprolegniaceae*, which comprise several pathogens of fish, crustaceans and plants (Leclerc *et al.*, 2000).

A. invadans is the causative agent of Australian red spot disease, which is synonymous with Epizootic Ulcerative Syndrome (EUS) in Southeast Asia, mycotic granulomatosis from Japan (Egusa and Masuda, 1971) (McKenzie and Hall, 1976; Callinan *et al.*, 1995) and Ulcerative Mycosis in North America (Dykstra *et al.*, 1986).

EUS continues to spread through South East Asia into India and Pakistan, and has been implicated in mortalities of menhaden in the USA, (Blazer *et al.*, 2002). In Australia, *A. invadans* affects ornamental fish, freshwater and estuarine fish in particular silver perch, black bream and barramundi (Figure 3-1). Work by Dr Richard Callinan (NSW) and others fulfilled Henle-Koch's postulates and thus established that the fungus *A. invadans* is the primary pathogen in the syndrome, following an initial insult – usually acid sulphate soil run-off. The acidified water damages the skin increasing the fish's susceptibility to infection with *A. invadans* (Callinan *et al.*, 1995; Sammut *et al.*, 1996). The disease causes high mortality, and up to 80% of the fish may be affected. The large red ulcerated lesions make the fish unsaleable. Fish that are affected (both wild caught and aquaculture) accounted for a production of 6,546 tonnes at a value of $288 million in 2002-03 (Australian Fisheries Statistics, 2003).

Figure 3-1. Black bream showing ulcer associated with EUS.

Crayfish Plague (Figure 3-2), caused by *Aphanomyces astaci*, is a devastating disease affecting freshwater crayfish and in countries where the disease occurs outbreaks may be associated with 100% mortality. Crayfish plague is listed as significant by the Office Internationale des Epizooties (OIE). It is endemic in Europe but exotic to Australia where local crayfish are highly susceptible (Unestam and Ajaxon, 1976; Alderman, 1996). The fungus penetrates and grows extensively within the crayfish cuticle by both

enzymatic and physical means, causing death within a few weeks (Bangyeekhum *et al.*, 2001). Affected individuals may show protruding fungal hyphae and melanin deposits on the carapace (Alderman *et al.*, 1987).

Both diseases may be spread through water via production of motile zoospores that infect other fish or crayfish, by affected individuals, or from contaminated fishing equipment (Alderman *et al.*, 1987; Nylund *et al.*, 1993). Thus once established the fungi are almost impossible to eradicate.

EUS continues to enter Australia through the ornamental fish trade. Although Australia is free from Crayfish Plague, continued success to maintain freedom relies on quarantine restrictions and, as recommended by the OIE, prohibition of importation of live crayfish from areas where the disease is either reported or its presence cannot be ruled-out.

In Europe attempts to protect native stocks from Crayfish Plague by banning the importation of crayfish, have been unsuccessful.

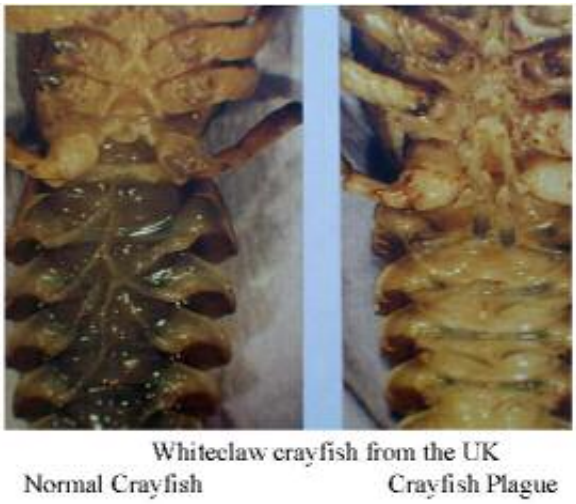

Figure 3-2. Differences in appearance between a normal crayfish, and a crayfish infected with crayfish plague.

Photographs courtesy Dr D. Alderman.

The difficulty of excluding any highly infectious disease means that it is crucial to have the ability to maintain surveillance of current populations, and to accurately and rapidly detect or monitor disease outbreaks with rapid, reliable tests. Currently, detection of both diseases relies on clinical diagnosis, histological examination and culture of the fungus (Alderman and Polglase 1986, Jones and Buller 2000, Roberts *et al.*, 1993, OIE Manual 2004). Many of the laboratories involved in diagnosis of fish diseases may not have the capability and experience of laboratory culture for the identification of these two diseases, and a diagnosis would rely heavily on clinical signs and pathological findings. As explained in Section 4, NEED, there are drawbacks to these methods.

This project attempted to improve diagnostic capabilities not only for the detection, but also for the definitive identification of both *A. invadans* and *A.*

astaci by molecular methods using Polymerase Chain Reaction (PCR), and Fluorescent *In-Situ* Hybridization (FISH) probes to detect fungal DNA.

4. NEED

There is a need for improved detection and identification techniques that will improve laboratories' diagnostic capabilities, provide accurate and rapid disease assessment, improve importation/exportation testing times and confidence, and allow prevalence studies to be undertaken.

Detection and identification of both diseases relies on histology and culture. These methods are time-consuming because of the length of time for culture (up to 15 days in the case of *A. astaci*), and do not provide definitive differentiation from other *Aphanomyces* species.

The culture technique (Figure 4-1) is unreliable due to the difficulty in growing *Aphanomyces* species, and the unreliability of the fungus sporulating in artificial media; a requirement for distinguishing morphological features and thus the identification of the fungus (Alderman and Polglase 1986, Roberts *et al.*, 1993, and in the authors experience). Many of the diagnostic laboratories involved in fish diseases do not have access to media preparation facilities (discussed at Fish Bacteriology workshop, AAHL, 2001), and as specific growth media is required for both *A. invadans* and *A. astaci* respectively, disease caused by these fungi may be mis-reported.

Also, with histological diagnosis, fungal elements may not be seen until the lesion is well advanced. With experience, *A. invadans* is easy to differentiate because of the "larger than normal" size of the hyphae as seen in histology slides (Figure 4-2). However, this does not constitute a definitive differentiation from other *Aphanomyces* species or even other fungal species.

The current methods of diagnosis are entirely reliant on the experience of the investigating scientists. The majority of laboratories have access to a thermocycler and a fluorescent microscope. Thus the diagnostic methods proposed in this submission may be more accessible to laboratories than the current methods involving culture, clinical and histological experience.

Thus, rapid, reliable, diagnostic methods will reduce testing time for export requirements, for example, export of fresh yabbies to Asia and Switzerland, and increase assurance of Australia's disease-free status for Crayfish Plague in the case of exportation requirements. Improved detection capabilities will allow a more correct assessment of EUS in Australia and give confidence to the industry in laboratories' capabilities.

Figure 4-1. Five-day growth of *A. invadans* on IM medium.

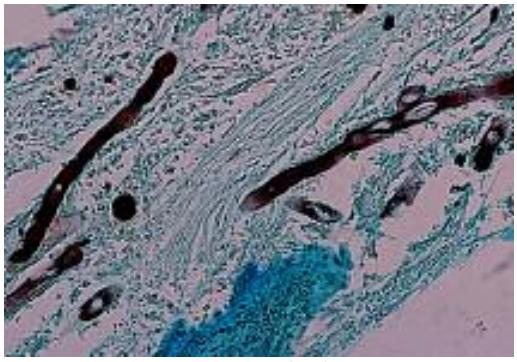

Figure 4-2. Histopathology section showing tissue infected with EUS (Grocott-Gomorri stain).

Photograph courtesy Dr B. Jones.

5. OBJECTIVES

1 To develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

2 To develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

3 To develop a rapid molecular diagnostic test for the detection of *Aphanomyces invadans,* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

4 To develop a rapid molecular diagnostic test for the detection of *Aphanomyces astaci,* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

5 To publish Australian Standard Diagnostic Techniques based on the above tests and in the format supplied by AFFA.

6. METHODS

6.1 Development of a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR), and for its use with fresh or dead tissue samples

6.1.1 Fungal isolates and diagnostic material

6.1.1.1 Fungal reference strains and clinical strains.

In order to design and test primers that were specific to *A. invadans* and *A. astaci*, a number of individuals and organisations were contacted for cultures, DNA or fixed tissues. The cultures, their details and the person or organisation who supplied the cultures are listed in Table 6-1. The original source of these cultures is acknowledged under the column headed "origin" as separate from the person or institution who supplied the cultures for this project.

Table 6-1. List of Oomycete fungal cultures

Isolate name	Isolate ID	AHL Lab ID	Disease	Origin	Supplier to AHL
A. invadans	UM3	#38	Menhaden, USA, 1998	Dr Blaser, USA	Dr Chinabut, AAHRI, Thailand
A. invadans	T99G2 (IMI 836084)	#39	EUS, Giant Gourami, Thailand, 1999	Varinee Panyawachira	Dr Chinabut, AAHRI, Thailand
A. invadans	G2PA	#40	EUS, Three-spot Gourami, 1994, Thailand	J. H. Lilley	Dr Chinabut, AAHRI, Thailand
A. invadans	RF8	#41	EUS, Striped snakehead, Thailand, 1992	L. G. Willoughby	Dr Chinabut, AAHRI, Thailand
A. invadans	RF6 (IMI 836082)	#42	EUS, Striped snakehead, Thailand,1992	L. G. Willoughby	Dr Chinabut, AAHRI, Thailand
A. invadans	30P	#43	EUS, Striped snakehead, Philippines, 1991	J. O. Paclibare	Dr Chinabut, AAHRI, Thailand
A. invadans	4P (IMI 836086)	#44	RSD, yellowfin bream, NSW, 1989	R. Clarence, Australia	Dr Chinabut, AAHRI, Thailand
A. invadans	NJM9701	#45	MG, Ayu, Japan, 1997	K. Hatai	Dr Chinabut, AAHRI, Thailand
A. invadans	PA8	#46	EUS, Striped snakehead, 1995, Thailand	J. H. Lilley	Dr Chinabut, AAHRI, Thailand
A. invadans	B99C	#47	EUS, *Cirrhinus reba*, Bangladesh, 1999	J. H. Lilley, M. H. Khan	Dr Chinabut, AAHRI, Thailand
A. invadans	WIC	#54	MG, Menhaden, USA	Dr V. Blaser	Dr V. Blaser, USA
A. invadans	WIC2	#55	MG, Menhaden, USA	Dr V. Blaser	Dr V. Blaser, USA

Table 6-1, continued

Isolate name	Isolate ID	AHL Lab ID	Disease	Origin	Supplier to AHL
A. *invadans*	A1S2	#23	EUS, NSW, 2002	R. Callinan, M. Landos	Dept of Fisheries, NSW
A. *invadans*	A2S2	#24	EUS, NSW, 2002	R. Callinan, M. Landos	Dept of Fisheries, NSW
A. *invadans*	AS-03-0417	#53	EUS, WA, 2002	AHL	Ian Vaughan, WA
Saprolegnia species	AS-01-3195-1	#9		AHL	DAWA
Saprolegnia species	AS-01-3195-2	#11		AHL	DAWA
Unidentified fungus	AS-01-4189	#12		AHL	DAWA
Saprolegnia	AS-00-3014	#14	Lesion, Black bream, WA, 2000	AHL	DAWA
Saprolegnia ferax/ diclina	AS-02-1803		Lesion on fish.	AHL (identified by partial sequencing of ITS region)	DAWA
Cladosporium sp.	AS-03-2247-1			AHL	DAWA
Pythium sulcatum	WAC 8470	#22	1997	Plant Pathology	DAWA
Unidentified fungus	AS-02-3995	#36	Lesion, Black bream, WA, 2003	AHL	DAWA
Achyla diffusa	ATCC 16111	#51*			ATCC
Aphanomyces species	ATCC 62427*	#27*			ATCC
Aphanomyces frigidophilus	ATCC 204464	#26*			ATCC
Saprolegnia australis	ATCC 38487	#49*			ATCC
Saprolegnia parasitica	ATCC 46241	#25*			ATCC
Saprolegnia salmonis	MYA-399	#52*			ATCC
Saprolegnia diclina	ATCC 36144	#50*			ATCC
Trichoderma sp.		#28		Dr A. Thomas	DPI QLD
A. invadans	34P	#29*		Dr A. Thomas	DPI QLD
A. invadans	3P	#30*		Dr A. Thomas	DPI QLD
A. invadans	33P	#31*		Dr A. Thomas	DPI QLD
A. invadans	4P	#32*		Dr A. Thomas	DPI QLD
Saprolegnia sp.	45710	#33*		Dr A. Thomas	DPI QLD
A. invadans		#21$^{\delta}$		Dr Chris Burke	Tasmania
Aspergillus niger			Plate contaminant	AHL	DAWA

Table 6-1, continued					
Isolate name	**Isolate ID**	**AHL Lab ID**	**Disease**	**Origin**	**Supplier to AHL**
Aspergillus fumigatus			Plate contaminant	AHL	DAWA
Penicillium sp			Plate contaminant	AHL	DAWA

AHL = Animal Health Laboratories; ATCC = American Type Culture Collection; DAWA = Department of Agriculture Western Australia; DPI QLD = Department of Primary Industries, Queensland; EUS = Epizootic Ulcerative Syndrome; ID = identification; MG = Mycotic Granulomatosis; RSD = Red Spot Disease, *Cultures were not viable. $^{\delta}$the culture received was a fungal contaminant and the *A. invadans* was no longer viable.

The cultures supplied by Dr Thomas were sent with the knowledge that they were unlikely to be viable due to time in storage, however it was thought that they could be used as a source of DNA.

6.1.1.2 Tissue material infected with EUS

Archival material in the form of paraffin embedded tissue was available from the Fish Health Unit, Department of Fisheries Western Australia, which is located at Animal Health Laboratories, Department of Agriculture Western Australia. The PCR that was developed was used to detect *A. invadans* from this type of sample. The samples that were tested are listed in Table 6-2, along with the clinical diagnosis and the fungus that was subsequently cultured from these samples. Frozen fish tissue that had lesions diagnostic for EUS were tested by PCR and are listed in Table 6-3.

Table 6-2. Paraffin-embedded tissue from cases of EUS

Case Number	**Diagnosis**	**Fungus cultured**
AS-00-3014	Lesions on body, EUS	None
FH-02-019a	Lesions on body, EUS	*Trichoderma* species
A2S2 culture only	Seeded sample	*A. invadans*
A2S2 + fish tissue	Seeded sample	*A. invadans*

Table 6-3. Frozen stored fish tissue from cases of EUS

Case Number	**Diagnosis**	**Fungus cultured**
AS-00-3014	Lesions on body, EUS	None
AS-03-0417, #53	Lesions on body, EUS	*A. invadans*
AS-03-0342	Lesions on body, EUS	none
FH-02-019a	Lesions on body, EUS	*Trichoderma* species

Mock samples of paraffin-embedded tissue were also prepared using fish tissue blocked-out (*i.e.* co-embedded in paraffin wax for histology) with *A. invadans* fungal hyphae grown in a broth culture (Table 6-2). Culture material from a five day broth culture was fixed in formalin for 24 hours. It was placed into a Petri dish and then molten wax was poured over the fixed fungal hyphae. The wax was set by placing the Petri dish on ice. A square of wax containing the fungal hyphae in wax was cut out and then processed according to normal histological procedure.

6.1.2 Procedures for the design and evaluation of primers

6.1.2.1 Sequence Information available in Genbank
http://www.ncbi.nlm.nih.gov/PubMed/

A reasonable amount of sequence information for *A. invadans* and other aquatic Oomycete fungi was available on Genbank. The genetic information that is known for *A. invadans* is represented by the schematic in Figure 6-1 and is composed of the region of the ribosomal genes from the 18S across both internally transcribed spacer regions including the 5.8S rRNA and the 28S rRNA.

Also, the sequence information for many of the aquatic Oomycete fungi that were likely to be a potential problem for cross-reaction in the PCR or FISH were also available, therefore making it possible to select regions for the design of specific primers.

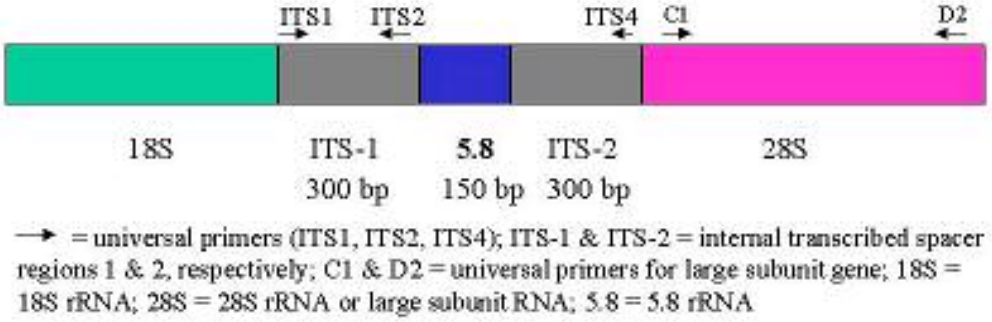

Figure 6-1. Schematic of known sequence information for *A. invadans*.

Table 6-4. Aphanomyces sequences available on Genbank.

Culture	Accession no.	Genome Region	Source or isolate ID
A. invadans	AF349602	18S rRNA gene, partial sequence	RF6
A. invadans	AF349603	18S rRNA gene, partial sequence	10D
A. invadans	AF349604	18S rRNA gene partial sequence	NJM9030
A. invadans	AF349605	18S rRNA gene	3P
A. invadans	AF349614	ITS1	WIC isolate
A. invadans	AF349613	ITS1 partial sequence	NJM9701
A. invadans	AF349612	ITS1 partial sequence	24P
A. invadans	AF349611	ITS1	BH
A. invadans	AF349610	ITS1	S1PA
A. invadans	AF396684	18S-ITS1-5.8S-ITS2-28S (partial) rRNA genes	Menhaden
A. invadans	AY082908	ITS1-5.8S-ITS2-28S (partial) rRNA genes	Catfish
A. invadans	AY082907	ITS1-5.8S-ITS2-28S (partial) rRNA genes	Channel catfish
A. piscicida	AF235941	28S rRNA, partial sequence	= *A. invadans* (in Japan)
Aphanomyces species	AF396683	18S rRNA, (partial sequence)-ITS1-5.8S-ITS2-28S (partial) rRNA genes	Menhaden, ATCC 62427
A. astaci	AF235940	28S rRNA, partial sequence	

Table 6-4, continued

Culture	Accession no.	Genome Region	Source or isolate ID
A. astaci	AF320583	28S rRNA, partial sequence	Hoe
A. astaci	AF320583	28S rRNA	FDL I isolate
A. astaci	AF355099	Subtilisin-like serine proteinase precursor (SP1) gene	(Bangyeekhum *et al.*, 2001)
A. astaci	AF355100	Trypsin proteinase precursor (SP2) gene	(Bangyeekhum *et al.*, 2001)
A. astaci	AJ416354	Partial chi 1 gene for putative chitinase	(Andersson and Cerenius, 2002)
A. laevis	AY283648	18S-ITS1-5.8S-ITS2-28S rRNA (partial) gene	
Achlya aquatica	AF218150	ITS1-5.8S-ITS2-28S rRNA gene	
Saprolegnia diclina	AF218166		
S. ferax	AF218167		
S. ferax	AJ238633	18S rRNA partial gene	
S. ferax	AF036542	ITS1	
S. ferax	AF036543	ITS2	
S. ferax	X01545	5.8S rRNA	
Saprolegnia sp	AY270033	18S-ITS1-5.8S-ITS2-28S rRNA	
Saprolegnia species strain 2	AF218168		
Saprolegnia species strain 1	AF218169		
S. parasitica	AF218170		
S. monoica	AF218171		

ID = identification code; ITS = internally transcribed spacer; no. = number.

6.1.2.2 Resources used for the design of primers

Using the Clustal Alignment software (http://www.ebi.ac.uk/clustalw/ Clustal alignment program (Thompson *et al.*, 1994)), the rRNA sequence information for *A. invadans*, *A. astaci*, other *Aphanomyces* species and closely related fungal species were aligned. Regions of DNA specific to *A. invadans* and *A. astaci*, respectively, were selected for primer design.

Primers were designed using the freeware program Primer3 available on the web at the following address.

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

The primers were tested for amplification efficiency using the electronic PCR program Amplify (for MacIntosh computers only).

Primers were ordered from the company, Qiagen (Qiagen Pty Ltd, Australia). Oligonucleotides with a fluorescein molecule added at the 5 prime end were used in the FISH assay and were also purchased from Qiagen.

Primers were designed within the ITS region of *A. invadans*. Sequence information that was available for *A. invadans* was combined to make a consensus DNA sequence that spanned the entire 18S to 28S rDNA region. This consensus sequence was composed of AF396684 and AY082907. It was used in alignments with other fungal sequences to identify areas that were potentially specific to *A. invadans*.

6.1.2.3 Universal and published primers tested in this project

Table 6-5 lists the universal primers (ITS1, ITS2, ITS4, C1, D2, EF4, fung5) that were used in the course of this project. The primers AIFP1, AIFP2, ASP1, ASP2, APH3 and APH4 were published after the start of this project and had been reported as suitable for the detection and identification of *A. invadans* or in the case of ASP1 and ASP2; *A. astaci*. These primers were tested in this project and compared to primers that were designed in the course of this project.

Table 6-5. List of published primers used in this project

Primer ID	Sequence 5 prime to 3 prime	Reference
ITS 1	TCCGTAGGTGAACCTGCGG	(White *et al.*, 1990; Leclerc *et al.*, 2000)
ITS 4	TCCTCCGCTTATTGATATGC	(White *et al.*, 1990; Leclerc *et al.*, 2000)
ITS 2	GCTGCGTTCTTCATCGATGC	
C1	ACCCGCTGATTTAAGCAT	(White *et al.*, 1990; Leclerc *et al.*, 2000)
D2	TCCGTGTTTCAAGACGG	(White *et al.*, 1990; Leclerc *et al.*, 2000)
EF4	GGAAGGGRTGTATTTATTAG (where R = G/A)	(Smit *et al.*, 1999; Borneman and Hartin, 2000)
Fung5	GTAAAAGTCCTGGTTCCCC	(Smit *et al.*, 1999; Borneman and Hartin, 2000)
AIFP1	AAGGCTTGTGCTGAGCTCACACTC	(Blazer *et al.*, 2002)
AIFP2	GATGGCTAAGGTTTCAGTATGTAG	(Blazer *et al.*, 2002)
ASP1	AATATGCATTTCCTGCCCTG	(Oidtmann *et al.*, 2002)
ASP2	CCACGCTTTCACGGTTTGTA	(Oidtmann *et al.*, 2002)
APH3	ATAAGGCTTGTGCTGAGC	(Lilley, Hart et al. to be submitted)
APH4	CATTTCTGATGGCTAAGG	(Lilley, Hart et al. to be submitted)

ID = identification

ITS1, ITS2 and ITS4 are universal primers that amplify fungal ribosomal DNA genes. The combination of ITS1 and ITS2 primers spans the region known as the internal transcribed spacer 1 (ITS-1) located between the 18S rDNA and the 5.8S rDNA and is approximately 300 bp in size. The universal primers ITS1 and ITS4 amplify a 800 base pair region of DNA from the 18S rDNA to the 28S rDNA (large subunit DNA), which includes the spacer regions 1 and 2 and the 5.8S rDNA, Figure 6.1.

C1 and D2 are universal primers that amplify the large subunit rDNA (28S rDNA) of all fungal species, Figure 6-1.

The universal primers EF4 and fung5 amplify a 550 bp fragment from the 18S rDNA region of all fungi (Smit *et al.*, 1999). This primer pair had been used to amplify the 18S rDNA of Oomycetes fungi (Borneman and Hartin, 2000).

The primers AIFP1 and AIRP2 amplify a 98 bp fragment of the internally transcribed spacer 1 (ITS-1) which lies between the 5.8S rDNA and the 18S

rDNA of *A. invadans* (Blazer *et al.*, 2002). In the original paper these primers had the prefix P1 and P2, respectively. For this project the prefix AIF and AIR were used to denote primers for *A. invadans* (AI) and either forward or reverse primer (F or P).

The primer pair AHP3 and AHP4 amplify a 107 base pair region within the internally transcribed spacer region 1.

Primers ASP1 and ASP2 amplify a 1052 bp fragment of the 28S rDNA in *A. astaci*. This primer pair is not specific and also amplifies the same region in other Oomycete fungi such as *A. laevis*, *A. helicoides*, *Achlya recemosa*, *A. colorata*, *Leptolegnia* species, *Saprolegnia parasitica*, *S. monilifera* (syn *Isoachlya tourlades*), *S. diclina*, and *S. litoralis*. The primers do not amplify a fragment from *Aphanomyces irregulare*. The primers were designed to be used in conjunction with a restriction digest using a number of enzymes to differentiate *A. astaci* from the other Oomycete fungi that were amplified with the primers ASP1 and ASP2 (Oidtmann *et al.*, 2002). The original paper named the primers P1 and P2, but for clarification the prefix AS was used in this project to denote *A. astaci*.

The sequence for *A. invadans* and the location of the primers is detailed in Appendix F.

6.1.2.4 Sequencing of DNA

The sequencing reaction was carried out with universal primers (Table 6-5), and the amplified product was purified using a PCR Purification Kit Protocol for Sequencing (Geneworks). The purified DNA was used in the sequencing reaction using the protocols recommended by PE Applied Biosystems for the ABI PRISM® Big Dye™ Terminator v3.1. Basically, 5.5 µl of DNA at a concentration of 30 ng/µl was added to 4 µl of PCR Dye Terminator Sequencing Mixture and 0.5 µl of primer. The sequencing PCR was performed on a Corbet FTS 320 using an initial denaturation step of 96°C for 2 min 20 secs, followed by 25 cycles of denaturation for 30 sec, annealing at 60°C for 30 secs with a final annealing step of 60°C for 4 min. The dye terminators were removed by precipitating the DNA in ethanol. The material for sequencing was sent to the State Agricultural Biotechnology Centre (SABC) at Murdoch University, Western Australia and the sequencing reaction carried out on a 373 DNA sequencer (Applied Biosystems).

The sequence data information was analysed using the BioEdit software program that is available on the web.
http://www.mbio.ncsu.edu/Bioedit/bioedit (Hall, 1999)

The Blast program was used to search for sequence similarity and for the alignment of 2 sequences.

http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html (Altschul *et al.*, 1997).

6.1.2.5 PCR protocols

The PCR reaction was performed in 0.6 ml tubes (BioRad) on a Corbet FTS 320 thermocylcer. A universal protocol was used for all universal primers and was also used when first testing designed primers. Each 25 µl reaction volume contained 5 µl of DNA, 2.5 µl of 10 x PCR buffer, 2.5 µl of a 2 mM

mixture of all four deoxynucleotide triphosphates (adenine, cytosine, guanine, thymine) with a final concentration of 200 µM, *Taq* DNA polymerase enzyme (PE Applied Biosystems) at a final concentration of 1 Unit, $MgCl_2$ at a final concentration of 2 mM, and 20 pmol of each primer.

When optimising reagent concentrations for different primer sets, $MgCl_2$ was tested at final concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4 mM. The dNTP's were tested at concentrations of 100 µM and 200 µM, and the primers were tested at 20 pmol, 40 pmol, and 60 pmol.

Basic cycling conditions were performed using an initial denaturation temperature of 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. A final cycle was done using an extension temperature of 72°C for 10 min, followed by holding at 4°C. When optimising cycling conditions, especially the annealing temperature, the reaction was done on an iCycler (BioRad). This thermocycler has a gradient program that allows a range of annealing temperatures from 50°C to 68°C to be tested in the one run.

6.1.2.6 Gel electrophoresis

5 µl of amplified product plus 5 µl of 2x loading buffer was placed into the wells of a 2% gel prepared with chromosomal grade agarose (BioRad) and 0.5M TBE buffer. Electrophoresis was carried out at 80 volts for 90 minutes. Gels were stained for 1 hr by immersion in a one litre solution of ethidium bromide in distilled water at a concentration of 50 µl/l. The gel was stained with ethidium bromide and digitally photographed over ultraviolet light using an Alpha Innotech gel documentation system. Alternatively, ethidium bromide (0.8 µl to 90 ml buffer and agarose solution) was added to the gel at the time of preparation.

6.1.2.7 Testing PCR specificity

The specificity of the primers both published and those designed in this project were tested against the fungal cultures listed in Table 6-1. If cross-reactions occurred, the PCR reaction was repeated using a range of $MgCl_2$ concentrations, a dNTP concentration of 100 µM and 200 µM, a range of primer concentrations and a range of annealing temperatures as indicated in the optimisation of the PCR reaction under PCR protocols, 6.1.2.5. Primers that had been published were tested under the conditions stated in the appropriate reference, however if adverse reactions were obtained then the PCR protocol was optimised as previously indicated.

The PCR was also tested against strains of *A. invadans* from other parts of Australia, Thailand, Japan and USA to determine the possibility of cross-reaction between *A. astaci* and *A. invadans*. The primers that were developed for *A. invadans* were tested against all strains of *A. invadans* from the aforementioned countries to determine if the primers were capable of amplifying DNA from all of these strains.

6.1.2.8 Testing PCR sensitivity

Primers that were specific were then tested for sensitivity of the reaction. DNA that had been extracted from broth culture was serially diluted and each dilution tested in the PCR reaction.

6.1.3 DNA extraction methods

6.1.3.1 DNA extraction from fungal culture

A number of different methods were trialled to determine the method most suitable for extraction of DNA from cultured material. The methods were tested on fungi grown on plate media and broth culture. The methods that were used included manual methods and the use of commercially available DNA extraction kits.

Cultures were grown on GP or IMTAP plates for 3-5 days. Broth cultures were performed using 10 ml of GP broth in disposable MacCartney bottles. Material from the growing edge of a culture from a plate was subcultured to a broth. Cultures were incubated at 25°C for 4-7 days. The fungal growth was removed from the broth using flame-sterilized forceps and the liquid drained before placing in either a 1.5 ml microfuge tube or mortar. Fungal material that wasn't being treated immediately was stored at –20°C until required.

Media recipes and instructions for growth and isolation of fungi are listed in Appendix C.

A. *Boiling method*

Fungal culture from a broth was removed and placed into a 1.5 ml microfuge tube. The tube was placed into a boiling waterbath and boiled for 3 minutes. Glass beads (106 micron) were added and the mixture vortexed for 3-5 minutes. Boiling and vortexing steps were repeated once. The material was centrifuged for 5 min at 10,000 rpm and the supernatant was used in the PCR reaction.

B. *Grinding in liquid nitrogen*

Grinding in liquid nitrogen was assessed to determine whether it improved the amount of DNA extracted from the Oomycete fungi. It was also used as a preliminary treatment prior to use of fungal material in other DNA extraction methods.

Culture material from a broth culture (about the size of a 20 cent piece) or a plate culture was placed into an ice-cold mortar. Liquid nitrogen was added and the fungal hyphae ground to a powder using a pestle.

Grinding using a micro pestle and a microfuge tube was also assessed. Growth from a broth culture was placed into a 1.5 ml microfuge tube to a depth of 1/3 of the tube. The tube was either placed into liquid nitrogen for 3 minutes until the contents were frozen, or liquid nitrogen was added to the contents of the microfuge tube and then ground with a sterile micro pestle.

C. *Addition of sodium dodecyl sulphate (SDS)*

The use of 2% SDS to breakdown the fungal hyphae was also tested. The use of SDS on its own, or as an initial step prior to application to a commercial DNA extraction kit was assessed. Fungal mycelia were placed into a 1.5 ml microfuge tube to a depth of 1/3 of the tube. 400 µl of TE buffer was added and mixed by vortexing. Then 80 µl of 10% SDS was added and mixed by vortexing. The solution was boiled for 10 min in a steaming waterbath.

The lysate was used directly in the PCR reaction, or used as an initial step prior to extraction of the DNA using the Dneasy Qiagen kit.

D. *Addition of lysozyme*

The use of lysozyme as an aid to the destruction of fungal hyphae to expose the DNA was assessed. It was used in combination with other methods. Lysozyme was used at a final concentration of 1 mg/ml. In the DNeasy Qiagen method, lysozyme was used at step 2 with 8 µl of stock lysozyme (50 mg/ml) added to 400 µl of Buffer AP1.

E. *Addition of proteinase K*

The effect of Proteinase K was assessed by incorporating an incubation step with proteinase K following the addition of Buffer AP1 and Rnase in the Qiagen DNeasy mini plant tissue kit method. 10 µl of 1.4 mg/ml of Proteinase K was added, and the tube incubated at 50°C overnight. The mixture was heated at 80°C for 15 min to inactivate the proteinase K. The method was continued according to the protocol at the 65°C incubation step.

F. *Qiagen DNeasy mini plant tissue kit*

The DNeasy Plant mini kit was recommended by technical personnel at Qiagen for the isolation of DNA from Oomycete fungi. The protocol was followed as recommended in the kit for the extraction of DNA from plant tissue and fungal tissue. Fungal growth from a broth culture was ground to a powder in a cold mortar and pestle without allowing the liquid nitrogen to evaporate. To approximately 100 mg of wet weight of ground material was added 400 µl of Buffer AP1 and 4 µl of Rnase stock solution (100 mg/ml). The solution was vortexed vigorously. The mixture was incubated at 65°C for 10 min with 2-4 inversions during the incubation time. 130 µl of Buffer AP2 was added to the lysate, mixed and incubated on ice for 5 min. The tube was centrifuged at maximum speed for 5 min and the supernatant applied to the QIAshredder spin column. The tube was centrifuged for 2 min at maximum speed and the flow-through transferred to a new tube. 1.5 ml volume of Buffer AP3/E was added and mixed by pipetting. 650 µl of the mixture was applied to the DNeasy spin column and centrifuged for 1 min at 8,000 rpm. The flow-through was discarded. 500 µl of Buffer AW was added to the spin column and centrifuged for 1 min at 8,000 rpm. The flow-through was again discarded and 500 µl of Buffer AW added to the column and centrifuged on maximum for 2 min. 50 µl of preheated (65°C) Buffer AE was added to the column and centrifuged for 5 min at 8,000 rpm. The eluted DNA was collected and stored at –20°C until required.

G. *E.Z.N.A™. plant DNA miniprep kit*

The E.Z.N.A. plant miniprep kit was recommended for Oomycete fungi. The protocol was followed as recommended by the manufacturer for fresh or frozen samples. Basically the tissue or fungal culture was placed into a 1.5 ml microfuge tube and snap frozen using liquid nitrogen. The material was ground using a disposable pellet pestle. Pestles were soaked in a bleach solution after each use and washed and sterilized before re-use. 600 µl of Buffer P1 was added to 100 mg of ground material followed by 10 µl of 2-mercaptoethanol. The mixture was vortexed until all lumps were dispersed. The sample was incubated at 65°C for 10 minutes, and mixed by inversion twice during the incubation time. 140 µl of Buffer P2 was added and mixed by vortexing. It was then centrifuged at 10,000 rpm for 10 min. The lysate was aspirated into a fresh microfuge tube and 0.7 volumes of isopropanol was added. The DNA was precipitated by vortexing. The DNA was pelleted by centrifuging at 10,000 rpm for 2 min. The supernatant was decanted and 300 µl of sterile ultrapure water previously heated to 65°C was added to the pellet of DNA. The DNA was resuspended by vortexing. 150 µl of Buffer P3 was added to the DNA followed by 300 ul of absolute ethanol and vortexing. The sample was then applied to the supplied HiBind™ DNA column and centrifuged for 1 min at 10,000 rpm. The column was transferred to a new microfuge tube and 750 µl of wash buffer was added to the DNA bound to the column. The wash buffer had been prepared by dilution with absolute ethanol. The column was centrifuged at 10,000 rpm for 1 min and the flow-through discarded. The wash step was repeated and then the column was dried by centrifuging at the maximum speed for 2 min. The column was transferred to a new microfuge tube and 100 µl of 10mM Tris buffer pH 9.0 (pre-warmed to 65°C) was added and incubated at room temperature for 1 minute. The DNA was eluted by centrifuging at 10,000 rpm for 1 min. The elution step was repeated with a further 100 µl of Tris buffer. The DNA was stored at –20°C until required.

H. *DNAzol reagent*

DNAzol (Invitrogen, Life Technologies) is a commercially available reagent for the isolation of genomic DNA from samples from animal, plant, yeast and bacteria and is based on a guanidine-detergent lysing solution, which selectively precipitates the DNA from the cell lysate. The protocol was followed according to the manufacturers instructions. 25-50 mg of fungal hyphae was added to 700 – 800 µl of DNAzol reagent in a 1.5 ml microfuge tube. The material was homogenised for 2-3 min using a sterile disposable hand held microfuge pestle. (The lysate/homogenate can be stored for 18 h at 15-30°C or for 3 days at 2-8°C, if required, at this stage). The homogenate was centrifuged for 10 min at 13,000 rpm and the supernatant transferred to a fresh labelled tube. The DNA was precipitated by adding 400 µl of 100% ethanol, and mixing by inversion. The tube was centrifuged for 5 min at 13,000 rpm and the supernatant decanted. The pellet was washed by adding 600 µl of 75% ethanol and then resuspended in ethanol, before centrifuging for 5 min at 13,000 rpm. The supernatant was decanted. The wash step was repeated. The pellet was air dried so that all the residual ethanol was evaporated. The DNA was dissolved in 8 mM NaOH. If pellet was >2 mm in

diameter 150 µl of 8mM NaOH was added. If pellet was small 50-100 µl of 8mM NaOH was added. As recommended by the manufacturer, heating assisted in dissolving the pellet.

I. *Puregene DNA extraction kit*

The Puregene kit (Gentra) was assessed for the extraction of DNA from fungal material. The protocol for plant tissue was followed. Fresh fungal culture was ground in liquid nitrogen using a mortar and pestle. The ground material was placed into a 1.5 ml microfuge tube. 300 µl of Cell Lysis buffer was added. The tube was incubated at 65°C for 60 minutes. At 30 and 60 minutes the tube was mixed by inverting the tube 10 times. After the sample was cooled to room temperature, 100 µl of Protein Precipitation solution was added to the cell lysate. The mixture was mixed by vortexing at high speed for 20 seconds. If the sample contained high polysaccharides the mixture was put on ice for 15-60 minutes. The lysate was centrifuged for 3 min at 10,000 rpm. The DNA in the supernatant was removed to a new 1.5 ml microfuge tube containing 300 µl of 100% isopropanol. The mixture was gently mixed by inversion. The DNA was pelleted by centrifugation at 13,000 rpm for 1 min. The pellet was washed in 300 µl of 70% ethanol at 13,000 rpm for one minute, and then the ethanol removed and the DNA pellet allowed to dry. The DNA was hydrated using 50 µl of DNA Hydration solution and incubating at 65°C for 1 hour. The DNA was stored at –20°C.

J. *InstaGene matrix*

InstaGene matrix (BioRad) is recommended for bacteria, but was tested to determine its suitability for extraction of DNA from fungal cultures.

Fungal growth was washed once in distilled water, by centrifugation at 10,000 rpm for 1 minute. The supernatant was removed and replaced with 200 µl of InstaGene matrix. The mixture was incubated at 56°C for 15-30 minutes. The mixture was vortexed, and then incubated in a boiling waterbath for 8 minutes. The mixture was again vortexed for 10 seconds on high speed and then centrifuged for 3 minutes at 10,000. The supernatant was tested for fungal DNA.

K. *PrepMan reagent*

The PrepMan reagent (Applied Biosystems) is recommended for extraction of DNA from food pathogens. It was assessed for its potential for extraction of DNA from Oomycte fungi.

The reagent was mixed gently to re-suspend the reagent as recommended by the manufacturer. 200 µl of reagent was added to fungal hyphae in a 1.5 ml microfuge tube. The mixture was vortexed on high speed and then incubated in a boiling waterbath for 10 minutes. The samples were cooled to room temperature and then centrifuged for 2 minutes at 13,000 rpm. 50 µl of sample was transferred to a fresh 1.5 ml microfuge tube containing 50 µl of sterile distilled water. This mixture was used to assess DNA extraction.

6.1.3.2 DNA extraction from fresh tissue

Fungal DNA from *A. invadans* was extracted from fish tissue stored at either –20°C or –80°C using similar methods to those used previously.

A. *DNAzol reagent*

A 20 mg piece of tissue was added to 800 µl of DNAzol reagent and homogenised using a sterile micro pestle. The rest of the protocol was followed as detailed in 6.1.3 part H.

B. *DNeasy Qiagen mini plant tissue kit*

The tissue was ground in liquid nitrogen and the material then treated according to the protocol as recommended by the manufacturer, section 6.1.3, part F.

C. *Manual method* 1 (Lee and Taylor, 1990)

Fresh tissue or fungal mycelia (0.1 – 0.3 g) were ground in liquid nitrogen using a cold mortar and pestle. The ground material was placed into a 1.5 ml microfuge tube. 400 µl of lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl, 1% sodium dodecyl sulphate) was added to the material and incubated for 1 hour at 65°C. 400 µl of chloroform:phenol (1:1, v:v) was added and vortexed. The mixture was centrifuged for 13,000 rpm for 1min at room temperature. The upper aqueous phase (300 – 350 µl) was transferred to a fresh tube, and 10 µl of 3M NaOAc added followed by 0.54 volumes of isopropanol. The phases were mixed by inversion, and then centrifuged for 15 min at 13,000 rpm. The supernatant was removed and the pellet rinsed with 70% ethanol. The ethanol was removed and the DNA pellet dried. The pellet was re-suspended in 100 µl of TE buffer.

D. *Manual method* 2 (Fish Health Unit, Dept of Fisheries WA)

Fish tissue was stored frozen at –80°C or in an alcohol preservative. A 100 mg piece of tissue was placed into a 1.5 ml microfuge tube and 450 µl of digestion solution and 50 µl of proteinase K (2 mg/ml) were added. The tubes were kept on ice. The tissue was homogenised using a disposable sterile micro pestle. The homogenate was centrifuged for 5 min and supernatant removed to a fresh tube. The supernatant was incubated at 65°C for 1 hour or overnight. Proteinase K was inactivated by heating at 80°C for 15 min. Then, 600 µl chloroform/isoamyl alcohol (24:1) was added and the mixture vortexed gently for 20 secs. The tube was centrifuged for 5 min at 10,000 rpm. The upper layer was removed to a fresh tube. The chloroform/isoamyl alcohol step was repeated and the upper layer removed to a fresh tube. 400 µl of cold isopropanol was added to the aqueous part and the tube mixed by inverting the tube carefully until the solution was clear. The DNA was precipitated by centrifugation for 5 min at 6,500 rpm. The precipitate was washed with 400 µl of 70% alcohol, twice using centrifugation at 6,500 rpm for 5 min. A final wash was done in redistilled 95% alcohol for 5 mins at 6,500 rpm. The DNA was air-dried before reconstituting with 20 µl sterile milliQ water.

<u>Stock digestion solution</u>.

1 ml of 1M NaCl, 0.1 ml of Tris-HCl (pH 8.0), 1 ml of 0.2 M EDTA (pH 8.0), 1% SDS. Make up to 10 ml.

6.1.3.3 DNA extraction from paraffin-embedded tissue

Two methods were used to extract DNA from paraffin-embedded tissue. Early in the project the following method (Bateman *et al.*, 1997) was used to extract DNA from paraffin-embedded tissue infected with crayfish plague.

(i). Two 12 micron sections were cut and placed into a 0.6 ml microfuge tube. The sections were dewaxed in 500 µl of xylene and mixed for 5 min. The xylene was removed the dewaxing step repeated twice. 500 µl of 100% ethanol was added to the tube and mixed by gentle inversion for 5 min. The ethanol was removed. The material was treated with ethanol twice more. The sample was completely dried. The material was then used in the DNeasy Qiagen Mini Plant Tissue kit with a proteinase K digestion step added as described in section 6.1.3 part E and part F.

(ii). Later in the project, the extraction of DNA from paraffin-embedded tissue from diagnostic cases of EUS and from seeded samples was performed using a second method (Miller *et al.*, 1997; Whittington *et al.*, 1999). A new microtome blade was used for each block and the microtome contact surfaces were wiped with ethanol between each block to prevent cross-contamination. The block was wiped with 100% ethanol and the first few sections discarded. The blade, operator gloves and applicator stick were changed, and then two 5 micron sections were cut and placed into a 1.5 ml screw-capped centrifuge tube. The tube was centrifuged at 10,000 rpm for 1 min to pellet the tissue. 200 µl of sterile water and 0.5% v/v of Tween 20 was added to the pellet and boiled for 10 min. The boiled tissue was snap frozen in liquid nitrogen. The boiling and freezing were repeated three times. After a final 10 min boil the tissue was centrifuged at 3,000 rpm for 20 min. The supernatant was removed to a fresh tube. For use in the PCR, 5 µl of diluted, and undiluted, supernatant was used in the reaction.

6.1.4 Maintenance of oomycete fungal cultures

Oomycete fungi are unable to be stored using the traditional storage methods used for bacteria and true fungi, such as freeze-drying or storage at –80°C. They are killed by freezing and when undergoing the freeze-drying process. It is recommended that cultures be maintained by subculturing onto fresh agar slopes every 6 months. During the course of acquiring cultures of *Aphanomyces* species and *A. invadans* for use in the PCR, and for specificity studies, it came to our attention that many culture collections were unable to maintain their collections of Aphanomyces. Six isolates were requested from the American Type Culture Collection (ATCC) in America. The ATCC sent cultures but these were non-viable on arrival at our laboratory and had been transported on dry ice and hence frozen. No amount of correspondence could persuade ATCC and their transportation company Cryosite to freight the cultures at room temperature. Requests to transport the cultures on agar slopes would have incurred a further charge of $US400 per culture plus freight costs and would have taken another 8 months to do so. It had already taken 8 months to obtain the non-viable cultures.

Enquiries were made to the CABI (Centre for Agriculture and Biosciences International, UK) for other isolates. However, CABI was unable to provide

the isolates, as they had been unable to maintain the viability of the cultures donated to them.

It is tedious and time consuming to subculture a large number of isolates every 6 months to maintain viability. Therefore, in this project a number of methods were assessed to determine the most appropriate method for storage of *A. invadans* and related fungi for the long and short term. The type of media, temperature and length of time of storage for each factor were investigated.

The cultures used in the storage assessment study are listed in Table 6-6.

The storage media that were assessed were Glucose Peptone (GP) plates, GP slopes, Isolation Medium (IM) plates, and distilled water (DW). Two temperatures were investigated (room temperature and 4°C), and the assessment was conducted for 12 months. Media is detailed in Appendix C.

Table 6-6. Cultures used in storage assessment

Group Designation	Isolate Name	Isolate Number/Notes
Group 1	*Pythium sulcatum*	WAC 8470
Group 2	*A. invadans*	NSW isolate (A2S2, #24)
Group 3	*A. invadans*	Isolates obtained from AAHRI (T99G2,G2PA,RF8, RF6, 4P, NJM9701, PA8, B99C)
Group 4	*A. invadans*	USA isolate (#54)
Group 5	*A. invadans*	WA isolate (#53)
Group 6	*Saprolegnia* sp.	Project Ref No. #9, #11
Group 7	Unidentified fungus	AS-01-4189

6.1.4.1 Method 1. Slope media

A. Without oil overlay

This method was based on the storage method recommended by Dr. D. Alderman, CEFAS, UK. Dr Alderman recommends storage of duplicate cultures at 4°C and 7°C. However, we were unable to obtain an incubator or refrigerator that could be set at 7°C. Therefore, the tests were assessed at 4°C and room temperature.

Universal bottles (30 ml) were used with a length of slope from the bottom of the tube to near the rim to obtain a maximum surface area. The lid was kept tightly closed to prevent evaporation. Agar slopes were prepared using GP medium. Blocks of agar were transferred by inverting them face down onto a new medium. A strong loop or a sterile needle was used to take out a plug of agar from the old medium and transfer the plug growth-side down onto a fresh slope. Duplicate cultures were placed at room temperature in the dark and at 4°C in the dark.

B. *With paraffin oil overlay*

This method is based on the recommendations of the Centraalbureau voor Schimmelcultures (Netherlands). Duplicate fungal cultures were grown on agar slopes prepared with GP medium, and before growth reached the sides

of the tube, the tube was sealed to the top with sterile paraffin oil. One sealed tube was placed at 4°C and the other at room temperature. Both were kept in the dark.

6.1.4.2 Method 2. Storage under sterile distilled water

This method was used for oomycete fungi isolated from plants and was recommended by Dominie Wright, Plant Pathologist, Dept of Agriculture Western Australia. This method enables the recovery of plant oomycete fungi six years after initial storage.

A large plug of agar containing fungal growth was cut from the original medium using a sterile scalpel blade, or forceps, and placed into a MacCartney bottle containing 10 ml of sterile distilled water. The lid was closed tightly and sealed with parafilm to prevent loss of water thorough evaporation. A duplicate culture was sealed with a sterile paraffin oil overlay.

6.1.4.3 Method 3. Storage on plate media

Fungal cultures were grown on GP and IM media in Petri dishes. Duplicate plates were sealed with parafilm and stored at room temperature and 4°C.

6.2 Development of a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

6.2.1 Sources of A. astaci

AQIS approval was given to import formalin fixed tissue infected with the agent of crayfish plague, paraffin embedded infected tissue, and purified DNA obtained from culture material. All these samples were obtained from Dr. David Alderman, Centre for Environment, Fisheries and Aquaculture Science (CEFAS), United Kingdom.

The formalin-fixed freshwater crayfish were narrowclaw crayfish (*Astacus leptodachylus*) from laboratory-acquired infections stored in formalin since 1985, and Whiteclaw (*Austropotambius pallipes*) from natural infection from March 2002.

Two paraffin blocks containing infected crayfish tissue and a pellet of purified DNA obtained from a broth culture of *A. astaci* were also sent from the CEFAS. These were given the AHL laboratory case number of AS-02-1993.

6.2.2 Design and evaluation of primers for A. astaci

6.2.2.1 Sequence Information available in Genbank

http://www.ncbi.nlm.nih.gov/PubMed/

Some sequence information for *A. astaci* was available on Genbank as represented by the coloured areas in Figure 6-2. Partial sequence information for the chitinase gene and two serine proteases, trypsin and subtilisin were available, as were the 18S and the 28S rRNA regions. However, sequence information on the region that spans between these genes, the internally transcribed spacer regions 1 and 2, and the 5.8S rRNA were not known.

Sequence information for other aquatic oomycete fungi that were likely to be a potential problem for cross-reaction in the PCR or FISH assays were also available, as presented in Figure 6-2.

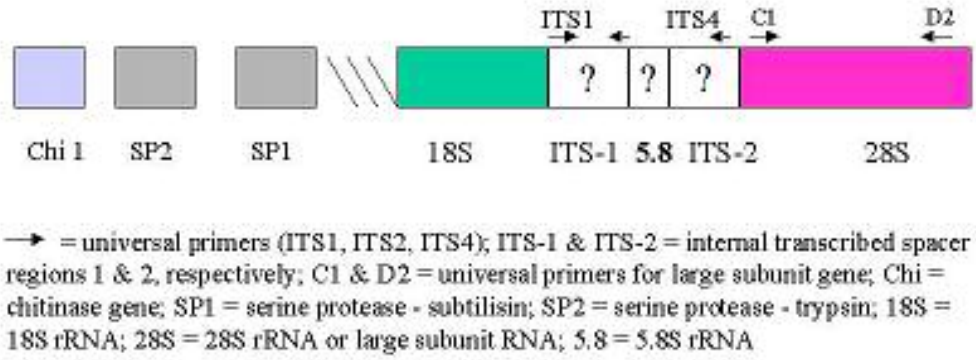

Figure 6-2. Schematic of known genetic sequence for *A. astaci*.

The design of primers was based on the following putative virulence genes (Subtilisin-like serine proteinase precursor (SP1) gene, Trypsin proteinase precursor (SP2) gene and Partial chi 1 gene for putative chitinase gene, Table 6-7. The protocols for designing primers are detailed in 6.1.2.

The PCR protocols are detailed in section 6.1.2.5, and testing of PCR specificity is described in section 6.1.2.7.

Table 6-7. *Aphanomyces astaci* sequences used for designing specific primers.

Culture	Accession number	Genome Region	Source or isolate ID
A. astaci	AF355099	Subtilisin-like serine proteinase precursor (SP1) gene	(Bangyeekhum *et al.*, 2001)
A. astaci	AF355100	Trypsin proteinase precursor (SP2) gene	(Bangyeekhum *et al.*, 2001)
A. astaci	AJ416354	Partial chi 1 gene for putative chitinase	(Andersson and Cerenius, 2002)

ID = identification

6.2.3 DNA extraction methods for A. astaci-infected material

6.2.3.1 DNA extraction from paraffin-embedded tissue

Two paraffin-embedded wax blocks were received from the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in the UK. DNA was extracted according to section 6.1.3.3.

6.2.3.2 DNA Extraction from formalin-fixed tissue

A small piece (size of 5 cent piece) of formalin fixed crayfish tissue was selected and placed into 10 ml of sterile distilled water for one hour to remove the formalin. A further two water washes were performed and then the tissue was left for 2 days at 4°C. A final water replacement was done and the tissue stored at –20°C until DNA extraction was carried out.

Two commercial kit methods were tested as per the manufacturer's instructions and with modifications to determine if the yield of DNA could be improved.

A. *DNAzol reagent (Invitrogen, Life Technologies)*

20 mg of washed tissue was ground with a micro pestle in the DNAzol reagent. The method was followed according to the manufacturer's instructions, which are also detailed in section 6.1.3.1.

B. *DNeasy Qiagen mini plant tissue kit – no modifications*

DNA was extracted using the DNeasy Qiagen mini plant tissue kit (Invitrogen, Life Technologies) according to the manufacturer's instructions. The tissue was placed into a 1.5 ml microfuge tube and AP1 buffer and Rnase were added as specified. The tissue was ground finely in the buffer using a micropestle. The instructions are detailed in section 6.1.3.1.

B (i) *Prior treatment with liquid nitrogen*

The formalin-fixed tissue was frozen with liquid nitrogen and ground in a mortar and pestle. This material was then used in the extraction procedure.

B (ii) *Added proteinase K digestion*

The Dneasy Qiagen kit was also used with a proteinase K step added to the protocol as detailed in section 6.1.3.1, part E and part F.

6.3 Development of a rapid molecular diagnostic test for the detection of *Aphanomyces invadans,* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

6.3.1 Preparation of specific fluorophore

Primers that were specific in the PCR were prepared with fluorescein isothiocyanate added to the 5 prime end of the primer, and were tested to determine their suitability for use as a specific fluorophore in the fluorescent in-situ hybridization (FISH) method. The fluorophores were prepared by Qiagen.

6.3.2 Preparation of fluorophore for positive control

Universal primers C1 and D2 were prepared with a fluorescein added to the 5 prime end and used to determine their suitability as a fluorophore to be used as a positive control.

6.3.3 Cultures used in the development and specificity testing for FISH

The cultures listed in Table 6-8 were used to test the specificity of the fluorophores designed for the detection of *A. invadans*.

Table 6-8. Cultures used for *in-situ* hybridization

Isolate name	Isolate ID	AHL Lab ID
A. invadans	A2S2 (NSW)	#24
A. invadans	UM3	#38
A. invadans	T99G2 (IMI 836084)	#39
A. invadans	4P	#44
Saprolegnia sp.	AS-03-3416	AS-03-3416
Saprolegnia sp.	AS-03-3827-4	AS-03-3827-4
Saprolegnia sp.	AS-03-3827-5	AS-03-3827-5
Unidentified fungus	AS-01-4050-6	AS-01-4050-6

ID = identification

6.3.4 Fluorescent in-situ hybridization using broth culture

The following method was adapted from methods suggested at website http://www.pasteur.fr/recherche/unites/biophyadn/e-fish.html

An isolate was grown in GP broth for 4–7 days until the colony was about the size of a 50 cent piece. Approximately 20-25 mg (wet weight) of cultured hyphae were placed into a 0.6 ml microfuge tube and 200 µl of formamide (Sigma chemicals) was added to the mass of hyphae. The tube was incubated at 70°C for 3 hours. The formamide was removed by aspiration before adding 5 µl of 100 pmol of fluorescent probe. The probe was allowed to hybridize overnight at 55°C. All reactions involving the probe were protected from the light. The stock solution and working solution of the probe were kept in the dark and the tube wrapped in tin foil to prevent exposure of the fluorophore to the light when being used on the bench. After hybridization the hyphae were washed in wash buffer (20mM Tris-HCl, 0.9 M NaCl, pH 7.2) for one minute. A second wash step was carried out using a skim milk wash for one minute. These two washes were repeated, followed by a final wash in buffer for 30 minutes. The hyphae were removed from the microfuge tube, and placed on a glass microscope slide and allowed to drain. Mounting medium (permaflor) was applied to the hyphae on the glass slide, which was covered with a coverslip, and examined using a fluorescent microscope.

All samples were treated with the test probe and the positive control probe.

A number of variations to the method and procedure were tried to determine the easiest way of dealing with the fungal material and for optimising the reagents and the method.

A. *Preparation of hyphae on a glass slide.*

As an alternative to incubating the probe in a microfuge tube the hyphae were placed onto a glass slide. The probe was added to an area marked with a chinagraph pencil and then placed into a wet chamber, in the dark. The wet chamber was prepared using damp filter paper in the lid of a Petri dish and the slide supported by two small pieces of 'orange stick'. Formamide exposure and incubation to the probe were all carried out on the glass slide.

B. *Use of skim milk as blocking agent.*

Skim milk is used as a blocking agent in probe methodologies. A skim milk wash was tested to determine if cross-reactions could be reduced, or the strength of the hybridisation reaction for *A. invadans* could be improved.

C. Probe hybridization time

Probe hybridisation time was tested at times of 1, 2, 3, hours, and overnight to determine the effects on specificity and sensitivity.

D. Hybridisation temperature

Probe hybridization temperatures were tested at 37°C, 55°C and 60°C to determine if specificity and sensitivity of the reaction could be improved.

E. Different incubation times of formamide

Different incubation times in formamide were tested. These were 1, 2, 3, 4 hours, or overnight.

6.4 Development of a rapid molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

The FISH for *A. astaci* was unable to be achieved in this project because the lack of DNA meant that primers were unable to be assessed for specificity and sensitivity in the PCR reaction. Primers that were found to be specific would then have been used to develop the FISH method.

6.5 Publish Australian Standard Diagnostic Techniques based on the above tests and in the format supplied by AFFA.

The Australian and New Zealand Standard Diagnostic Techniques manual will be written once validation for the *A. invadans* PCR has been completed and the primers for the *A. astaci* PCR have been assessed. This work will be completed in FRDC project 2004-091.

7. RESULTS

7.1 Development of a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

7.1.1 DNA extraction methods

7.1.1.1 DNA extraction from fungal culture

A number of different DNA extraction methods were tested to determine their suitability for oomycete fungi. The methods were also tested against true fungi such as *Penicillium* species, as many of the kits and methods had been used for these fungi and reported in the literature. Testing the methods using true fungi provided a check on the method, and compared the ease of DNA extraction from true fungi as opposed to oomycete fungi.

Table 7-1. Results of DNA extraction methods on culture material.

Destruction method	DNA extraction method	DNA obtained
None	DNeasy Qiagen kit	Yes, but results variable
None	DNeasy Qiagen kit + proteinase K	Yes
Liquid nitrogen grinding with micro pestle	DNeasy Qiagen kit + proteinase K	Yes, strong band chromosomal DNA
Liquid nitrogen grinding with Mortar + pestle	DNeasy Qiagen kit + proteinase K	Yes, strong band chromosomal DNA. Results could be variable
Liquid nitrogen grinding with Mortar + pestle	DNeasy Qiagen kit	Yes, strong band chromosomal DNA
Lysozyme	DNeasy Qiagen kit	Improved result
SDS + Lysozyme	DNeasy Qiagen kit + proteinase K	No chromosomal DNA
Liquid nitrogen grinding with Mortar + pestle	DNAzol kit	No chromosomal DNA seen
Grinding with micro pestle in reagent	DNAzol kit	Yes, weak band chromosomal DNA
Freezing at –20°C	DNAzol kit. Grind in reagent with micropestle	No chromosomal DNA seen, but product amplified with universal primers
Liquid nitrogen grinding with Mortar + pestle	EZNA kit	Yes, strong band chromosomal DNA. Results variable - some extractions did not produce DNA
None	PureGene kit	Chromosomal DNA from *A. invadans*. *Penicillium* sp and *Saprolegnia* sp. No product when used in PCR
None	PrepMan	No DNA from oomycetes or true fungi
None	InstaGene	No DNA from oomycetes or true fungi
Liquid nitrogen grinding with Mortar + pestle	Manual Method 1	Faint band of chromosomal DNA seen with *Saprolegnia Penicillium* sp and *A. invadans*

Table 7-1, continued

Destruction method	**DNA extraction method**	**DNA obtained**
Boiling	None	No DNA from oomycetes or true fungi
Boiling and vortexing with glass beads	None	No DNA
Boiling and vortexing with glass beads	DNeasy Qiagen kit	No DNA
Boiling with SDS	None	No DNA
SDS	None	No DNA
None	Phenol chloroform extraction	No DNA
Liquid nitrogen grinding with Mortar + pestle	Phenol chloroform extraction	No DNA
None	lysozyme	No DNA
Liquid nitrogen grinding with Mortar + pestle	lysozyme	DNA seen
Liquid nitrogen grinding with Mortar + pestle	No lysozyme	No DNA
Liquid nitrogen grinding with micro pestle	lysozyme	DNA seen
Liquid nitrogen grinding with micro pestle	No lysozyme	No DNA seen
Liquid nitrogen grinding with Mortar + pestle + SDS		No DNA seen
Liquid nitrogen grinding with micro pestle + SDS		No DNA seen
Liquid nitrogen grinding with micro pestle + buffer + mercaptoethanol	None	No DNA seen

Most methods used for the extraction of DNA from true fungi and from oomycete fungi recommend grinding the hyphae in liquid nitrogen. These results, shown in Table 7-1, confirmed that grinding in liquid nitrogen was required for the majority of DNA extraction methods. However, the method could be difficult to use. Any moisture from the broth culture or agar from a plate culture froze immediately when the liquid nitrogen was added to the material in the mortar and pestle, or into the microfuge tube. The frozen material stuck to the sides of the mortar, which made grinding to a fine powder, difficult. The method recommends grinding the hyphae without allowing the material to thaw out, however, this was found to be impossible to achieve because the moisture was unable to be removed from the culture that had been grown in a broth. It also was impossible to pick out fungal material from the agar plate media because *Aphanomyces invadans* grows through the agar and does not produce aerial hyphae as other true fungi do.

It is necessary to use a mortar and pestle that is ice-cold to reduce the "spitting" of the liquid from the hyphae when the liquid nitrogen is added to the mortar. This is a possible source of cross-contamination if a number of DNA extractions are being performed at the same time.

A recent paper suggested drying the fungal hyphae between pieces of filter paper (Oidtmann *et al.*, 2004), however this method was not tested in this project.

The DNeasy Plant Mini kit from Qiagen gave variable results with different fungi tested. The plant oomycete, *Pythium sulcatum* produced a weak band of chromosomal DNA, as did the true fungus *Penicillium* species (Table 7-1). Different extraction runs for *A. invadans* gave variable results. Grinding in liquid nitrogen was required with this method, as without this step, no chromosomal DNA was seen on the gel (Figure 7-1). Proteinase K and lysozyme were added as extra steps in the DNeasy Qiagen mini plant tissue kit, as this kit produced small amounts of DNA when other methods were unable to produce DNA. It was hoped that the yield of DNA could be improved when these extra steps were used in the extraction process. The use of proteinase K and lysozyme did appear to produce more DNA than when these reagents were not used, although results were variable in some extractions (data not shown). On the whole, the yield of DNA was more consistent when a proteinase K digestion step was added. Chromosomal DNA could be seen on a gel when a proteinase K, or lysozyme digestion step was added to the DNeasy protocol (Figure 7-1). Using proteinase K and lysozyme together did not appear to improve the yield of DNA over the reagents used individually with the DNeasy protocol.

Other reagents were trialled to determine if they had an improved effect for breaking up the hyphae of oomycete fungi. These were the use of SDS, boiling, or boiling and vortexing the hyphae with glass beads, prior to the use of the DNeasy Qiagen mini plant tissue kit. Prior treatment with SDS, or simple treatment such as boiling and vortexing the fungal hyphae did not improve the yield of DNA (Table 7-1).

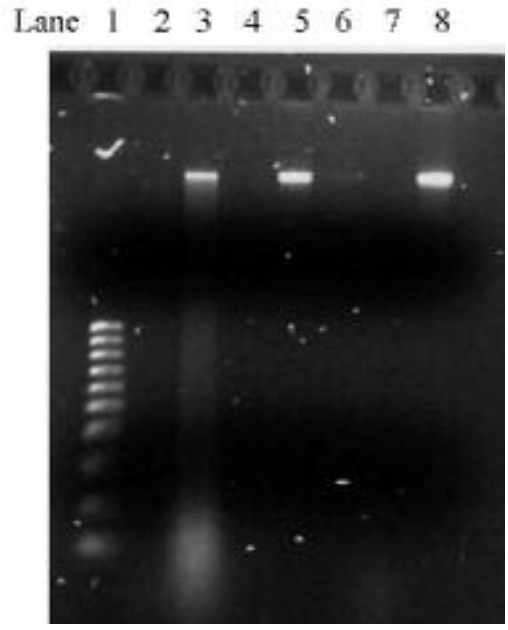

Figure 7-1. Results for chromosomal DNA extraction.

Lane 1 = 100 base pair molecular weight marker (Geneworks); Lane 2 = *A. invadans* #46 DNA extracted using boiling and glass beads; Lane 3 = *A. invadans* #44 DNA extracted using EZNA fungal DNA extraction kit; Lane 4 = *A. invadans* #46 DNA extracted using Boil & Beads & DNeasy Plant tissue kit (Qiagen); Lane 5 = *A. invadans* #39 DNA extracted using liquid nitrogen (LN_2) grind & Qiagen DNeasy (No PK); Lane 6 = *A. invadans* #24 DNA extracted using -20^{o}C, DNAzol, Grind; Lane 7 = *A. invadans* #40 DNA extracted using LN_2 Grind, DNAzol (Invitrogen); Lane 8 = *A. invadans* #41 DNA extracted using LN_2 Grind in mortar & pestle, DNeasy, Proteinase K (PK) overnight.

The manual method 1 (Lee and Taylor, 1990) produced DNA from fungal hyphae from the oomycete fungi *A. invadans* and *Saprolegnia*, and also from the true fungi *Penicillium*. In all cases shearing of the chromosomal DNA occurred, and was seen as a streak on the gel.

The PureGene kit produced chromosomal DNA from *A. invadans*, *Penicillium* sp. and *Saprolegnia* sp. However, no amplified product was seen when this DNA was used in PCR reaction with universal primers.

The E.Z.N.A kit was specifically promoted for extraction of DNA from oomycete fungi. A strong band of chromosomal DNA with some shearing was seen (Figure 7-1). However, the results were variable and there were occasions when no band for chromosomal DNA was seen using DNA extracted from *A. invadans* and other oomycetes such as *Saprolegnia* species, *Pythium sulcatum*, or the true fungus – *Penicillium* sp.

The simple methods of boiling, or use of boiling and vortexing with glass beads to physically break up the fungal hyphae, did not produce DNA when tested on oomycete fungi or true fungi.

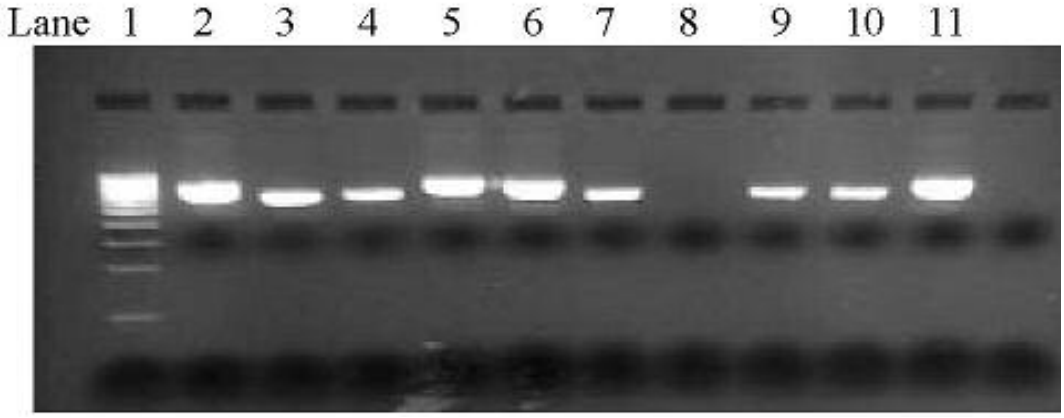

Figure 7-2. Results of extraction of culture-derived DNA using DNAzol reagents and amplification with universal primers C1 + D2.

Lane 1 = Hyper IV molecular weight marker; Lane 2 = AS-01-3195 *Saprolegnia* sp.; Lane 3 = AS-01-4050 Unknown fungus; Lane 4 = AS-01-4050 *Penicillium* sp.; Lane 5 = WAC 8470 *Pythium sulcatum*; Lane 6 = 45710 *Saprolegnia* sp.; Lane 7 = ATCC 36144 *S. diclina* (non-viable); Lane 8 = ATCC 16111 *A. diffusa* (non-viable); Lane 9 = IV-d Unknown fungus; Lane 10 = IV-r Unknown fungus; Lane 11 = A2S2 (#24) *A. invadans* (NSW); Lane 12 = negative DNA control.

The DNAzol reagent (Invitrogen) was used to extract DNA from broth cultures of a number of oomycete fungi and True fungi (Figure 7-2). The fungal hyphae grown in GP broth were treated with DNAzol reagent as described in 6.1.3.1, part H. Very little DNA was seen when 5 µl was loaded to a 2% gel. However, when the same samples were amplified with the universal primers C1 + D2, which amplify the large subunit gene, then a strong band of DNA was seen (Figure 7-2). Therefore, this method was suitable for the extraction of DNA from fungal hyphae that had been grown in GP broth for 4 to 8 days. The DNAzol reagent produced DNA without the need for the prior treatment of grinding the hyphae in liquid nitrogen. Although when used prior to the

DNeasy kit, more chromosomal DNA was obtained with the DNeasy method than with the DNAzol reagent, the latter had the advantage of being quicker and easier to use.

Conclusion:

The findings from the assessment of different DNA extraction methods indicated that the DNAzol reagent was the easiest and quickest method to use, and suitable amounts of DNA were produced from a 4-7 day old broth culture. Grinding in liquid nitrogen was not required. The DNeasy Qiagen mini plant tissue kit also produced good quantities of DNA, and the addition of proteinase K and/or lysozyme digestion steps improved the yield of DNA. Grinding in liquid nitrogen was required with this method. Chromosomal DNA may, or may not be seen when 2-5 µl is loaded directly to a gel, therefore, to check that DNA has been extracted the extract should be amplified with universal primers, C1 + D2.

7.1.2 Results of testing universal primers against A. invadans

A number of universal primers were tested against *A. invadans*, *A. astaci*, other oomycete fungi, and True fungi (Table 7-2). Some of these primers were used to assess whether DNA had been extracted by the different methods, particularly when no chromosomal DNA was seen on a gel. The primers also were used to assess the methods for the extraction of DNA from paraffin-embedded material, and formalin-fixed tissue. Amplification with universal primers indicated that fungal DNA was present, and that the DNA had been extracted.

Table 7-2. Results for universal primers tested with *A. invadans* and other fungal species

Primers	Sample	Product size bp	Comment
C1 + D2	AS-01-3635, paraffin-embedded	700	Strong band
C1 + D2	#24, A2S2	750	Strong band
ITS1 + C1 RC	#24 broth culture	700 & 500	No improvement with Tween 20. Two bands produced.
ITS1 + C1 RC	#46 broth culture	700 & 500	Two bands produced
ITS1 + ITS2	#24 broth culture	300	Strong band
ITS1 + ITS2	#46 broth culture	500	With Tween 20 (no band at 500 bp without Tween)
ITS1 + ITS4	#24	800 ++++	No improvement with Tween 20
ITS1 + ITS4	#46	800 ++++	No improvement with Tween 20
EF4 + fung5	FH-02-019a, *Trichoderma* sp	0	No result with DNA extracted from broth culture
EF4 + fung5	AS-02-1803, *Saprolegnia* sp.	0	No result against paraffin embedded

bp = base pair; ID = isolate identification number; RC = reverse complement; ITS1 = universal primer for internally transcribed spacer region 1; ITS2 = universal primer for internally transcribed spacer region 2; ITS4 = universal primer for internally transcribed spacer region 4.

Tween 20 was used in the PCR master mix to try and improve the integrity of the PCR product. In some instances amplicons were only obtained when Tween 20 was used (primers ITS1 + ITS2 and #46), whereas with primers ITS1 + ITS4 the use of Tween 20 did not improve the result.

The universal primers EF4 + fungi 5 that are universal for True fungi and plant oomycete fungi were unable to amplify DNA from *Trichoderma* species or *Saprolegnia* species.

7.1.2.1 Results of DNA sequencing

A number of sequencing reactions were carried so as to provide an identification of some of the fungi that were cultured from samples of fish tissue that had lesions suspect for EUS. In many of these cases, contaminant fungi were cultured rather than *A. invadans* because the fish or tissue samples were not fresh by the time they were received at the laboratory. In other cases the fish had been frozen, and freezing can destroy *A. invadans*. The contaminant fungi that were cultured were used in specificity assays.

The culture of *A. invadans* (A2S2, #24) that was received from Dr Callinan and Dr Landos, DPI NSW was also sequenced using universal primers to the large subunit gene (28S rRNA). The 707 base pair region that was sequenced had a 98% homology to *A. piscicida* AF235941 (Japanese strain, said to be the same as *A. invadans*), and a 96% homology to both *A. piscicida* AF235940 and *A. astaci* AF218197.

Universal primers to the large subunit (28S rDNA) regions were used to identify a *Saprolegnia* species that had been isolated from a case of EUS. The AHL case number was AS-02-1803. Universal primers C1+D2 were used to amplify this region, and then the individual primers were used as forward and reverse primers in the sequencing reaction, detailed in section 6.1.2.4. The fungus was identified by this method as *Saprolegnia ferax/diclina*. Sequencing was unable to differentiate between the two subspecies of *Saprolegnia*. The culture was then used in specificity studies.

A fungus that had been isolated from a case of EUS in black bream, case number FH-02-109a (Fish Health Unit, AHL) was sequenced using the D2 primer to the large subunit gene. Sequencing identified this fungus as *Trichoderma* species. It was then used in specificity studies.

7.1.3 Results of testing published primers specific for A. invadans

7.1.3.1 Assessment of primers AIFP1 and AIRP2

Early in the project a paper detailing specific primers (AIFP1 and AIRP2) for *A. invadans* was published (Blazer *et al.*, 2002). These primers were tested to determine their suitability for use in detecting and identifying *A. invadans* from culture, wax-embedded sections and fresh and stored fish tissue.

Table 7-3. Results for published primers AIFP1 + AIRP2 for *A. invadans*

Primers and isolate ID	Sample	Product size bp	Comment
AIFP1+AIFP2	A2S2 culture, (#24, NSW)	92	Weak band
AIFP1+AIFP2	A1S2 culture (#23, NSW)		smear
AIFP1+AIFP2	Wax block	92	Faint band
AIFP1+AIFP2	*A. astaci* (AS-02-1993), paraffin-embedded	92	Faint band

bp = base pair; ID = identification

The *A. invadans*-specific primers (AIFP1 and AIRP2) (Blazer *et al.*, 2002) produced a weak band at 92 bp when tested against DNA derived from broth culture of *A. invadans*. The PCR was performed using two different primer concentrations (20 pmol and 0.2µM). From four replicates, one produced a band at the correct molecular weight using 0.2uM of primer, whereas the remaining three replicates produced a smear of DNA regardless of the primer concentration used.

The AIFP1 and AIFP2 primers, said to be specific for *A. invadans*, also produced a band at the same molecular weight as the amplicon for *A. invadans* when tested against DNA extracted from a paraffin-embedded section of crayfish tissue infected with crayfish plague. Amplified product was seen using a primer concentration of 20 pmol, but no amplification occurred using a concentration of 0.2µM of primer (Figure 7-3).

Specificity testing for primers (AIFP1 + AIRP2)

The *A. invadans* primers (AIFP1 + AIRP2) (Blazer *et al.*, 2002) were tested for specificity. Amplified product was seen at the correct product size (92 bp) for *A. invadans*, *A. astaci* (very weak product), *Saprolegnia diclina*, *Saprolegnia* species, *Pythium sulcatum* (plant oomycete), three unknown true fungi and the true fungus *Penicillium* species, Figure 7-3. An annealing temperature of 60°C was used.

The results were sometimes difficult to interpret because the molecular weight of the amplicon at 92 bp was very similar in size to the excess primer material seen at the end of the electrophoresis gel. If the gel concentration was too low, or the electrophoresis run time was too short to allow adequate band separation, then a true positive reaction was difficult to separate from the excess primer material, as shown in Figure 7-3.

True fungi such as *Penicillium* species, and other unidentified true fungi, were included in specificity tests as these might be present in tissue samples as contaminating fungi, or may occur as plate contaminants.

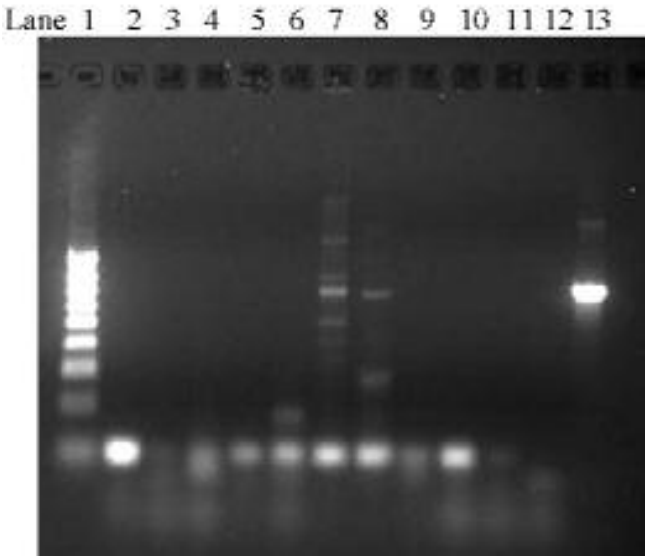

Figure 7-3. Specificity testing for primers AIFP1 and AIFP2.

Lane 1 = Molecular weight marker Hyper IV; Lane 2 = *A. invadans* (A2S2 #24); Lane 3 = *A. astaci*; Lane 4 = AS-01-3195 (*Saprolegnia* sp.); Lane 5 = AS-01-4050 (unknown fungus); Lane 6 = AS-01-4050 (*Penicillium* sp.); Lane 7 = WAC 8470 (*Pythium sulcatum*); Lane 8 = 45710 (*Saprolegnia* sp.); Lane 9 = ATCC 36144 (*S. diclina*); Lane 10 = IV-d (unknown fungus); Lane 11 = IV-r (unknown fungus); Lane 12 = negative DNA control; Lane 13 = *A. invadans* #24 with C1 + D2 primers.

Reagent concentrations and PCR conditions were optimised, however the specificity of the primers could not be improved.

7.1.3.2 Assessment of primers APH3 and APH4

A second set of primers that had been reported to be specific for *A. invadans* (APH3 and APH4) (Lilley *et al. to be submitted*) were also tested in this project. An amplicon of the correct molecular weight (107 bp) was obtained for *A. invadans*. Weakly amplified product was also obtained at the same molecular weight for *Saprolegnia diclina*, *Saprolegnia* species, *Pythium sulcatum*, an unidentified true fungi and *Penicillium* species. Extraneous bands were seen in the PCR results (Figure 7-4), and these were reported also by Lilley *et al.* The tests were repeated on DNA extracted from fresh broth cultures in case contamination of the cultures or DNA had occurred. However, the repeat testing still indicated cross-reaction between the same fungal species as previously tested.

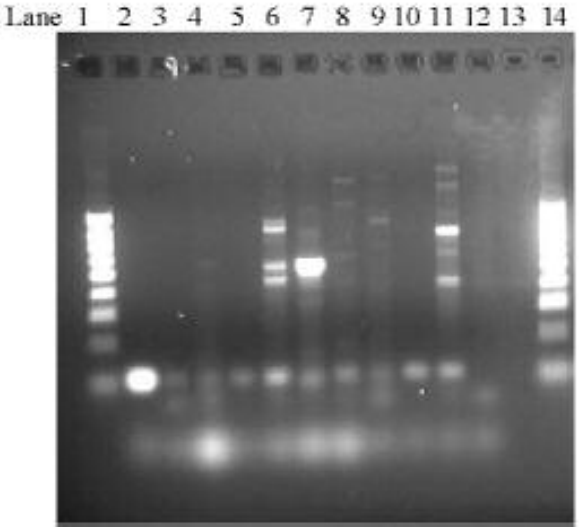

Figure 7-4. Testing primers APH3 + APH4.

Lane 1 = Molecular weight marker Hyper IV; Lane 2 = *A. invadans* (A2S2 #24); Lane 3 = *A. astaci*; Lane 4 = AS-01-3195 (*Saprolegnia* sp.); Lane 5 = AS-01-4050 (unknown fungus); Lane 6 = AS-01-4050 (*Penicillium* sp.); Lane 7 = WAC 8470 (*Pythium sulcatum*); Lane 8 = 45710 (*Saprolegnia* sp.); Lane 9 = ATCC 36144 (*S. diclina*); Lane 10 = IV-d (unidentified fungus); Lane 11 = IV-r (unknown fungus); Lane 12 = negative DNA control; Lane 13 = Molecular weight marker Hyper IV.

7.1.4 Results for primers designed in this project for A. invadans

The only sequence information that was known for *A. invadans* was in the region of the internally transcribed spacer regions. This area is often used to differentiate between species, as it is an area that is evolving more quickly than conserved areas on the genome. Alignment of sequence information from this area from *A. invadans* and other oomycete fungi using the clustalW program indicated that this region is closely related between these species. Regions of only one or two base pair differences were seen. Therefore, a number of different primers were designed and then tested for specificity to *A. invadans*.

Altogether, thirteen different primer sets were designed and tested for the amplification of *A. invadans*. The results of the primers that were the most specific (AIFP14 + AIRP10) are presented in Table 7-4. Other primers that showed potential specificity are presented in Tables 7-5 and 7-6.

Some of these primers produced weakly-staining amplicon for DNA from *A. invadans*, yet had produced a strongly-staining amplicon with universal primers, or other primers that had been designed and tested. Results from primers that were designed, but proved to be non-specific, or only produced a weakly staining amplified product, are presented in Table 7-7. The location of the primers in relation to the internally transcribed spacer region is shown in Appendix F, Figure F-1. The sequence information for the primers is listed in Appendix F, Table F-1.

Photographs of the gels, other than those presented in this section, are presented in Appendix F, Figures F-2, F-3, F-4, and F-5.

Overall three primer sets were specific for the isolates tested. These were primers AIF14 + AIR10, AIF14 + AIR11, and AIF15 + AIR10. Investigations into primer pair AIF13 + AIR9b subsequently proved that the primer pair were non-specific, section 7.1.4.6.

7.1.4.1 Investigation of primer pair AIF14 + AIR10

The primer pair AIF14 + AIR10 was selected for investigation by testing against a wide range of *A. invadans* cultures and other Oomycetes and true fungi. These results are detailed in Table 7-4.

Table 7-4. Results for PCR reaction with primers AIFP14 + AIRP10 specific for *A. invadans*

Primers	Sample	Product size bp	Comment on amplification
AIFP14 + AIRP10	*A. invadans* #24 (NSW)	554	band
AIFP14 + AIRP10	*A. invadans* #39 (T99G2, IMI 836084)	554	Strong band
AIFP14 + AIRP10	*A. invadans* #41 (RF8)	0	No band
AIFP14 + AIRP10	*A. invadans* #44 (4P)	0	No band
AIFP14 + AIRP10	*A. invadans* #45 (NJ9701)	554	Strong band
AIFP14 + AIRP10	*A. invadans* #46 (PA8)	0	No band
AIFP14 + AIRP10	*A. invadans* #54 (WIC, USA)	554	Strong band
AIFP14 + AIRP10	*A. invadans* #55 (WIC, USA)	554	Strong band
AIFP14 + AIRP10	*Saprolegnia* sp. #9 (AS-01-3195-1)	0	No band
AIFP14 + AIRP10	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIFP14 + AIRP10	*Saprolegnia* sp #12 (AS-01-4189)	0	No band
AIFP14 + AIRP10	*Saprolegnia* sp. (45710, QLD)	0	No band
AIFP14 + AIRP10	Unknown #36 (AS-02-3995)	0	No band
AIFP14 + AIRP10	*Pythium sulcatum* #22	0	No band
AIFP14 + AIRP10	*Achlya. diffusa* (non-viable) ATCC 16111	0	No band
AIFP14 + AIRP10	*Penicillium* sp. AS-01-4050	0	No band
AIFP14 + AIRP10	*Aspergillus niger*	0	No band
AIFP14 + AIRP10	*Aspergillus fumigatus*	0	No band
AIFP14 + AIRP10	Formalin-fixed crayfish tissue	<100 bp	Weak band

bp = base pair

7.1.4.2 Optimisation of PCR for primers AIFP10 + AIRP14

Primer pair AIFP10 + AIRP14, which produced good amplification results for *A. invadans* was selected for optimisation studies. At an annealing temperature of 50°C the reaction was not specific, however at 55°C and up to 60°C the reaction was specific against the different fungal species tested.

7.1.4.3 Specificity testing for primers AIFP10 + AIRP14

Primers AIFP10 + AIRP14 were tested for specificity against a wide range of oomycete fungi and true fungi (potential plate contaminants and contaminating flora on tissue lesions), to determine the specificity level of the primers. The annealing temperature was tested at 55°C. These results are presented in Figure 7-5.

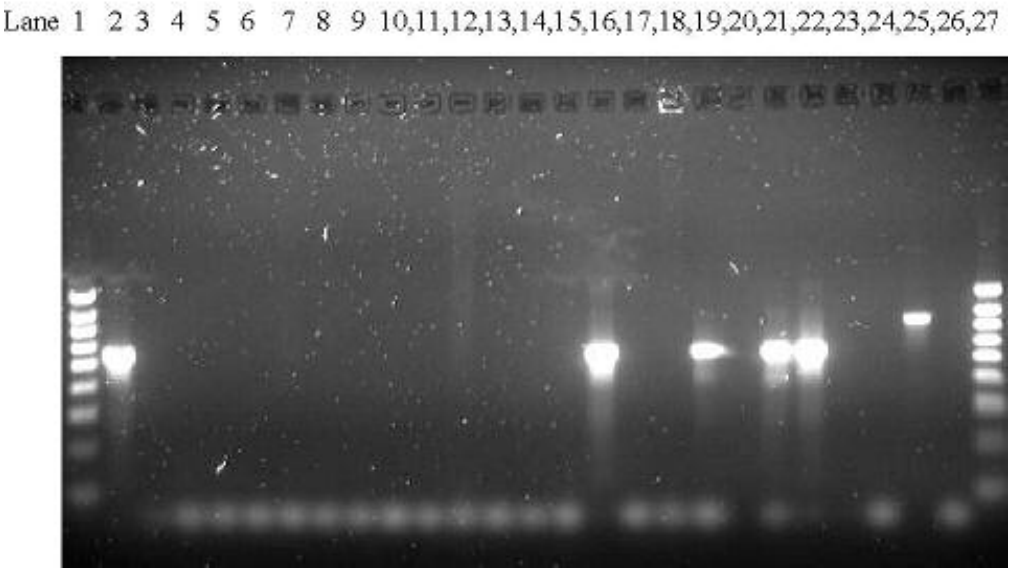

Figure 7-5. Specificity testing for primers AIFP14 + AIRP 10.

Lane 1 = Hyper IV Molecular weight marker; Lane 2 = *A. invadans* (#24, A2S2, NSW); Lane 3 = *A. astaci* (Formalin-fixed tissue); Lane 4 = *Saprolegnia* sp. (AS-01-3195); Lane 5 = unidentified fungus (AS-01-4050); Lane 6 = *Penicillium* sp. (AS-01-4050); Lane 7 = *Pythium sulcatum* (WAC 8470); Lane 8 = *Saprolegnia* sp. (45710); Lane 9 = *S. diclina* (ATCC 36144); Lane 10 = *Achlya diffusa* (ATCC 161111); Lane 11 = unidentified fungus (IV-r); Lane 12 = *Saprolegnia* sp. (AS-01-3195-1, #9); Lane 13 = unidentified fungus (AS-01-3195-2, #11); Lane 14 = unidentified fungus (AS-01-4189, #12); Lane 15 = unidentified fungus (AS-02-3995, #36); Lane 16 = *A. invadans* (T99G2, IMI 836084, #39); Lane 17 = *A. invadans* (RF8, #41); Lane 18 = *A. invadans* (4P, IMI 836086, #44); Lane 19 = *A. invadans* (NJM 9701, #45); Lane 20 = *A. invadans* (PA8, #46); Lane 21 = *A. invadans* (WIC, #54); Lane 22 = *A. invadans* (WIC, #55); Lane 23 = *Aspergillus niger*; Lane 24 = *Aspergillus fumigatus*; Lane 25 = *A. invadans* with C1+D2 primers; Lane 26 = negative DNA control; Lane 27 = Hyper IV Molecular weight marker.

Primers AIFP14 + AIRP10 were specific against the oomycete and true fungi tested. No amplicons were detected for the oomycete fungi *Saprolegnia diclina*, *Saprolegnia* species, *Achlya diffusa* and *Pythium sulcatum*. Likewise no amplicons were detected for true fungi that may be seen as either contaminating flora on tissue lesions, or as plate contaminants on laboratory media. Plate contaminants are an issue because of the length of time (15 days) that a plate needs to be incubated before a negative result for *A. invadans* is issued. However, as for previous primers that were designed and tested for *A. invadans* there were some interesting results for the different species of *A. invadans*. Not all *A. invadans* species were amplified by the primers AIFP14 + AIRP10 (Figure 7-5). Cultures of *A. invadans* that were not amplified by the primers were RF8 (#41), 4P, IMI 836082 (#44), and PA8 (#46). When tested with primers AIF13 + AIR9b, the DNA from these cultures produced weak amplicon. Interestingly, the cultures of RF8 (#41), and #40 (G2PA) produce a different colony morphology than usually seen for *A. invadans*. This is discussed in section 7.1.7.

7.1.4.4 PCR master mix for AIFP10 + AIRP14

The PCR cycling conditions and reagent concentrations were optimised for primers AIFP10 + AIRP14. The primers were tested against commercially

available master mix from Promega, and against an in-house PCR master mix.

For the Promega master mix, the primers performed well at a range of primer concentrations. 20 pmol, 40 pmol, 60 pmol, 80 pmol and 100 pmol all gave identical results with strongly staining amplicon at all primer concentrations. The primers also performed well at a range of annealing temperatures from 53°C up to 60°C. After 60°C decreased strength of amplicon was seen and the likelihood of double bands and non-specific amplification increased.

The primers also performed well with the in-house master mix under varying reagent concentrations and reaction conditions. Primer concentrations from 20 to 100 pmol all produced good amplicon, as did $MgCl_2$ concentrations from 1.5 to 4.5 mM. Dinucleotide concentrations did not affect the PCR, and concentrations of 100 µM or 200 µM did not adversely affect the end result. The volume of *Taq* polymerase was not critical for volumes from 0.1 µl to 3 µl in a single reaction tube. Results similar to those seen for the Promega master mix were also seen for the conventional in-house master mix. Strongly staining amplicon was produced at a range of annealing temperature from 53°C to 60°C. At a higher temperature there was a decrease in the amplicon, and the occurrence of double bands.

Other commercially available PCR master mixes that were trialled included Failsafe from Epicentre, Hot start from Qiagen, IQ Supermix (BioRad). All master mixes performed well, however, the advantage of the Promega master mix was the cost, as it was the cheapest.

Promega master mix for primers AIF14+AIR10

Reagent	**Volume (µl) for single tube**
Promega PCR master mix	12.5
Primer AIFP10 (20 pmol)	0.5
Primer AIRP14 (20 pmol)	0.5
Water	9.5
DNA	2

In-house PCR master mix for primers AIF14+AIR10

Reagent	**Volume (µl) for single tube**
Water	14.3
10 X PCR buffer	2.5
DNTP mix	2.5
$MgCl_2$	1.5
Primer AIFP10 (20 pmol)	1
Primer AIRP14 (20 pmol)	1
Taq enzyme	0.2
DNA	2

Final concentration of dNTPs = 200 µM.

PCR cycling conditions

Performed as per method in 6.1.2.5, but with an annealing temperature of 55°C.

7.1.4.5 Sensitivity testing for A. invadans PCR with primers AIFP14 + AIRP10
Purified DNA from *A. invadans* (#24, A2S2, NSW isolate) was serially diluted, and each dilution tested in the PCR reaction to determine the smallest amount of DNA that could be detected. The lowest concentration of DNA that could be detected was 19.5 picograms/ml using the Promega PCR master mix. The in-house PCR master mix was a thousand-fold less sensitive at detecting DNA, and the lowest concentration of DNA that could be detected was 19.5 nanograms/ml.

7.1.4.6 In-depth investigation of primers AIF13 + AIR9b.
Other primers that produced a strong band of amplified DNA were selected and tested for specificity and optimisation of the PCR conditions and reagent concentrations. Primer pair AIF13 + AIR9b initially showed strong product amplification, however, further specificity studies indicated that there was the potential for cross-reaction with other oomycete fungi, in particular *Saprolegnia* species. The results of the investigation into specificity are presented in Table 7-5 and in Appendix F, Figure F-4 and F-5. Optimisation of the reagent concentrations and the cycling conditions was unable to improve the specificity of these primers, and *Saprolegnia* species always produced strong amplicon. For example even with high annealing temperature of 60°C, cross-reaction was still obtained from *Saprolegnia* sp. and the amplicon was still produced in the same amounts as that from *A. invadans*, Appendix F, Figure F-4.

Optimised conditions for primers AIF13 + AIR9b proved to be a primer concentration of 20 pmol, $MgCl_2$ (24 mM) volume of 1.5 µl, 200 µM dNTP in a 25 µl reaction volume. Cycling conditions were optimised at an initial denaturation at 94°C for 5 minutes for one cycle followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 30 sec at 60°C, and elongation for 30 sec at 72°C. A final elongation step was carried out for 10 min at 72°C.

Table 7-5. Results for primers AIF13 + AIR9b *A. invadans*

Primers	Sample	Product size bp	Comment on amplification
AIF13 + AIR9b	*A. invadans* #24 (NSW)	238	Strong band
AIF13 + AIR9b	*A. invadans* #39 (T99G2)	238	Strong band
AIF13 + AIR9b	*A. invadans* #40 (G2PA)	238	weak band, variable result
AIF13 + AIR9b	*A. invadans* #41 (RF8)	0	No band
AIF13 + AIR9b	*A. invadans* #44 (4P)	238	weak band, variable result
AIF13 + AIR9b	*A. invadans* #46 (PA8)	238	Very weak band
AIF13 + AIR9b	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF13 + AIR9b	*Saprolegnia* sp #12 (AS-01-4189)	238	Band
AIF13 + AIR9b	*Saprolegnia* sp. (45710, QLD)	238	Strong band
AIF13 + AIR9b	*S. diclina* ATCC36144		Non-specific band
AIF13 + AIR9b	*Pythium sulcatum* #22	0	No band (non-specific band)
AIF13 + AIR9b	Unknown fungus AS-01-4050	0	No band
AIF13 + AIR9b	Unknown fungus IV-d	238	Faint band
AIF13 + AIR9b	Unknown fungus IV-r	0	No band
AIF13 + AIR9b	*Penicillium* sp. AS-01-4050	238	Weak band
AIF13 + AIR9b	Crayfish plague formalin-fixed	0	No band

bp = base pair

An interesting observation from the results with primers AIF13 + AIR9b was that some isolates of *A. invadans* did not produce strongly-staining amplicon with these primers. Weak results were obtained for isolates 4P (#44), PA8 (#46). These can be seen in the primer optimisation gel in Appendix F, Figure F-4.

7.1.4.7 Results for other primers designed for A. invadans that have potential specificity

Some primers that were designed and tested for *A. invadans* showed potential as specific primers (Table 7-6). These were tested against fungal species likely to cause a problem with cross-reaction in the PCR test. Because in-depth specificity testing of primers AIF10 + AIR14 produced good results, the other primers (Table 7-6) were not tested further.

Table 7-6. Results for other primers that showed specificity for *A. invadans*

Primers	Sample	Product size bp	Comment on amplification
AIF14 + AIR11	*A. invadans* #24 (NSW)	581	Strong band
AIF14 + AIR11	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF14 + AIR11	*Pythium sulcatum.* #22 (WAC 8470)	0	No band
AIF14 + AIR11	*Saprolegnia* sp. (45710, QLD)	0	No band
AIF14 + AIR11	*S. diclina* ATCC36144	0	No band
AIF14 + AIR11	Formalin-fixed crayfish tissue	<100 bp	Strong band
AIF15 + AIR10	*A. invadans* #24 (NSW)	490	Strong band
AIF15 + AIR10	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF15 + AIR10	*Pythium sulcatum.* #22 (WAC 8470)	0	No band
AIF15 + AIR10	*Saprolegnia* sp. (45710, QLD)	0	No band
AIF15 + AIR10	*S. diclina* ATCC36144	0	No band
AIF15 + AIR10	Formalin-fixed crayfish tissue	0	No band

bp = base pair

7.1.4.8 Results for primers designed for A. invadans that were non-specific
Nine primer pairs that were designed and tested proved to be non-specific for *A. invadans* and cross-reacted with other oomycete fungi, Table 7-7.

Primer pair AIFP18 and AIRP14 produced a weak amplicon for *A. invadans* (#24, A2S2) from NSW. Cross-reactions were also found against *Saprolegnia* species, the plant oomycete *Pythium sulcatum*, and an unidentified true fungus also produced a weak amplicon at the same molecular weight as *A. invadans*, Table 7-7. These and other primers in Table 7-7 were considered unsuitable.

Table 7-7. Results for primers that showed non-specificity for *A. invadans*

Primers	**Sample**	**Product size bp**	**Comment on amplification**
AIF5 + AIR9b	*A. invadans* #24 (NSW)	452	Strong band
AIF5 + AIR9b	*Saprolegnia* sp. #11 (AS-01-3195-2)	452	Strong band
AIF5 + AIR9b	Crayfish plague formalin-fixed	0	No band
AIF5 + AIR9b	*Pythium sulcatum.* #22 (WAC 8470)	0	Non-specific bands
AIF5 + AIR9b	*Saprolegnia* sp. (45710, QLD)	452	Strong band
AIF15 + AIR11	*A. invadans* #24 (NSW)	517	Strong band
AIF15 + AIR11	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF15 + AIR11	*Pythium sulcatum.* #22 (WAC 8470)	517	weak band
AIF15 + AIR11	*Saprolegnia* sp. (45710, QLD)	0	No band
AIF15 + AIR11	*S. diclina* ATCC36144	0	No band
AIF15 + AIR11	Formalin-fixed crayfish tissue	0	No band
AIF16 + AIR10	*A. invadans* #24 (NSW)	476	Strong band
AIF16 + AIR10	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF16 + AIR10	*Pythium sulcatum.* #22 (WAC 8470)	476	weak band
AIF16 + AIR10	*Saprolegnia* sp. (45710, QLD)	476	weak band
AIF16 + AIR10	*S. diclina* ATCC36144	0	No band
AIF16 + AIR10	Formalin-fixed crayfish tissue	0	No band
AIF16 + AIR11	*A. invadans* #24 (NSW)	503	Very strong band
AIF16 + AIR11	*Saprolegnia* sp. #11 (AS-01-3195-2)	503	Weak band
AIF16 + AIR11	*Pythium sulcatum.* #22 (WAC 8470)	503	Strong band
AIF16 + AIR11	*Saprolegnia* sp. (45710, QLD)	503	Strong band
AIF16 + AIR11	*S. diclina* ATCC36144	503	Weak band
AIF16 + AIR11	Formalin-fixed crayfish tissue	0	No band
AIF17+ AIR19	*A. invadans* #24 (NSW)	186	Strong band
AIF17+ AIR19	*Saprolegnia* sp. #11 (AS-01-3195-2)	186	Band
AIF17+ AIR19	*Pythium sulcatum.* #22 (WAC 8470)	0	No band
AIF17+ AIR19	*Saprolegnia* sp. (45710, QLD)	186	Band
AIF17+ AIR19	*S. diclina* ATCC36144	0	No band
AIF17+ AIR19	*A. astaci* (formalin-fixed tissue)	186	Very faint band
AIF18 + AIR14	*A. invadans* #24 (NSW)	165	Very weak band
AIF18 + AIR14	*Saprolegnia* sp. #11 (AS-01-3195-2)	165 & 300bp	Very weak band
AIF18 + AIR14	*Pythium sulcatum.* #22 (WAC 8470)	165	Very weak band
AIF18 + AIR14	*Saprolegnia* sp. (45710, QLD)	165 & 500	Very weak band
AIF18 + AIR14	*S. diclina* ATCC36144	500	Very weak band

Table 7-7, continued			
Primers	**Sample**	**Product size bp**	**Comment on amplification**
AIFP18 + AIRP14	*A. invadans* #24 (NSW)	165	Weak band
AIFP18 + AIRP14	Crayfish plague formalin-fixed	0	No band
AIFP18 + AIRP14	*Saprolegnia* sp. #11 (AS-01-3195-2)	165	Weak band
AIFP18 + AIRP14	*Pythium sulcatum* #22	165	Weak band
AIFP18 + AIRP14	*Saprolegnia* sp. (45710, QLD)	165	Weak band
AIFP18 + AIRP14	*S. diclina* ATCC36144	165	Weak band
AIF18 + AIR14	*A. astaci* (formalin-fixed tissue)	0	No band
AIF18 + AIR15	*A. invadans* #24 (NSW)	228	Strong band
AIF18 + AIR15	*Saprolegnia* sp. #11 (AS-01-3195-2)	228	Very weak band
AIF18 + AIR15	*Pythium sulcatum*. #22 (WAC 8470)	228	weak band
AIF18 + AIR15	*Saprolegnia* sp. (45710, QLD)	228	weak band
AIF18 + AIR15	*S. diclina* ATCC36144	228	weak band
AIF18 + AIR15	*A. astaci* (formalin-fixed tissue)	228	Very faint band
AIF18 + AIR16	*A. invadans* #24 (NSW)	244	Strong band
AIF18 + AIR16	*Saprolegnia* sp. #11 (AS-01-3195-2)	0	No band
AIF18 + AIR16	*Pythium sulcatum*. #22 (WAC 8470)	244	Weak band
AIF18 + AIR16	*Saprolegnia* sp. (45710, QLD)	244	Weak band
AIF18 + AIR16	*S. diclina* ATCC36144	0	No band
AIF18 + AIR16	*A. astaci* (formalin-fixed tissue)	0	No band

bp = base pair

7.1.4.9 Results for nested and hemi-nested PCR reactions

Primers were nested to determine if the sensitivity of the PCR could be improved by using a two-stage PCR reaction. Five primer pairs were used in a first round reaction and various combinations of other primers were tested in the second (nested or hemi-nested) reaction. All produced amplicons at the correct size in the nested reaction, however other extraneous bands were also produced, which could make interpretation difficult. The use of nested and hemi-nested PCR was not investigated further.

7.1.5 Results for PCR of A. invadans using frozen tissue material infected with EUS

Frozen fish with lesions resembling EUS that had been stored at –80°C were tested for the presence of *A. invadans* DNA using the PCR and primers AIF14 + AIR10. The DNA was extracted using DNAzol reagent (section 6.1.3.1 part H). The master mix was prepared using the Promega PCR master mix, section 7.1.4.4. The results of the samples tested are presented in Table 7-8 and Figure 7-6.

DNA was amplified from a lesion on a black bream that had been caught in the Swan River, Perth (case number AS-03-0417). The lesion was in the process of healing when the fish was caught. *Aphanomyces invadans* was also cultured from this fish (culture number #53). However, the other lesion samples were negative for *A. invadans* when tested in the PCR. Some of these lesions had been sampled quite thoroughly for culture, so there is the possibility that no fungal material remained in the lesion.

Case number FH-02-019a was from a black bream that had a lesion that was typical of EUS, Figure 3-1. However, *A. invadans* was not cultured from this sample, although a *Trichoderma* species was cultured and was possibly part of the contaminating flora in the lesion.

Table 7-8. Results for PCR on frozen stored fish tissue from cases of EUS

Case Number	Fish	Diagnosis	Fungus cultured	PCR result
AS-00-3014	Black bream	Lesions on body, EUS	None	negative
#53 (AS-03-0417)	Black bream	Lesions on body, EUS	*A. invadans*	positive
AS-03-0342	Black bream	Lesions on body, EUS	Unknown	negative
FH-02-019a	Black bream	Lesions on body, EUS	*Trichoderma* species	negative

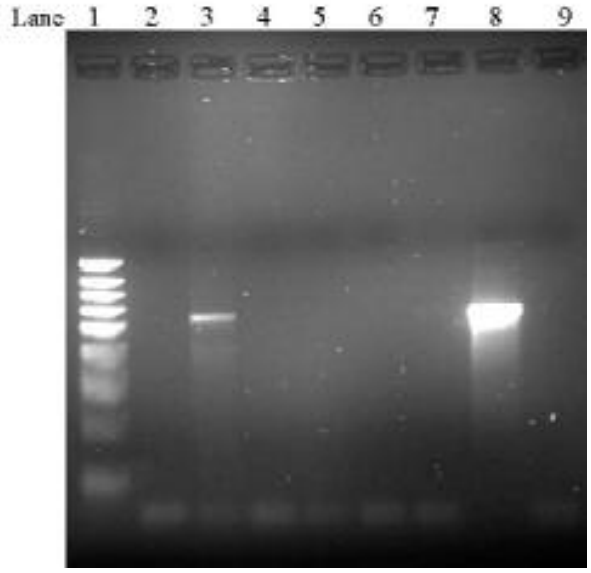

Figure 7-6. Results for PCR using primers AIF14 + AIR10 on paraffin-embedded tissue or fish tissue stored frozen.

Lane 1 = Hyper IV molecular weight marker; Lane 2 = AS-00-3014 paraffin-block; Lane 3 = tissue AS-03-0417 (#53); Lane 4 = tissue AS-03-0342; Lane 5 = FH-02-019a tissue; Lane 6 = AS-00-3014 tissue; Lane 7 = AS-01-4189 #12 (pure DNA); Lane 8 = positive DNA control (*A. invadans* DNA); Lane 9 = negative DNA control.

7.1.6 Results for DNA extraction from A. invadans *from paraffin-embedded tissue material infected with EUS*

A. *Using universal primers*

DNA was extracted from paraffin-embedded tissue using the DNeasy Qiagen Mini Plant tissue kit with an added step of treatment with proteinase K, (described in 6.1.3.1 part E). No amplification was obtained when using universal primers (ITS1 + ITS4) to the internally transcribed spacer regions (ITS1 and ITS2). A positive control using DNA extracted from *Saprolegnia* species isolated from the tissue of case AS-02-1803 produced amplified product at the correct molecular weight. However, the paraffin-embedded

tissue from this same case did not produce product (Table 7-9). Increasing the annealing and denaturing times did not improve the result.

In contrast, DNA extracted from paraffin-embedded tissue using this same method was amplified from all cases when the universal primers (C1 and D2) to the large subunit (28S rDNA) were used (Table 7-9). A band at approximately 650-700 base pairs was produced from these primers, and the ITS1 and ITS4 primers produced a band of approximately 750 base pairs.

Table 7-9. Results for amplification of DNA from paraffin-embedded tissue using universal primers.

Case No.	Diagnosis	DNA Extraction method	Primers	Result
AS-00-3014	EUS	Dneasy + PK	ITS1 + ITS4	Negative
AS-00-3014	EUS	Dneasy + PK	C1 + D2	Positive
AS-01-3195	Lesion, *Saprolegnia* cultured	Dneasy + PK	ITS1 + ITS4	Negative
AS-01-3195	Lesion, *Saprolegnia* cultured	Dneasy + PK	C1 + D2	Positive
AS-01-3635	EUS	Dneasy + PK	ITS1 + ITS4	Negative
AS-01-3635	EUS	Dneasy + PK	C1 + D2	Positive
FH-02-019a	EUS	Dneasy + PK	ITS1 + ITS4	Negative
FH-02-019a	EUS	Dneasy + PK	C1 + D2	Positive
AS-02-1803	Lesion, *Saprolegnia* cultured	Dneasy + PK	ITS1 + ITS4	Negative
AS-02-1803	Lesion, *Saprolegnia* cultured	Dneasy + PK	C1 + D2	Positive
AS-02-1993	Crayfish plague	Dneasy + PK	ITS1 + ITS4	Negative
AS-02-1993	Crayfish plague	Dneasy + PK	C1 + D2	Positive

EUS = epizootic ulcerative syndrome; No. = number

B. *PCR using primers AIF14 + AIR10*

DNA from paraffin-embedded tissue from clinical cases of EUS was extracted according to the second method in section 6.1.3.3 and subjected to PCR using primers AIF14 + AIR10 (Table 7-10). Seeded paraffin-embedded sections were prepared as described in section 6.1.3.3 and also subjected to PCR.

Table 7-10. Paraffin-embedded tissue from cases of EUS

Case Number	Fish	Diagnosis	Fungus cultured	PCR result
AS-00-3014	Black bream	Lesions on body, EUS	None	Negative
FH-02-019a	Black bream	Lesions on body, EUS	*Trichoderma* species	Negative

EUS = epizootic ulcerative syndrome

No amplification of DNA was achieved using primers AIF14 + AIR10 (Table 7-10). All samples were tested at neat, 1:10 and 1:100 dilutions with bovine serum albumin (BSA) added to the PCR master mix, yet amplicon was still not detected. The PCR using primers AIF14 + AIR10 on diagnostic case

AS-03-0417 (#53) from black bream was positive on frozen fresh tissue, but negative on the paraffin-embedded section (Table 7-11).

Table 7-11. Paraffin-embedded tissue and seeded samples using primers AIF14 + AIR10

Case Number	Sample	Diagnosis	Fungus cultured	PCR result
AS-00-3014	PE tissue	Lesions on body, EUS	None	Negative
A2S2 #24	PE culture	Seeded sample	*A. invadans*	Negative
A2S2 #24	PE culture + fish tissue	Seeded sample	*A. invadans*	Negative
AS-03-0417 (#53)	PE tissue	Lesions on body, EUS	*A. invadans*	Negative

PE = paraffin-embedded.

Other primer pairs that produced smaller molecular weight amplicon were tested on the paraffin-embedded tissue. One primer pair (AIF14 + AIRR15) produced an amplicon of the correct molecular weight (84 bp) when tested against culture material embedded into paraffin, and fish tissue seeded with a culture of *A. invadans* (A2S2, #24) (Table 7-11). An improved result was obtained when bovine serum albumin (BSA) was added to the PCR master mix. However, these primers were not specific for *A. invadans*, and produced strong amplicon for strains of *Saprolegnia* and *Aspergillus*. The same result was obtained using primers AIF14 + AIR16, which produced an amplicon of 100 bp. These primers were also able to amplify DNA from paraffin-embedded material, however they were not specific for *A. invadans*.

Conclusions:

Paraffin-embedded tissue was positive for fungal DNA using universal primers (C1 + D2) that amplified the large subunit gene (28S rRNA), but amplified product was not obtained from these same samples using universal primers for the internally transcribed spacer region (ITS), or primers (AIF14 + AIR10) specific for *A. invadans*. Paraffin-embedded tissue seeded with *A. invadans* fungal culture did not produce amplified product using primers AIF14 + AIR10). Primers C1 + D2 produced amplicon of 650 to 700 bp, primers ITS1 + ITS4 produced amplicon of 750 bp, and primers AIF14 + AIR10 produced amplicon of 554 bp.

7.1.7 Different PCR reactions and growth morphologies seen with different strains of *A. invadans*

During the course of this work differences were noted between some isolates of *A. invadans*. Differences in growth morphology were seen on IM plates for isolates RF8 (#41), G2PA (#40), and PA8 (#46). Photographs showing growth of these isolates are presented in Figures 7-7 to 7-13. Isolate G2PA was isolated from EUS in Three-spot Gourami in Thailand in 1994, and isolates RF8 and PA8 (#46) were isolated from EUS in striped snakehead in Thailand in 1992 and 1995, respectively. Isolate 4P, IMI 836086 (#44) grew noticeably faster than the other *A. invadans* isolates and completely covered the plate

medium in 9 days. Other *A. invadans* isolates covered half, or less than half of the surface area of the plate in the same time. Isolate 4P, IMI 836086 was originally isolated from red-spot disease in yellowfin bream from NSW, Australian in 1989. Background information on isolates is listed in Table 6-1. The isolates were some of the *A. invadans* isolates kindly provided by Dr Chinabut, AAHRI, Thailand.

Cultures T99G2, IMI 836084 (#39) from Thailand, A2S2 (#24) from NSW and WIC (#54) from USA all had similar morphologies and grew at the same rate as each other. Isolate T99G2 was isolated from EUS in giant Gourami in Thailand in 1999. Isolate A2S2 (#24) was isolated in NSW in 2002 and kindly provided by Dr Callinan, and isolate WIC (#54) was isolated from menhaden in the USA and kindly provided by Dr Blaser.

The differences in morphology were reflected in the differences noted in amplification of DNA in the PCR reaction using two different primer sets. Primer set AIF13 + AIR9b produced variable amplification that was either weak or negative in different test runs using isolates #40, #44 and #46, Figure F-4, Appendix F. These primers were not specific, as they produced amplicon of variable strength for *Saprolegnia* species.

Primer set AIFP14 + AIRP10 did not produce amplicon for isolates #41 and 44. Variable results were obtained with isolate #46, as amplicon was not produced when using an in-house PCR master mix, yet produced a faint band using Promega PCR master mix.

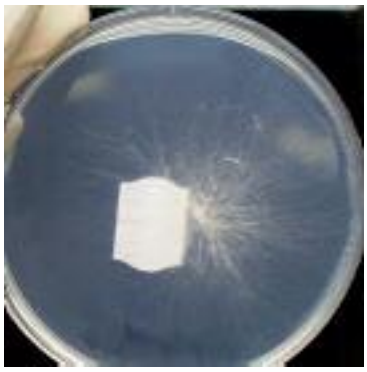

Figure 7-7. *A. invadans* G2PA (#40), 9 days growth on IM medium

Figure 7-8. *A. invadans* RF8 (#41), 9 days growth on IM medium

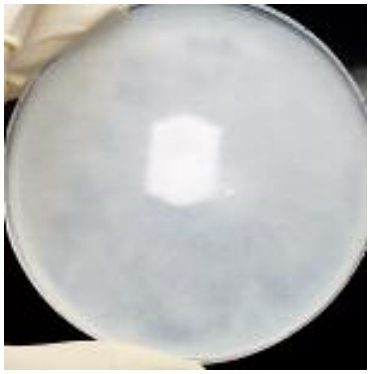

Figure 7-10. *A. invadans* 4P, IMI 836086 (#44), 9 days growth on IM medium

Figure 7-9. *A. invadans* PA8 (#46), 9 days growth on IM medium

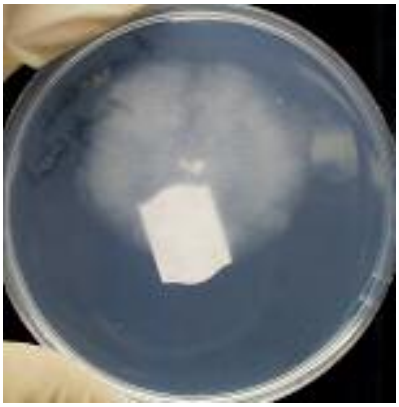

Figure 7-12. *A. invadans* A2S2 (#24), 9 days growth on IM medium

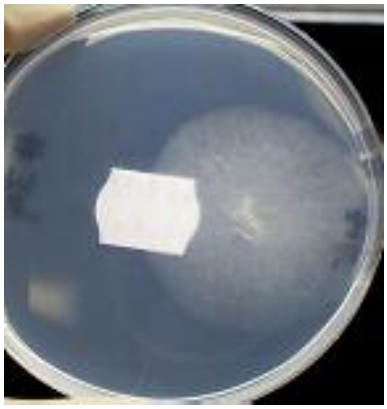

Figure 7-11. *A. invadans* T99G2, IMI 836084, (#39), 16 days on IM medium

Figure 7-13. *A. invadans* WIC, USA (#54), 10 days on GP plate medium

7.2 Development of a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

7.2.1 Results for testing universal primers against A. astaci

DNA was amplified from paraffin-embedded material (AS-02-1993) using universal primers (C1 + D2) to the large subunit gene (28S rDNA). The amplicon was of a high strength at a molecular weight of 650-700 bp. Amplicon was not obtained from the universal primers EF4 + fung5, and variable amplification results were obtained with primers ITS1 + ITS4.

A weakly staining amplicon was obtained from formalin-fixed tissue using universal primers C1 + D2, ITS1 + ITS2, and ITS1 + ITS4. Tween 20 was added to the master mix to determine if the strength of the amplicon could be improved, however the results were inconsistent. At times, the signal strength was improved with the use of Tween 20, whereas for other reactions a better result was achieved without the use of Tween 20 (Figure 7-14). The amplicon from formalin-fixed tissue could not be re-amplified when this product was used as template in a second PCR reaction.

Table 7-12. Results for testing *A. astaci* against universal primers

Primer ID	Gene Target and product size	Sample	Result & comments
C1 + D2	Universal primer to LSU	Formalin-fixed crayfish tissue	Faint band, 750 bp, Figure 7-14
C1 + D2	Universal primer to LSU	AS-02-1993, Paraffin-embedded material	Strong band produced 650-700 bp
ITS1 + C1 RC	ITS regions	Formalin-fixed crayfish tissue	Two weak bands at 600 + 700 bp
ITS1 + ITS2	ITS-1	Formalin-fixed crayfish tissue	Band seen, 300 bp.
ITS1 + ITS4	ITS-1 5.8S rDNA and ITS-2	Formalin-fixed crayfish tissue	Weak band seen, 700 bp.
EF4 + fung5	Universal fungal primers	AS-02-1993, Paraffin-embedded material	No band

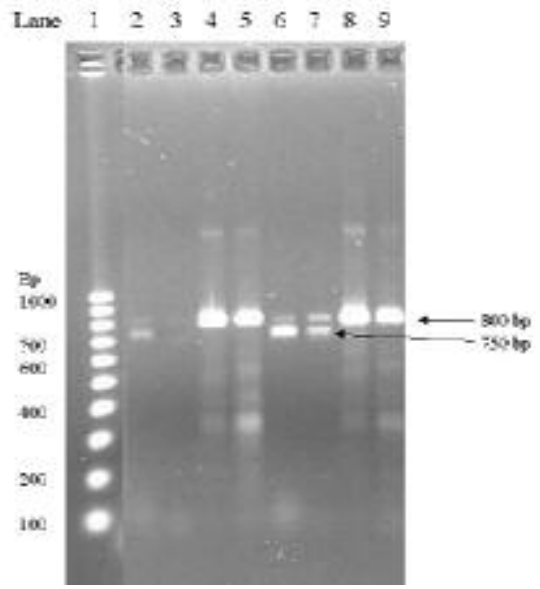

Figure 7-14. Results for testing universal primers C1 + D2 against formalin-fixed crayfish tissue with and without the addition of Tween 20 to the master mix.

Lanes 2 to 4 are with Tween 20 and Lanes 5 to 9 are without the addition of Tween 20. Lane 1 = 100 base pair molecular weight marker (Geneworks); Lane 2 = DNA from crayfish plague tissue (CP-1); Lane 3 = DNA from crayfish plague tissue (CP Q-A); Lane 4 = *A. invadans* #24; Lane 5 = *A. invadans* #54; Lane 6 = DNA from crayfish plague tissue (CP-1); Lane 7 = DNA from crayfish plague tissue (CP Q-A); Lane 8 = *A. invadans* #24; Lane 9 = *A. invadans* #54.

DNA amplified from *A. invadans* A2S2 (#24) from NSW and WIC (#54) from USA using universal primers C1 + D2 to the large subunit gene (28S rDNA) produced an amplicon at 800 bp, whereas the amplicon for DNA from formalin-fixed tissue infected with crayfish plague produced a product at 750 bp (Figure 7-14).

7.2.1.1 Results of DNA sequencing

DNA extracted from the paraffin-embedded tissue provided by Dr Alderman, CEFAS, UK was amplified using universal primers C1 and D2, and used in the sequencing reaction. When the D2 primer was used in the sequencing reaction, the results suggested a 90% homology to *A. astaci* and *A. piscicida*.

Product sequenced using the C1 primer had 83% homology to *A. piscicida* AF235941, and *Aphanomyces* species AF119585. There was 80% homology to *A. astaci* AF235940 and AF218197. A high proportion of mis-reads were obtained in the sequence results, but the sequencing reaction was not repeated.

DNA from formalin-fixed crayfish was unable to be extracted at a high enough concentration to be used in the sequencing reaction. Product amplified with either ITS1+ ITS4, or C1+D2 primers could not be re-amplified, even when the amplicon was used as template in a second PCR reaction. Concentrating the product could not be achieved using DNA concentration techniques, such as a manual method (ethanol precipitation), or using commercial kits.

7.2.2 Results of testing published primers against A. astaci

Primers ASP1 and ASP2 were used to amplify a 1050 base pair region of the 28S rDNA of *A. astaci* (Oidtmann *et al.*, 2002). This region was amplified in other fungi such as *Saprolegnia* species, *Leptolegnia* and *Achlya* species. To differentiate *A. astaci* from these other oomycetes, the amplified product was digested with the enzyme *Alu* I to differentiate it from *Saprolegnia*, and with *Ava* I to differentiate it from *Achlya* and *Leptolegnia* species. Restriction with *Alu* I produced identical bands for both *Achlya*, *A. astaci* and *Leptolegnia* (Oidtmann *et al.*, 2002).

The ASP1 and ASP2 primers were tested in this project against DNA extracted from wax-embedded tissue infected with crayfish plague, but no amplified product was seen (Figure 7-15).

Primers (AIFP1 and AIRP2), that had been developed and reported for the detection of *A. invadans* (Blazer *et al.*, 2002), produced amplicon at the same molecular weight when tested against *A. astaci* DNA extracted from paraffin-embedded tissue. Duplicate samples were tested. One duplicate produced amplicon at 70-80 bp, whereas the other duplicate sample produced two bands; one at 92 bp and the second at 70-80 bp (Figure 7-15). These primers produce a band at 92 bp when tested against *A. invadans*.

Thus, in this project it was found that *A. astaci* cross-reacts with the primers (AIFP1 and AIRP2) that were developed for *A. invadans*.

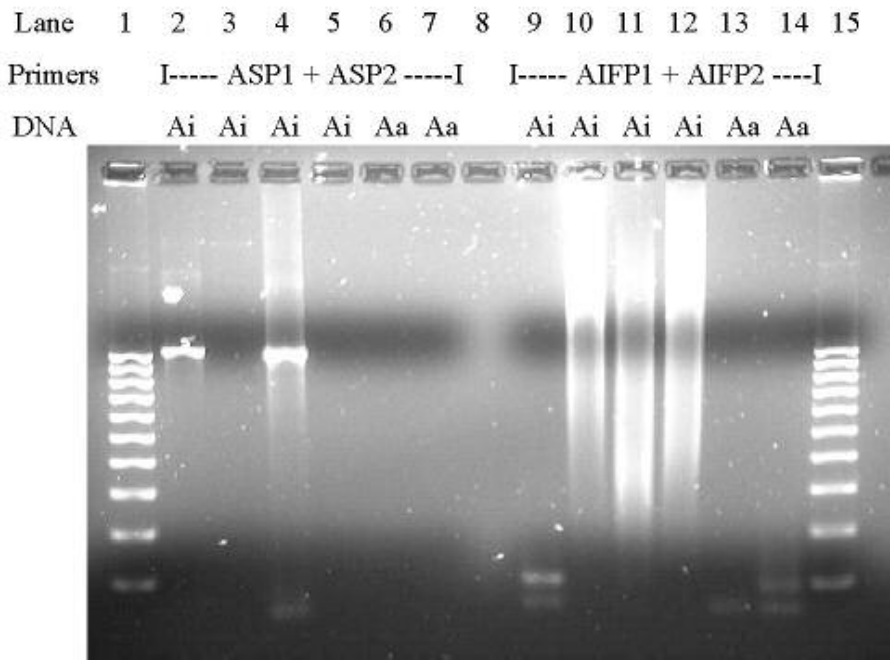

Figure 7-15. Gel showing amplification of *A. invadans* and *A. astaci* using primers ASP1+ASP2 (Lanes 2 to 7), and primers AIFP1+AIFP2 (Lanes 9 to 14).

Lane 1 = 100 base pair ladder; Lane 2 = *A. invadans* (A2S2, #24) primer concentration 12 pmol; Lane 3 = *A. invadans* (A2S2, #24) primer concentration 20 pmol; Lane 4 = *A. invadans* (A1S2, #23) primer concentration 12 pmol; Lane 5 = *A. invadans* (A1S2, #23) primer concentration 20 pmol; Lane 6 = *A. astaci* (AS-02-1993) primer concentration 12 pmol; Lane 7 = *A. astaci* (AS-02-1993) primer concentration 20 pmol; Lane 8 = blank; Lane 9 = *A. invadans* (A2S2, #24) primer concentration 0.2µM; Lane 10 = *A. invadans* (A2S2, #24) primer concentration 20 pmol; Lane 11 = *A. invadans* (A1S2, #23) primer concentration 0.2µM; Lane 12 = *A. invadans* (A1S2, #23) primer concentration 20 pmol; Lane 13 = *A. astaci* (AS-02-1993) primer concentration 0.2µM; Lane 14 = *A. astaci* (AS-02-1993) primer concentration 20 pmol; Lane 15 = 100 base pair ladder.

7.2.3 Primer sequences designed in this project for A. astaci

There was sufficient sequence information in Genbank from which to design specific primers for the detection of *A. astaci*. The areas that had been sequenced were putative virulence genes, namely a chitinase gene (Andersson and Cerenius, 2002), and two protease genes, subtilisin-like serine proteinase precursor (SP1) gene and trypsin proteinase precursor (SP2) gene (Bangyeekhum *et al.*, 2001). Six primers were designed to the subtilisin-like serine proteinase precursor (SP1) gene, eight primers were designed to the trypsin proteinase precursor (SP2) gene, and eight primers were designed to the putative chitinase gene chi1. The primers and their sequences are detailed in Table 7-13.

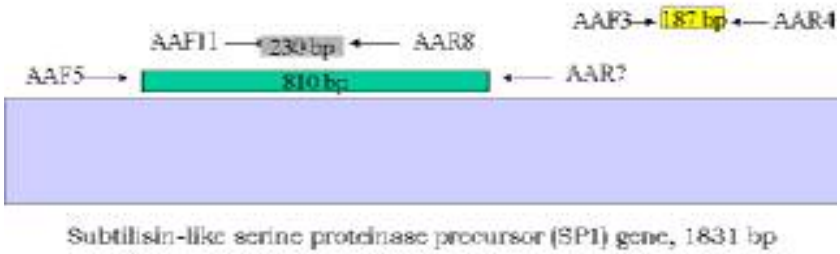

Figure 7-16. Schematic showing position of primers designed for amplifying regions of the SP1 gene.

Table 7-13. Sequence information for primers designed for *A. astaci* in this project

Primer ID	Sequence 5' to 3'	Genome region
AAF3	TGTACCTTGTGGACCAACGA	subtilisin-like serine proteinase precursor (SP1) gene
AAR4	CCCACTTGACCGATTTGTCT	subtilisin-like serine proteinase precursor (SP1) gene
AAF5	GAGTCGGTGGTTCATTCGTT	subtilisin-like serine proteinase precursor (SP1) gene
AAR7	CAGCAATCACTTGAGGGGAT	subtilisin-like serine proteinase precursor (SP1) gene
AAF11	ATGAAACCAAAAGCCACGAC	subtilisin-like serine proteinase precursor (SP1) gene
AAR8	CGTTGGGCAGTTCCGTGTTATCG	subtilisin-like serine proteinase precursor (SP1) gene
AAF	GGGGTGGAAGGAGAAAACTC	trypsin proteinase precursor (SP2) gene
AAR	GCACGCACAAGCTTGATTTA	trypsin proteinase precursor (SP2) gene
AAF12	TCTCGAGTGCTCAAGGAGGT	trypsin proteinase precursor (SP2) gene
AAR13	CGCTGACGCGTTCATAGATA	trypsin proteinase precursor (SP2) gene
AAF14	CTCGAGTGCTCAAGGAGGTC	trypsin proteinase precursor (SP2) gene
AAF15	ACACATCCCCAATCATCCAT	trypsin proteinase precursor (SP2) gene
AAR16	GGTGAAAGCGAGAAGGAGTG	trypsin proteinase precursor (SP2) gene
AAF17	GAGTGCTCAAGGAGGTCAGC	trypsin proteinase precursor (SP2) gene
AAF18	GGGACAAGATCACCCACATC	putative chitinase gene chi1
AAR19	CCAATGGACAGACCGAACTT	putative chitinase gene chi1
AAR20	AATGGACAGACCGAACTTGG	putative chitinase gene chi1
AAF21	GCGTCGGGAACTGTCATTAT	putative chitinase gene chi1
AAR22	GTGGGTGATCTTGTCCCAGT	putative chitinase gene chi1
AAF23	AAATACACGGACCACCAAGC	putative chitinase gene chi1
AAR24	GTCGGCTTGGTAAAGTTGGA	putative chitinase gene chi1
AAR25	AGATGGCCCATTCCATGTAG	putative chitinase gene chi1
AAF27	GCGTCGGGAACTGTCATTAT	putative chitinase gene chi1
AAR26	GCGTCGGGAACTGTCATTAT	putative chitinase gene chi1
AAF29	ACCTGGGGTTGGACTTTCTC	putative chitinase gene chi1
AAR28	GTAAAGTCCCAGTGGCGGTA	putative chitinase gene chi1

7.2.3.1 Results of testing primers designed for the detection of A. astaci

Six primers (three pairs) were designed to the SP1 gene as shown in Figure 7-16. Primers AAF5 + AAR7 also were designed to be used as the first reaction for a nested PCR, with primers AAF11 + AAR8 as the second primer pair. The amplified product size was 810 bp for the first reaction, and 230 bp for the nested PCR reaction. No amplified product was detected with any of the three primer pairs, or in the nested reaction when tested on formalin-fixed crayfish tissue that had been extracted with either the DNAzol method, or the Qiagen DNeasy plant mini kit. All primers were tested at concentrations of 100 µM and 200 µM dNTPs, but no amplification was seen at either concentration. All primers were tested for specificity using *A. invadans* (#24 and #39) and *Saprolegnia* species (#9).

In the nested reaction a strongly staining amplicon was seen at 230 bp for *A. invadans* #39, and *Saprolegnia* sp. #9, but not for *A. invadans* #24, and only when dNTPs were used at 200 µM concentration. In the single PCR reaction using primers AAF11 + AAR8, which produce amplicon of 230 bp, a faint band was seen at approximately 300 bp for *A. invadans* #39 and #24.

Table 7-14. Results for primers designed for the detection of *A. astaci*

Primer ID	Gene Target and product size	Sample	Result & comments
AAF3 + AAR4	SP1, 187 bp	Formalin-fixed crayfish tissue	Negative
AAF5 + AAR7	SP1, 810 bp	Formalin-fixed crayfish tissue	Negative
AAF11 + AAR8	SP1, 230 bp	Formalin-fixed crayfish tissue	Negative
AAF5 + AAR7 nested with AAF11 + AAR8	SP1, 810 bp and 230 bp	Formalin-fixed crayfish tissue	Negative for *A. astaci*. Product at 230 bp for *A. invadans*.
AAF13 + AAR4	SP1	Formalin-fixed crayfish tissue	Negative
AAF + AAR	SP2, 204 bp	Formalin-fixed crayfish tissue	No band
AAF12 + AAR13	SP2, 247 bp	Formalin-fixed crayfish tissue	No band (non-specific band at 850 bp and 100 bp)
AAF14 + AAR13	SP2, 246 bp	Formalin-fixed crayfish tissue	No band
AAF15 + AAR16	SP2, 165 bp	Formalin-fixed crayfish tissue	No band
AAF17 + AAR13	SP2, 243 bp	Formalin-fixed crayfish tissue	No band
AAF18 + AAR19	Chi, 204 bp	Formalin-fixed crayfish tissue	No band
AAF18 + AAR20	Chi1, 202 bp	Formalin-fixed crayfish tissue	No band
AAF21 + AAR22	Chi 1, 212 bp	Formalin-fixed crayfish tissue	No band
AAF23 + AAR24	Chi 1, 196 bp	Formalin-fixed crayfish tissue	No band
AAF21 + AAR25	Chi 1, 162 bp	Formalin-fixed crayfish tissue	No band
AAF27 + AAR26	Chi 1, 963 bp	Formalin-fixed crayfish tissue	No band
AAF27 + AAR26	Chi 1, 963 bp	Formalin-fixed crayfish tissue	? band at 800 bp with Tween 20 added to master mix
AAF29 + AAR28	Chi 1, 213 bp	Formalin-fixed crayfish tissue	No band
AAF27 + AAR26 AAF29 + AAR28	Chi 1, 963 bp 213 bp	Formalin-fixed crayfish tissue	No amplicon when used in a nested PCR reaction
ASP1 + ASP2		Wax block	No band
AIFP1 + AIFP2	100 bp	Wax block	Faint band. Primers designed for *A. invadans* (Blaser *et al.* 2002)
AIF17 + AIR19	ITS, 186 bp	Formalin-fixed crayfish tissue	Faint band at correct molecular weight for primers designed for *A. invadans*
AIF18 + AIRR15	ITS, 228 bp	Formalin-fixed crayfish tissue	Faint band at correct molecular weight for primers designed for *A. invadans*

SP1 = subtilisin-like serine proteinase precursor gene; SP2 = Trypsin proteinase precursor gene; LSU = Large subunit gene (28S rRNA)

The expected amplicon size for *A. astaci* was 230 bp. No amplicon was seen using DNA extracted from formalin-fixed crayfish tissue and tested against

primers in a single PCR reaction, or in the nested reaction. Amplified product of a size similar to the expected amplicon (for *A. astaci*) was seen for a strain of *A. invadans* and a strain of *Saprolegnia* species. This means that the nested reaction may not be suitable for detection of *A. astaci*, unless optimisation could be done to increase the specificity of the test.

Two of the three primer pairs (AA5 + AAR7 and AAF11 and AAR8) tested in a single PCR reaction, produced extraneous bands when tested with *A. invadans* isolates #39 and #24. All initial PCR reactions were performed at a lower annealing temperature of 50°C.

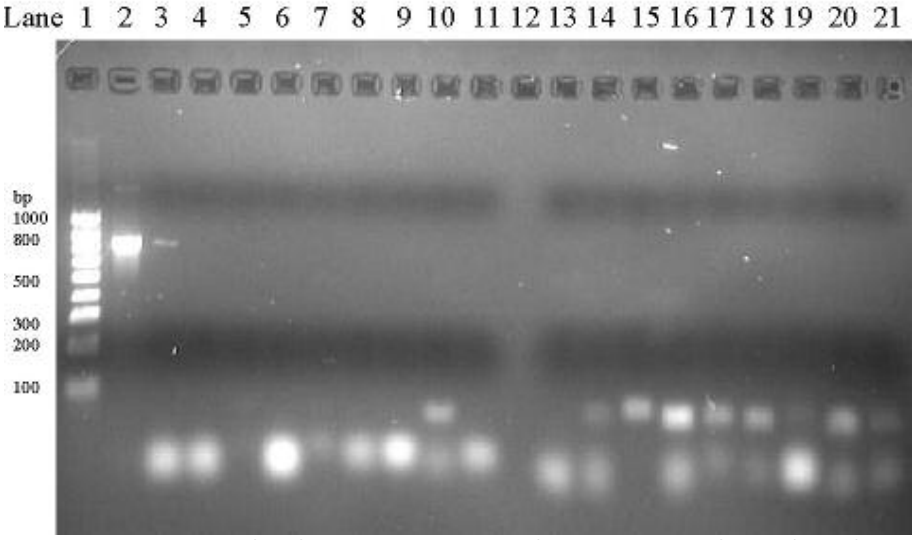

Figure 7-17. Results for primers tested on tissue infected with crayfish plague.

Lane 1 = HyperIV molecular weight marker; Lane 2 = *A. invadans* with C1+D2 primers; Lane 3 to Lane 21 = crayfish plague formalin-fixed tissue; Lane 3 to Lane 11 using Promega master mix; Lanes 13 to Lane 21 using in-house master mix. Lane 3 = primers AAF12+AAR13; Lane 4 = primers AAF14+AAR13; Lane 5 = primers AA15+AAR16; Lane 6 = primers AAF17+AAR13; Lane 7 = primers AAF18+AAR19; Lane 8 = primers AAF18+AAR20; Lane 9 = primers AAF21+AAR22: Lane 10 = primers AAF23+AAR24; Lane 11 = primers AAF21+AAR25; Lane 12 = blank; Lane 13 = primers AAF12+AAR13; Lane 14 = primers AAF14+AAR13; Lane 15 = primers AA15+AAR16; Lane 16 = primers AAF17+AAR13; Lane 17 = primers AAF18+AAR19; Lane 18 = primers AAF18+AAR20; Lane 19 = primers AAF21+AAR22: Lane 20 = primers AAF23+AAR24; Lane 21 = primers AAF21+AAR25.

A range of primers were designed (Table 7-13) and tested against DNA extracted from formalin-fixed crayfish tissue, Table 7-14, Figure 7-17. The primers were tested using two different master mixes; Promega PCR master mix, and an in-house master mix. No amplicon at the correct molecular weight was seen for any of the primers tested using DNA extracted from formalin-fixed tissue infected with crayfish plague. A number of non-specific bands at a molecular weight of less than 100 bp were seen. A greater number of non-specific bands were seen for the in-house master mix compared to the Promega master mix.

Conclusion:

No amplified product was obtained from DNA extracted from formalin-fixed tissue infected with crayfish plague, using the primers designed in this

project, and using DNA extracted either the DNAzol reagent or the Qiagen DNeasy plant mini kit.

7.2.4 Results for DNA extraction methods using formalin-fixed crayfish tissue

Formalin-fixed crayfish infected with crayfish plague were supplied by Dr David Alderman from the CEFAS laboratory in the UK. DNA was extracted using two kit methods, one with modifications as detailed in section 6.2.5. The DNA was tested by universal primers for the large subunit gene (C1 + D2) and by primers (AAF + AAR) designed to detect the trypsin proteinase precursor gene (SP2). No amplification was seen with the primers designed for the trypsin proteinase precursor gene. However, faint bands of amplified product were seen for the universal primers to the large subunit gene (Figure 7-18).

Amplicon from the universal primers was unable to be re-amplified using a second-round PCR with the same universal primers.

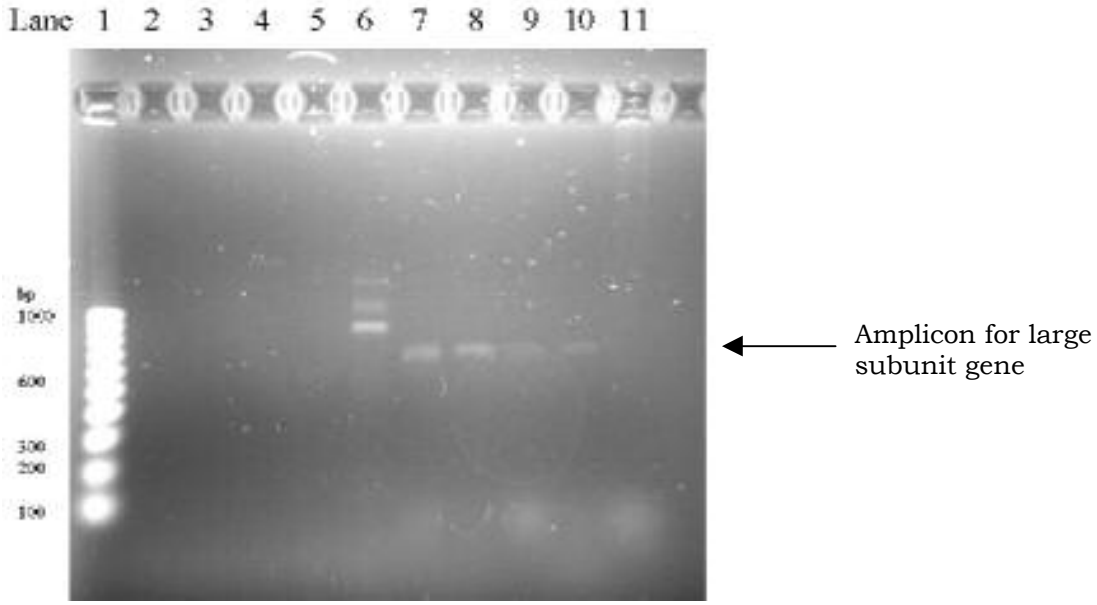

Figure 7-18. Gel showing the results of primers AAF & AAR, and universal primers C1 + D2 using DNA extracted from formalin-fixed tissue by different methods.

Lanes 2 to 6 = primers AAF + AAR; Lanes 7 to 11 = primers C1 + D2; Lane 1 = 100 base pair molecular weight marker; Lane 2 = DNAzol extraction; Lane 3 = DNeasy-1; Lane 4 = DNeasy-2; Lane 5 = DNeasy-3; Lane 6 = negative DNA control; Lane 7 = DNAzol extraction; Lane 8 = DNeasy-1; Lane 9 = DNeasy-2; Lane 10 = DNeasy-3; Lane 11 = negative DNA control

None of the primers designed for the specific detection of *A. astaci* produced amplicon from DNA extracted from formalin-fixed crayfish tissue (Table 7-14). However, amplicon at the correct molecular weight was achieved using the universal primers to the large subunit gene, Figure 7-18. DNA was amplified with these primers when the DNAzol reagent was used, and when the DNeasy Qiagen mini plant tissue kit was used with modifications. The modifications included grinding the tissue with a micropestle in AP-1 buffer (DNeasy-1), the use of grinding in liquid nitrogen (DNeasy-2), or adding a proteinase K digestion step (DNeasy-3). All methods gave equal results,

although amplicon was slightly stronger with the DNAzol reagent, and the DNeasy-1 method.

Conclusion:

These results proved that fungal DNA was present in the crayfish tissue that was selected for PCR, and that the DNA extraction method was suitable.

7.3 Development of a rapid molecular diagnostic test for the detection of *Aphanomyces invadans* based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material, within one hour.

7.3.1 Results for specific probe for A. invadans

The primers (AIF14 and AIR10) that proved to be specific in the PCR reaction for *A. invadans* were used to investigate their suitability for development of a fluorophore (fluorescent probe) for use in an *in-situ* hybridisation method. Each primer was prepared with a fluoroscein molecule added to the 5 prime end of the oligonucleotide. Fungal species that were tested are detailed in Table 6-8.

The fluorescent probes did not detect other fungi such as *Aspergillus*, which may be present on a sample as contaminating flora. However, at hybridisation temperatures of 37°C and 55°C both probes showed a weak positive fluorescence with *Saprolegnia* species. Optimisation of reaction conditions and reagent concentration did not improve specificity. At a hybridisation temperature of 62°C the fluorescence decreased for all fungal species that were tested. A temperature of 55°C was the highest temperature that could be used before the fluorescence signal decreased in strength.

The probes were tested individually and in combination. The use of two probes appeared to produce a better result, as the fluorescent signal increased, and the fluorescence was more consistent along the length of the hyphae. In many slides the fluorescence along the hyphae was inconsistent. The edges of the hyphae showed fluorescence, as did some of the internal components such as the spores. However, there were sections of hyphae that did not stain. This is consistent with the observation that DNA may be concentrated at the hyphae tips.

Preparation of hyphae on a glass slide

The simplest method for handling fungal hyphae grown from a broth culture was to perform all steps in a microfuge tube. This was the easiest method of handling the hyphae for the incubation and washing steps.

Use of skim milk as a blocking agent

A skim milk wash was performed to determine if the specificity of the method could be improved. However, skim milk did not seem to be an advantage in reducing background staining (Figure 7-19).

Optimisation of hybridization times
A range of different hybridisation times (1, 2, 3, and 24 hours) were tested. Optimal results were achieved with a hybridisation time of 3 hours or greater.

Optimisation of hybridization temperature
The test was carried out at hybridisation temperatures of 37°C, 55°C, 60°C, and 62°C using 100 pmol of probe in an attempt to reduce background staining and non-specific reactions with *Saprolegnia* species (Figure 7-20). The specificity of the test could not be improved, as fluorescence was seen for *Saprolegnia* species at all temperatures. The fluorescent signal for *A. invadans* was negative or very weak at 62°C, whereas at this temperature the fluorescence was still strong for *Saprolegnia* species.

Optimisation of incubation times with formamide
Different formamide incubation times were tested at 1, 2, 3, and 4 hours at 70°C. The fluorescent signal after 1 and 2 hour incubation appeared less than those samples incubated for 3 and 4 hours in formamide. Therefore, a minimum of three hours incubation in formamide was required to achieve the brightest fluorescent signal.

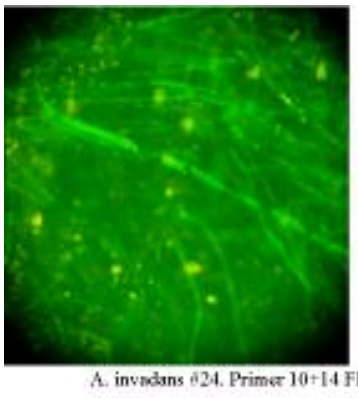

Figure 7-19. Fluorescent in-situ hybridisation with *A. invadans* A2S2, #24.

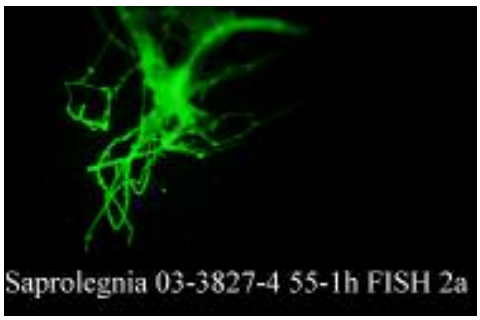

Figure 7-20. Cross reaction with *Saprolegnia* species using fluorophores 10+14 at hybridisation temperature of 55°C.

Conclusions:

Fluorescence was seen using fluorophores based on the primers AIF 14 and AIR 10, however the signal was weak for *A. invadans*. Strong cross-reaction

was seen with *Saprolegnia* species. The specificity could not be improved with the use of skim milk as a blocking agent, optimisation of incubation times for formamide, or optimisation of hybridisation temperature and time.

7.3.2 Results for universal positive probe

Two universal primers C1 and D2 that amplify the large subunit region (28S rDNA), were prepared with a fluoroscein molecule added to the 5' end, and tested for suitability as positive control in the FISH technique. Both probes detected *Aphanomyces invadans* and *Saprolegnia* species grown from broth culture. The probe using the C1 primer gave the best results. Optimal hybridization was achieves at 3 hours at 55°C.

7.4 Results of maintenance of Oomycete fungi culture collection

7.4.1 Fungal reference strains and clinical strains.

The seven Type strains purchased from the American Type Culture Collection (ATCC) were not viable on their arrival at Animal Health Laboratories, Department of Agriculture Western Australia. Repeat requests to ATCC and their transporting company Cryosite, not to freeze the cultures were ignored. Oomycete fungi are killed after 24 hours at –5°C, after 20 minutes at –20°C and after 10 minutes at –20°C. However, each batch of cultures was transported on dry ice and was frozen on arrival at the laboratory. This was still the case with replacement cultures, and they refused to supply cultures without dry-ice transportation. A request for actively growing cultures sent on sloped media would have taken an additional 8 months and would have involved a further charge of $US400 per culture. Therefore, it was decided to forego testing of Type strains held at ATCC.

Dr Jim Lilley, who based his PhD thesis on *Aphanomyces invadans*, had sent all his strains to the Mycological Institute in England (CABI), however none were viable when requests were made for purchase of cultures.

Cultures obtained from Dr Annette Thomas, DPI QLD, were not viable. They had been sent with the knowledge that they had not been regularly subcultured and therefore viability was not guaranteed. It was possible that they could have been used as a source of DNA, however the agar from the culture medium interfered with the PCR reaction. A culture of *Trichoderma* species was used in testing the specificity of the *A. invadans* primers.

All but one of the 10 cultures received from Dr Chinabut, AAHRI, Thailand, were viable. These cultures were sent on sloped medias under oil.

The duplicate cultures of *A. invadans* sent by Dr Vicki Blaser, USA, were viable and had been sent as actively growing cultures on sloped media.

The culture sent by Dr Chris Burke, University of Tasmania, was either not *A. invadans* or had been contaminated and overgrowth of the original *A. invadans* culture had occurred.

Table 7-15. Summary of viability of fungal isolates sourced for this project

Isolate ID	Lab ID	Identification	Source	Outcome
UM3	#38	*Aphanomyces invadans*	Dr Chinabut, AAHRI, Thailand	
T99G2	#39	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
G2PA	#40	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
RF8	#41	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
RF6	#42	*A. invadans*	Dr Chinabut, AAHRI, Thailand	
30P	#43	*A. invadans*	Dr Chinabut, AAHRI, Thailand	
4P	#44	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
NJM9701	#45	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
PA8	#46	*A. invadans*	Dr Chinabut, AAHRI, Thailand	Viable
B99C	#47	*A. invadans*	Dr Chinabut, AAHRI, Thailand	
WIC	#54	*A. invadans*	Dr V. Blaser, USA	Viable
WIC2	#55	*A. invadans*	Dr V. Blaser, USA	Viable
A1S2	#23	A. *invadans*	Dr R. Callinan, Dr M. Landos, NSW	Died out
A2S2	#24	A. *invadans*	Dr R. Callinan, Dr M. Landos, Dept of Fisheries, NSW	Viable
AS-01-3195-2	#11	*Saprolegnia*	WA	Viable
AS-01-4189	#12	unidentified	WA	Viable
AS-00-3014	#14	*Saprolegnia*	WA	Viable
AS-03-0417	#53	*A. invadans*	Ian Vaughan, WA	Viable
AS-02-1803		*Saprolegnia ferax/ diclina*	WA	Viable
WAC 8470	#22	*Pythium sulcatum*	Plant Pathology DAWA	Viable
ATCC 16111	#51	*Achyla diffusa*	ATCC	Non-viable
ATCC 62427*	#27	*Aphanomyces* species	ATCC	Non-viable
ATCC 204464	#26	*Aphanomyces frigidophilus*	ATCC	Non-viable
ATCC 38487	#49	*Saprolegnia australis*	ATCC	Non-viable
ATCC 46241	#25	*Saprolegnia parasitica*	ATCC	Non-viable
MYA-399	#52	*Saprolegnia salmonis*	ATCC	Non-viable
ATCC 36144	#50	*Saprolegnia diclina*	ATCC	Non-viable
	#28	*Trichoderma* sp.	Dr A. Thomas, DPI QLD	Viable
34P	#29	*A. invadans*	Dr A. Thomas, DPI QLD	Not viable
3P	#30	*A. invadans*	Dr A. Thomas, DPI QLD	Not viable
33P	#31	*A. invadans*	Dr A. Thomas, DPI QLD	Not viable
4P	#32	*A. invadans*	Dr A. Thomas, DPI QLD	Not viable
45710	#33	*Saprolegnia* sp.	Dr A. Thomas, DPI QLD	Contaminated
	#21	*A. invadans*	Chris Burke, Uni Tasmania	Not viable, contaminated

The DNA obtained from the paraffin embedded sections of crayfish infected with crayfish plague was used up rapidly when undertaking specificity studies for *A. invadans* and further paraffin embedded tissue was unable to be obtained.

7.5 Results of assessment of storage methods

The raw data is presented in Appendix C.

At the beginning of the project a number of different media were used to assess which was the best media for the maintenance of fungal cultures. GP-POX medium is recommended for initial isolation of *A. invadans* because it contains penicillin and oxolinic acid (Roberts *et al.*, 1993). These antibiotics assist in reducing the overgrowth by contaminating bacterial flora during the first few days of isolation of fungus from tissue samples. However, the antibiotics appeared to slightly reduce the growth of the *A. invadans* cultures, therefore it was not assessed as a maintenance medium.

Table 7-16. Summary of results of assessment of culture storage methods

Group Designation	Isolate Name	Isolate Number	Results
Group 1	*Pythium sulcatum*	WAC 8470	Viable for 12 m in all methods except –80°C. Plate method viable to 9 m, only.
Group 2	*A. invadans*	NSW isolate (A2S2, #24)	Variable depending on method. DW (12 m), GP slope (6 m) at RT
Group 3	*A. invadans*	Isolates obtained from AAHRI (T99G2,G2PA,RF 8, RF6, 4P, NJM9701, PA8, B99C)	RT best temperature, viable at 12 m on DW, GP slope and GP broth. Plate not viable at 4°C, and up to 6 m RT.
Group 4	*A. invadans*	USA isolate (#54)	RT best temperature, non-viable at 4°C, GP slope and broth to 6m, DW to 12 m.
Group 5	*A. invadans*	WA isolate (#53, AS-03-0417)	Viable by most methods (except –80°C & plate) to 12 m. GP slope of broth best. DW 12 m at RT only.
Group 6	*Saprolegnia* sp.	Project Reference No. #9, #11	Viable all methods to 12 m except plate. Variable results for GP broth. RT best
Group 7	Unidentified fish fungus		Viable in all storage methods. Plate method up to 9 m, only.

AAHRI = Aquatic Animal Health Research Institute, Thailand; DW = distilled water; GP = glucose peptone medium; m = months; RT = room temperature'

A range of results were seen for the different groups of oomycete fungi tested. The plant oomycete fungi, *Pythium sulcatum* WAC 8470 was the most hardy of all the fungi tested. It was viable in all storage methods including cryopreservation at –80°C in glycerol lab lemco broth. It could also be maintained on a plate medium for 9 months at room temperature, and for 12 months at 4°C.

Saprolegnia species (S. *diclina*/*ferax*), were the next hardiest oomycete fungi that were tested, and could be re-cultured from all methods after 12 months, except for storage at –80°C and for the GP plate. The GP slope was superior to GP broth and GP plate. *Saprolegnia* species were generally unaffected by storage temperature, and on the GP slope and DW it remained viable for 12 months at both 4°C and room temperature.

A. invadans seem to be the most sensitive of the oomycetes, however, there were differences within the groups, as isolates from different countries gave different results. There was variation between all the strains of *A. invadans* obtained from AAHRI, AHL, NSW WA and USA. This was noted for tolerance to temperature and type of media. The WA isolate was more tolerant to long term storage on a variety of media compared to the NSW isolate and the isolate from USA. The WA isolate was more tolerant to storage in DW at room temperature compared to the isolate from NSW and USA. It also was more robust and less temperature sensitive if kept on the most appropriate medium (GP slope or GP broth). The NSW and USA isolates were not viable on GP slopes after 6 months at room temperature with or without oil, and were not viable after 3 months at 4°C.

The GP plate produced the worst results with only group 6 (*Saprolegnia* sp.) being viable after 12 months at room temperature, although group 7 (unidentified fungus) was viable at 12 months when stored at 4°C. A majority of the isolates did not survive past 6 months. GP plate media could not be recommended for long-term storage. For cultures of aquatic *A. invadans* the growth should be subcultured regularly, and before the growth reaches the edge of the plate, as viability is severely reduced once the culture reaches the edge of the plate (Dr A. Thomas, DPI QLD, pers comm). This observation was also supported in the current study.

GP broth had little advantage over distilled water except with the isolate from WA. It was more viable in GP broth at 4°C than in distilled water, but no difference in viability of the two media was seen at room temperature.

The difference observed for storage temperature was interesting, as a longer storage time was achieved using room temperature, compared to 4°C. A greater number of fungal isolates were viable for a longer period of time at room temperature than at 4°C.

All isolates were viable in distilled water kept for 3 months at room temperature. This method would best serve as a short-term storage method when viable cultures were required for material for a project spanning several weeks. The oil overlay had the advantage of reducing the amount of contamination from air contaminants such as other fungi and bacteria. Use of a biological safety cabinet when handling cultures, and good aseptic

technique also assisted to reduce the contamination rate. It is recommended that all fungal growth taken from stored material should be cultured to a plate medium to assess the purity of the culture before inoculation into a broth medium.

The use of an oil overlay did not have much effect on viability when the cultures were stored at room temperature with the exception of the isolate from WA, which showed improved viability in DW when an oil overlay was used. No difference was seen in viability at 4°C with or without a paraffin oil overlay.

Overall, the best storage method was on a GP slope kept at room temperature. However, it is recommended that isolates are stored by a variety of methods, and that viability is checked every 6 months. Thus, all isolates should be stored at room temperature, and kept on a GP slope with and without oil, and in distilled water.

8. DISCUSSION

8.1 Develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples

8.1.1 DNA extraction methods

One of the factors required for successful amplification of DNA from samples is a dependable DNA extraction method. If DNA is not extracted in reliable and adequate quantities then false negative results may be obtained for the PCR. For this reason a number of methods that had been used to extract DNA from fungi, including true fungi and oomycete fungi, were assessed in this project.

The results indicated that it is more difficult to extract DNA from oomycete fungi than True fungi. Mechanical disruption of the fungal hyphae is recommended. With True fungi it is easier to gather the hyphae from plate media because True fungi tend to produce aerial hyphae, whereas most oomycetes, especially *Aphanomyces* species, grow within the agar and the hyphae cannot be separated from the agar prior to grinding. Agar contains inhibitors that interfere with the PCR reaction. Therefore, growth in a broth culture is recommended. However the moisture from the broth culture must be drained from the hyphae before grinding in liquid nitrogen. Any moisture that is present freezes, and prevents the hyphae from being ground to a fine powder. If the hyphae are placed direct into DNAzol reagent and ground with a micropestle, then any liquid carried over from the broth culture may dilute the DNAzol reagent. The hyphae must be ground for a sufficient length of time to ensure disruption of the hyphae. Chemical reagents such as SDS, and physical disruption techniques such as boiling or vortexing with glass beads, did not cause sufficient disruption of the hyphae for successful DNA extraction.

Freezing the hyphae in either liquid nitrogen or at –20°C prior to grinding in liquid nitrogen did appear to improve the yield of DNA. Two methods are recommended for the extraction of DNA from broth culture. One is the DNeasy Qiagen miniplant tissue kit with grinding in liquid nitrogen, as recommended by the manufacturer. The addition of proteinase K appeared to give more consistent results than without the use of proteinase K. The other technique that is recommended is the DNAzol reagent (Invitrogen, Life Technologies). This method did not require grinding in liquid nitrogen, however physical disruption was still required and was done by grinding the hyphae in the reagent using a micropestle and microfuge tube.

Amplification of DNA from paraffin-embedded material was variable using the primers AIFP14 + AIFP10, which produce an amplicon of 554 base pairs. The processing method used for paraffin-embedding tissue may cause shearing of the DNA. Shearing can result in small fragments of DNA that are then too small for amplification to proceed when the primers span a large molecular weight area. If this is the case then primers that produce a smaller product size will need to be developed. Further work is needed to

determine whether the variation in results is due to the large product size, or the DNA extraction method. Investigation into this will be performed in FRDC project 2004/091.

8.1.2 Testing universal primers against A. invadans

Universal primers for the amplification of fungal DNA are available and these were tested on DNA derived from a broth culture of *A. invadans*. Some of these primers, such as EF4 and fungi 5, had been reported to amplify DNA from the plant oomycete fungi, *Pythium* species. These primers did not amplify DNA from *A. invadans* and this suggests that there are significant sequence differences between the aquatic oomycetes and the plant oomycetes. The primers for the large subunit gene (28S rDNA) and primers that amplify the internally transcribed spacer regions 1 and 2 amplified DNA from *A. invadans*. These primers, therefore, can be used for identification of isolates by sequencing these regions. They can also be used as a test for assessing DNA extraction methods on a variety of tissues. The presence of fungal DNA in different types of tissue such as fish tissue or paraffin-embedded tissue can be confirmed using universal primers. In this way the universal primers can assist in the assessment of specific primers.

8.1.3 Testing published primers specific for A. invadans

Early into the project, two sets of primers were published for the detection of *A. invadans* (Lilley *et al.*, to be submitted; Blazer *et al.*, 2002). (In the case of the first reference, this was a draft paper that was presented to the research group). These primers were tested in the course of the project. The primers, in our hands, were found to cross-react with other *Saprolegnia* species, and with some true fungi that may occur on tissues or agar plates as contaminants. Although the amplicon seen on the gels from the cross-reacting species was not as brightly staining as the amplicon from *A. invadans*, the potential for cross-reaction would be of concern in samples where the concentration of DNA is low. This may be seen with tissue samples where DNA extraction methods may not yield a high quantity of fungal DNA. The primers may not be suitable as a diagnostic test because such a test needs to be robust with a high level of specificity and sensitivity.

8.1.4 Primers designed in this project for A. invadans

The internally transcribed spacer (ITS) regions are non-coding regions that evolve quickly, and as a result can be used to separate species within a genus. However, the ITS region amongst aquatic oomycete fungi seems to be more closely related than other fungi, as it proved difficult to identify regions that showed enough differences in the sequence to allow specific primers to be designed. Of thirteen primer pairs designed for *A. invadans*, only three primer pairs showed enough specificity to separate *A. invadans* from other aquatic oomycete fungi.

Primers published in the early stages of this project (AIFP1 + AIRP2, and APH3 + APH4) (Blazer *et al.*, 2002) were found to cross-react with other aquatic oomycete fungi when tested in specificity reactions. They also cross-reacted with true fungi such as *Aspergillus* species and *Penicillium* species

that are found in the environment and which can occur as contaminants on fish tissue lesions or as laboratory contaminants on plate media.

Restriction digest analysis of the internal transcribed spacer regions of *A. invadans*, *A. astaci* and saprophytic *Aphanomyces* species indicated that *A. invadans* and *A. astaci* were closely related and clustered in the same clonal group (Lilley *et al.*, 1997a). The menhaden isolate (84-1240) from the USA was found to be distinct from the other *A. invadans* isolates tested. Eight *A. invadans* isolates were tested, including *A. invadans* from Japan (NJM9030) and two isolates from red spot disease in Australia (3P and 24P). All were identical in their restriction fragment polymorphisms, indicating that the isolates were genetically identical (Lilley *et al.*, *to be submitted*). The *A. invadans* isolates from Australia, Japan, South East Asia and USA were all genetically related as indicated in restriction digests, and are likely to belong to the same clonal group (Lilley *et al.*, 1997a).

The fact that *A. invadans* and *A. astaci* clustered closely together indicates that they are genetically similar in the region of the internally transcribed spacer region. In this project we found that many of the primers that were said to be specific for *A. invadans* also produced a PCR product at the same molecular weight in *A. astaci* when specificity studies were done using these primers.

8.1.5 PCR for A. invadans using frozen tissue material infected with EUS

The primer pair AIF14 + AIR 10 was specific for *A. invadans* and also detected *A. invadans* in fish tissue. The direct detection by PCR of fungal material in tissue lesions provides a rapid diagnostic test without the need to culture viable fungus from the lesion. Therefore, the test time can be reduced from 15 days to two days.

In many cases, fish that have lesions suspicious of EUS are not sent to the laboratory within 24 hours. Any delay in the laboratory receiving the dead fish reduces the chances of culturing viable fungus. Also, fish may be frozen before being sent to the laboratory and as Aphanomyces are destroyed within minutes of freezing this also reduces the likelihood of culturing viable fungus. Thus, a method that can be used on fresh tissue and doesn't need viable fungus is an advantage for disease detection or confirmation.

8.1.6 PCR for A. invadans using paraffin-embedded tissue material infected with EUS

It was expected that primers that were specific for *A. invadans* DNA extracted from broth cultures could be used to amplify DNA extracted from paraffin-embedded material. This would allow a definitive diagnostic result using the same material that had been used for diagnosis by histopathology. However, variable results were obtained from amplification of the paraffin-embedded material. Primers for *A. invadans* (AIF14 + AIR10) did not produce amplicon, whereas the universal primers (C1+ D2) did produce amplicon. There appears to be no explanation as to why the C1 and D2 primers produced amplified product, whereas amplified product was not obtained using the specific primers. The same DNA source was used for both sets of

primers. The amplified product size is similar for both primer sets, therefore, the difference in results does not appear to be related to product size. The amplification of a smaller product size, but a negative result using primers that produced a larger product size may indicate that the DNA had been sheared into small fragments during the histological procedures for preparing the paraffin-embedded blocks. However, this theory is not supported here. Another reason may be due to a greater copy number of the large subunit regions compared to the regions that were used to design the specific primers.

Continued research into the use of paraffin-embedded material will be investigated in the next project FRDC 2004-091.

8.1.7 Different PCR reactions and growth morphologies seen with different A. invadans isolates

An unexpected finding in the project was the different amplification reactions observed with some strains of *A. invadans*. These strains were negative, or had weak reactions in the PCR for two sets of primers that were designed for the detection of *A. invadans*. These strains also had different cultural appearances on plate media.

No definite conclusions are drawn here, however it would appear that there are sequence differences in the internally transcribed spacer regions for some isolates of *A. invadans*, namely #40 (G2PA), #41 (RF8), #44 (4P, IMI 836086) and #46 (PA8). Previous studies using randomly amplified polymorphic DNA (RAPD) analysis of the internally transcribed spacer regions indicated that all isolates of *A. invadans* are clonal (Lilley *et al.*, 1997a). Cultural and growth differences have been noted in previous studies, but the isolates were different to those used in the present project (Lilley and Roberts, 1997).The purity of these cultures needs to be checked. Also, the morphology needs to be confirmed as being the same as when originally isolated. In the next phase of this project (FRDC project 2004-091), when the cultures are sent to AAHRI, Thailand for PCR validation tests, then the morphology of these isolates will be confirmed to ensure that no adverse differences have occurred.

8.1.8 Testing primers for A. astaci

Unfortunately, DNA from *A. astaci* was not available for most of the project, and as a result the primers designed in this project could not be properly assessed.

A PCR and restriction enzyme digest protocol that had been reported for *A. astaci* (Oidtmann *et al.*, 2002) was tested on DNA extracted from paraffin-embedded tissue. Unfortunately, no amplicon was obtained from this tissue, despite a strong amplicon produced with C1 + D2 universal primers. The amplicon size for the ASP1 + ASP2 primers was reported to be 1050 bp and the product size for the C1 + D2 primers was 750 bp. The large product size may have been the reason why amplification was not achieved with the *A. astaci* primers. If DNA is sheared or nicked as a result of the histological processing of the tissue, then this may prevent amplification of DNA.

No amplification was obtained using the primers on DNA extracted from formalin-fixed tissue. However, amplicon was obtained using the universal primers (C1 + D2) to the large subunit gene. This indicates that fungal DNA was present in the samples. The amount of amplicon from these reactions was low and was seen as a very faintly staining band on the gels. The amplicon size from the universal primers was 700 base pairs, and the primer pairs that had been designed produced amplicons that ranged in size from 162 bp to 810 bp. Because a 700 bp amplicon was produced from the universal primers it could be assumed that product size wasn't a reason for non-amplification using the designed primers. The primers were also used in a nested PCR reaction to improve the sensitivity of the test, but product was not detected. One reason why amplicon was seen for the universal primers but not the primers to the virulence genes may be due to copy number. If the virulence genes were in low copy number, it may explain why amplicon was not seen. However, a nested PCR should have increased the sensitivity to a level where amplicon could be detected.

Dr Birgit Oidtmann (University of Munich, Germany) has kindly provided seven cultures of *A. astaci*, and these have been sent to Mr Nick Gudkovs at the Australian Animal Health Reference Laboratory, Geelong, Victoria. DNA will be extracted from these cultures and sent to our laboratory. The primers will be tested in FRDC project 2004-091

8.1.9 DNA extraction methods formalin-fixed crayfish tissue

To be able to assess the primers designed for the detection of *A. astaci* from infected tissue that had been fixed in formalin, a reliable DNA extraction method was needed. The success of the extraction methods could be measured by amplification of the DNA using universal primers, C1 and D2, which amplify the large subunit gene (28S rDNA). Although a weak amplicon was seen, the methods could be assessed as suitable for extraction of DNA from this source. Positive amplicon using universal primers indicates that the DNA was extracted and that the piece of tissue selected did in fact contain fungal hyphae.

The most appropriate methods were the DNAzol reagent (Invitrogen, Life Technologies), and the DNeasy Qiagen miniplant tissue kit. Grinding in liquid nitrogen or the use of a proteinase digestion step did not appear to increase the yield of DNA.

8.2 Fluorescent i*n-situ* hybridization (FISH) applied to A. invadans

At this stage the FISH methods using the probes AIF14 or AIR10 cannot be used in a specific test for the detection of *A. invadans*. Although these primers produce a specific product in the PCR test, when applied to *in-situ* hybridization non-specific fluorescence was seen with *Saprolegnia* species.

The oomycetes fungi are closely related in the 18S rDNA and the 28S rDNA regions as determined by sequence alignment. However, it is also evident from the result of research undertaken in this project that the region of the internal transcribed spacer regions is also closely related. Background

staining, although weak, was seen in many of the fungal species that were tested. This background staining also occurred in clumps of hyphae and interpretation was difficult. The probes that were used were composed of 20 oligonucleotides and it may be possible to improve the specificity if a longer probe is used.

At this stage the FISH technique for the detection and identification of *A. invadans* can not be recommended as a diagnostic test for laboratories.

8.3 Fluorescent *in-situ* hybridization (FISH) applied to A. astaci

Because specific primers were unable to be identified for *A. astaci*, the FISH protocol could not be developed. This protocol will be developed in the course of FRDC project 2004-091.

8.4 Maintenance of an Oomycete fungi culture collection

The issue of the correct storage medium and conditions is an important one for laboratories involved in ongoing research or maintaining a culture collection. Further research into a long-term storage solution for the oomycetes and *A. invadans*, in particular, is still required because this study has only assessed these methods up to 12 months. It was necessary in this project to acquire as many isolates as possible in order to determine that the PCR primers could detect all isolates of *A. invadans* from different geographical locations. It was found in the course of this project that there are differences in the culture appearances and the PCR results for some of the *A. invadans* isolates. This needs further investigation, but is beyond the scope of this project.

Maintaining a culture collection is time consuming, as continual sub-culturing is needed to maintain the viability of the isolates. This brings into question the issue of genetic change that may occur because of this continual sub-culturing. Would continuous sub-culture change the integrity of the isolate over time, possibly affecting virulence? This is important for future research where these isolates are used. Therefore, any storage method that reduces the need for sub-culture would be an advantage.

9. BENEFITS

PCR for epizootic ulcerative syndrome (EUS, A. invadans)

Epizootic Ulcerative Syndrome (EUS) caused by the fungus *Aphanomyces invadans* affects freshwater finfish, mainly black bream, yellow-fin bream, silver perch and aquarium fish. The PCR for the detection and identification of EUS will improve the diagnosis for EUS. It will provide a rapid diagnostic test allowing results in two to three days rather than the 15 days it takes for diagnosis by the traditional method of culture and identification of spores. Diagnosis will be more accurate because the test is based on the detection of DNA that is specific to *A. invadans*, rather than the subjective diagnosis by clinical signs and histopathology that is reliant on the expertise of the pathologist. Rapid and accurate diagnosis of EUS will be of benefit to the fish farmers, recreational fisherman, the ornamental fish trade, researchers, diagnostic laboratories and regulators.

A rapid and accurate test will also be of benefit to the ornamental fish trade and assist in preventing the importation of strains of *A. invadans* from other countries. There is research evidence that suggests that some strains are more virulent than others, as well as some fish species being more susceptible to the disease than others. Although there are virulent strains in Australia, and research showed that red spot disease in Australia is synonymous with EUS in Asia and MG in Japan, a rapid test will assist in preventing strains of unknown virulence from entering Australia.

The tests developed in this project are likely to be used by overseas laboratories in Asia where EUS is endemic. This means that the diagnostic capacity of these laboratories will be improved and this will ultimately benefit the Australian industry. These benefits will be seen through improved disease diagnosis and detection, which may assist in preventing spread of the disease throughout the Australasian region. The validation of the PCR by laboratories in the region (Aquatic Animal Health Research Institute, Thailand and Ministry of Food and Fisheries, New Zealand) forms part of a future project FRDC 2004-091.

A test that provides an accurate diagnosis of EUS will be of benefit to future research to investigate aspects of the disease such as; the spread of the disease in Australia, determining an accurate assessment of the prevalence of EUS in the native populations of fish in the waterways across Australia, or aiding research into the virulence of the organism.

PCR for Crayfish Plague (A. astaci)

Crayfish plague affects freshwater crayfish and the disease is exotic to Australia. Currently, export of Western Australian yabbies to Europe, particularly Switzerland, requires a permit to assure the produce is free of crayfish plague despite Australia being free of the disease. An accurate and rapid PCR method for the detection of the disease will benefit the West Australian Yabby Producers, and other freshwater crayfish exporters. Test results will be provided in two days, rather than the two weeks that it takes for a result to be obtained using the current culture method. For exporters

this means a quick turn around time between submitting samples for testing, and the provision of an export permit.

Crayfish plague affects all freshwater crayfish in Australia, and should the disease enter Australia and get into the waterways it would cause an ecological disaster because the disease is impossible to eradicate. Should a disease incursion occur in Australia, through the importation of infected material, a PCR will assist in rapid disease diagnosis and emergency response to contain the disease. Therefore, it is important that a specific test for the detection of the disease continues to be investigated.

10. FURTHER DEVELOPMENT

All the issues that are discussed below will be addressed in a follow-on project FRDC 2004-091.

*PCR for EUS (*A. invadans*)*

The PCR for the identification of *A. invadans* (EUS) from broth culture material and fresh fish tissue is ready to be used by laboratories. The validation of this test will be performed by Australian and overseas laboratories that include the following; Department of Primary Industries Queensland, Department of Primary Industries Water and Environment Tasmania, Australian Animal Health Research Laboratory, Victoria, Aquatic Animal Health Research institute in Thailand, Ministry for Forests and Fisheries New Zealand, National Fish Health Research Laboratory, USA, CEFAS, UK, and Assiut University, Egypt.

PCR for EUS using paraffin-embedded (archival) material

The development of the PCR for use on paraffin-embedded tissue needs further work to determine why DNA was not amplified from this type of sample. One explanation may be the length of the amplicon, which would require the design of primers that produce a smaller amplicon. However, it will be difficult to identify a region on the genome (as currently known) because of the genetic similarity of the oomycete fungi in the internally transcribed spacer region.

*PCR for Crayfish Plague (*A. astaci*)*

The PCR for the detection of crayfish plague was unable to be completed in the course of this project because of a lack of DNA extracted from broth culture. DNA extraction from paraffin-embedded tissue and formalin-fixed tissue may contain substances that are inhibitory to the PCR reaction and therefore the primers that were designed for *A. astaci* were unable to be accurately tested on this type of material.

DNA will be extracted from broth culture by the Australian Animal Health Reference Laboratory and will be used to test primers that were designed in the course of the current project.

*Fluorescent in-situ hybridisation for EUS (*A. invadans*)*

The primers that were specific for *A. invadans* in the PCR were not specific when tested using the FISH method. The FISH assay will continue to be investigated in the next project, using longer primers.

Fluorescent in-situ hybridisation for Crayfish Plague (A. astaci)

Because primers were unable to be tested in the current project, this method will be developed in the next project if the results of the primers designed for the PCR prove to be specific.

Write method for the Australian and New Zealand Standard Diagnostic Procedures.

The procedure for the PCR and FISH for both EUS and CP will be written at the end of the next project (FRDC 2004-091) which validates the PCR

method for *A. invadans* and investigates the problems encountered for the tests for paraffin-embedded material and the FISH method.

11. PLANNED OUTCOMES

The planned outcomes of this project were to provide Australian laboratories with molecular diagnostic tests for the detection of two fungal diseases – Epizootic Ulcerative Syndrome (EUS) and Crayfish Plague (CP). The tests to be developed were a polymerase chain reaction (PCR) and a fluorescent *in-situ* hybridization method (FISH).

The output of a specific PCR for the detection of *A. invadans* that causes EUS has contributed to the planned outcomes. This rapid diagnostic test will assist in improved detection methods that can be used by laboratories for disease diagnosis of EUS using fresh fish tissue, or identification of fungal growth cultured from lesion samples.

The test will contribute to future research that will lead to a greater understanding of the disease, such as transmissibility studies, infection rate, dose etc. The test will facilitate surveillance for the disease and lead to improved aquaculture management.

Improved diagnostic capability will increase industry confidence for disease diagnosis.

12. CONCLUSIONS

12.1 Objective 1. Develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces invadans* in Australian fish, based on the polymerase chain reaction (PCR) for use with fresh, or dead tissue samples.

- A specific PCR for the detection of *Aphanomyces invadans* was developed.
- The PCR can be used to identify fungal growth cultured from lesion material obtained from infected fish.
- The PCR differentiates *A invadans* from other oomycete fungi such as *Saprolegnia* species that may also cause lesions, or be part of the contaminating flora in a skin lesion.
- The PCR differentiates *A. invadans* from true fungi such as *Aspergillus* species that may occur as laboratory contaminants.
- The PCR can be used on fungal DNA that has been extracted from fish tissue that is fresh, or has been stored frozen at –80°C.
- The PCR detected differences between different cultures of *A. invadans* (PA8, RF8, 4P, IMI 836082). These cultures also had different cultural morphologies to the other cultures of *A. invadans*.
- The PCR primers were unable to detect *A. invadans* in paraffin-embedded samples.

- DNA extraction could be achieved from fungal material derived from broth culture, using the DNAzol reagent (Invitrogen Life Technologies™) or the Qiagen DNeasy mini plant kit.
- DNA extraction could be achieved from fungal material derived from fish tissue, using the DNAzol reagent (Invitrogen Life Technologies™) or the Qiagen DNeasy mini plant kit.
- The commercially-available master mix from Promega produced the best results and was more sensitive than the in-house PCR master mix.
- The sensitivity of the test was 19.5 pg/ml using Promega master mix and DNA extracted from broth culture.

12.2 Develop a sensitive and specific molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the polymerase chain reaction (PCR) for use with fresh or dead tissue samples.

- Primers were designed to the virulence genes of *Aphanomyces astaci*; putative chitinase gene chi1, subtilisin-like serine proteinase precursor (SP1) gene, trypsin proteinase precursor (SP2) gene.
- The primers could not be tested properly because of a lack of fresh DNA derived from broth culture material.
- No amplification of DNA from formalin-fixed tissue was achieved using the primers designed to the virulence genes.
- Amplification of DNA from formalin-fixed tissue was achieved using universal primers to the large sub-unit gene 28S rDNA.
- Amplification of DNA from formalin-fixed tissue was achieved using universal primers to the internally transcribed spacer regions ITS1 and ITS2.
- Amplification of DNA from paraffin-embedded tissue was achieved using universal primers to the large sub-unit gene 28S rDNA.
- DNA extraction could be achieved from formalin-fixed tissue using the DNAzol reagent (Invitrogen Life Technologies™) or the Qiagen DNeasy mini plant kit.

12.3 Develop a rapid molecular diagnostic test for the detection of *Aphanomyces invadans*, based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

- Fluorophores were designed from primers (AIF14 + AIR10) that were specific to *A. invadans* in the PCR test.
- The fluorophores cross-reacted with *Saprolegnia* species and specificity could not be improved.
- Fluorophores were designed from the universal primers C1 and D2 for use as positive controls. The fluorophore using C1 gave the best results.

12.4 Develop a rapid molecular diagnostic test for the detection of *Aphanomyces astaci*, based on the technique of *fluorescent in situ hybridization* (FISH), which allows visualization of the fungus direct from lesion smears or culture material within one hour.

- The method for FISH was unable to be developed for *A. astaci* because the primers that were designed could not be tested due to lack of fresh DNA.

12.5 Write up Australian Standard Diagnostic Techniques based on the above tests and in the format supplied by AFFA.

- The ANZSDT will be written at the conclusion of a test validation project, FRDC 2004-091.

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Appendix A Intellectual Property

The intellectual property arising from this project is freely available to all.

Appendix B Staff List

Principal Investigator	Ms. Nicky Buller
Research Officer	Ms. Heather McLetchie
Co-Investigators	Dr Stan Fenwick
	Dr Phil Nicholls
	Dr Brian Jones
	Dr David Alderman

Appendix C : Formulae of Media, Buffers and Reagents

Media for *Aphanomyces invadans*

Glucose peptone (GP) Agar
(Roberts *et al.*, 1993).

Glucose	3 g
Peptone	1 g
$MgSO_4.7H_20$	0.128 g
KH_2PO_4	0.014 g
Ca	8 mg
Fe	0.5 mg
Mn	0.5 mg
Cu	0.1 mg
Zn	0.1 mg
Agar	15 g (1.5% w/v)
Distilled water	1000 ml

Prepare trace elements as a stock solution. Add 1 ml of trace element mix to 1l of DW. Add other ingredients to DW. Mix to dissolve and autoclave at 121°C for 15 min. Cool to 50°C and dispense into Petri dishes. Store media in plastic bags at 4°C.

Glucose peptone broth (GP)
(Roberts *et al.*, 1993).
As for GP agar but without the addition of agar. Autoclave at 121°C for 15 min. Dispense 10 ml aliquots into McCartney bottles. Store at 4°C.

GP broth and GP agar are used as primary growth media.

GP broth and GP agar supports the growth of *Aphanomyces* species, *Saprolegnia* species and other Oomycetes fungi.

GP-POX Broth
(Roberts *et al.*, 1993)

Glucose	3 g
Peptone	1 g
$MgSO_4.7H_2O$	0.128 g
KH_2PO_4	0.014 g
Ca	8 mg
Fe	0.5 mg
Mn	0.5 mg
Cu	0.1 mg
Zn	0.1 mg
Penicillin	0.1 g
Oxallinic acid	0.1 g
Water	1,000 mls

GPY-PS Agar
(Roberts *et al.*, 1993)

Glucose	3 g
Peptone	1 g
$MgSO_4.7H_2O$	0.128 g
KH_2PO_4	0.014 g
Yeast extract	0.5 g
Ca	8 mg
Fe	0.5 mg
Mn	0.5 mg
Cu	0.1 mg
Zn	0.1 mg
Penicillin	0.1 g
Streptomycin sulphate	0.1 g
Agar	15 g (1.5% w/v)
Water	1,000 mls

GPY-PS Broth
(Roberts *et al.*, 1993)

Glucose	3 g
Peptone	1 g
$MgSO_4.7H_2O$	0.128 g
KH_2PO_4	0.014 g
Yeast extract	0.5 g
Ca	8 mg
Fe	0.5 mg
Mn	0.5 mg
Cu	0.1 mg
Zn	0.1 mg
Penicillin	0.1 g
Streptomycin sulphate	0.1 g
Water	1,000 mls

Method for *Aphanomyces invadans* isolation
(Willoughby and Roberts, 1994)

Stage 1.
Isolate in liquid medium (GP-POX).
Cut thin slices of muscle tissue from the ulcerated region. Cut slices from successively deeper muscle. Transfer each slice to a separate dish of liquid resuscitation growth medium – GP-POX broth. Incubate 6 h at 22°C.

Stage 2.
Liquid medium
After 6 h incubation in stage 1, examine the tissue slices for newly formed hyphae. Make sure these hyphae have clear continunum with the hyphae in the muscle tissue. These often have a narrower diameter than the parent hyphae. Select these hyphae with the supporting tissue and transfer to a dish of fresh medium. The lower fish slices will have the newer hyphae.
These slices are incubated at 22°C for a further 24 h.

Stage 3
Should now see colonies about 4 mm in diameter. Larger colonies are likely to be an Aphanomyces saprophyte.

Stage 4
Transfer the 4mm colonies to solid agar. If done too early or too late, may not get growth or may get overgrowth of contaminants.
Transfer is to GPY Agar (glucose-peptone-yeast extract agar. (1.5% w/v agar). Containing penicillin G & streptomycin sulphate both at 100 mg/l with yeast extract at 0.5 g/l).
Examine hourly.

Stage 5.
Transfer colony margin to GPY broth. Stock cultures can be obtained and held at 10°C and subcultured every 4 to 5 weeks.

Media for *Aphanomyces astaci*

Isolation Medium (IM)
(Alderman and Polglase, 1986)

Oxoid Agar No1	2.4 g
Difco Yeast Extract	0.2 g
Glucose	1.0 g
Distilled Water	200 ml
Oxolinic Acid	2.0 mg
Penicillin G	200 mg

Combine all ingredients except for the antibiotics. Autoclave at 121°C for 15min. Cool to 50°C. Filter-sterilise the antibiotics and aseptically add to cooled medium. Store at 4°C.

If possible it is recommended that the distilled water is substituted with tap water or river water. Ensure that the river water is not collected from a chalk

and limestone area as the calcium and magnesium can cause strange colony forms or reduced growth of the fungus (Dr David Alderman pers comm).

This media supports the growth of *Aphanomyces* species, *Saprolegnia* and other Oomycetes fungi.

Appendix D Data for assessment of storage methods

Table D 1. Results of DW, GP plate, slope and broth at room temperature

	DW							**GP Plate**						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time														
3 months	V	V	V	V	V	V	V	V	N	V*	V*	V	V*	V
6 months	V	N	V	V*	V	V	V	V	N	V	V*	V	V*	V
9 months	V	N	V	N	V	V	V	N	N	N	N	V	V*	V
12 months	V	N	V	V*	V	V	V	N	N	N	N	N	V*	N
	GP Slope							**GP Broth**						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time														
3 months	V	V	V	V	V	V	V	V	N	N	V	V	V*	V
6 months	V	V	V	V*	V	V	V	V	V	V	V*	V	V*	V
9 months	V	N	V	N	V	V*	V	V	N	V	N	V	V*	V
12 months	V	N	V	N	V	V	V	V	N	V	N	V	V*	V

DW = distilled water; GP = glucose peptone slope; G = group number; N = non-viable; T = time in months of subculture; V = viable; V* = results for viability were variable.

Table D 2. Results of DW, GP plate, slope and broth at 4°C

	DW							**GP Plate**						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time														
3 months	V	N	V*	N	N	V	V	V	N	N	N	V	V	V
6 months	V	N	V*	N	N	V	V	V	N	N	N	V	V	V
9 months	V	N	V*	N	N	V	V	N	N	N	N	N	V*	V
12 months	V	N	V*	V*	N	V	V	N	N	N	N	N	V	V
	GP Slope							**GP Broth**						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time														
3 months	V	N	V	N	V	V	V	V	N	N	N	V	V	V
6 months	V	N	V	N	V	V	V	V	N	V*	N	V	V*	V
9 months	V	N	V	N	V	V*	V	V	N	N	N	V	N	V
12 months	V	N	V	N	V	V	V	V	N	V*	N	V	V*	V

DW = distilled water; GP = glucose peptone slope; Group = group number; N = non-viable; T = time in months of subculture; V = viable; V* = results for viability were variable.

Table D 3. Results of DW, GP slope and broth at RT with an oil layer

	DW							**GP Slope**							**GP Broth**						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time																					
3 months	V	V	V	V*	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
6 months	V	N	V	V	N	V	V	V	V	V	V	V	V	V	N	N	V	V	V	V*	V
9 months	V	V	V	V*	V	V	V	V	N	V	N	V	V	V	V	N	V*	N	V	V*	V
12 months	V	V	V	V	V	V	V	V	N	V	N	V	V	V	V	N	V*	N	V	V*	V

DW = distilled water; GP = glucose peptone slope; Group = group number; N = non-viable; T = time in months of subculture; V = viable; V* = results for viability were variable.

Table D 4. Results of DW, GP slope and broth at 4°C with an oil layer

	DW							GP Slope							GP Broth						
Group	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Time																					
3 months	V	N	V*	N	N	V	V	V	N	V*	N	V	V	V	V	N	V*	N	V	V*	V
6 months	V	N	V	N	N	V	V	V	N	V*	N	V	V	V	V	N	V	N	V	V*	V
9 months	V	N	V	N	N	V	V	V	N	V*	N	V	V	V	V	N	N	N	V	N	V
12 months	V	N	N	N	N	V	V	V	N	V*	N	V	V	V	V	N	N	N	V	V*	V

Table D 5. Results of Cryopreservation at –80°C

	-80°C						
Group	1	2	3	4	5	6	7
Time							
3 months	N	N	N	N	V	N	V
6 months	V	N	N	N	N	N	V
9 months	N	N	N	N	N	N	V
12 months	N	N	N	N	N	N	V

N = non-viable; V = viable

Appendix E List of Suppliers for Reagents

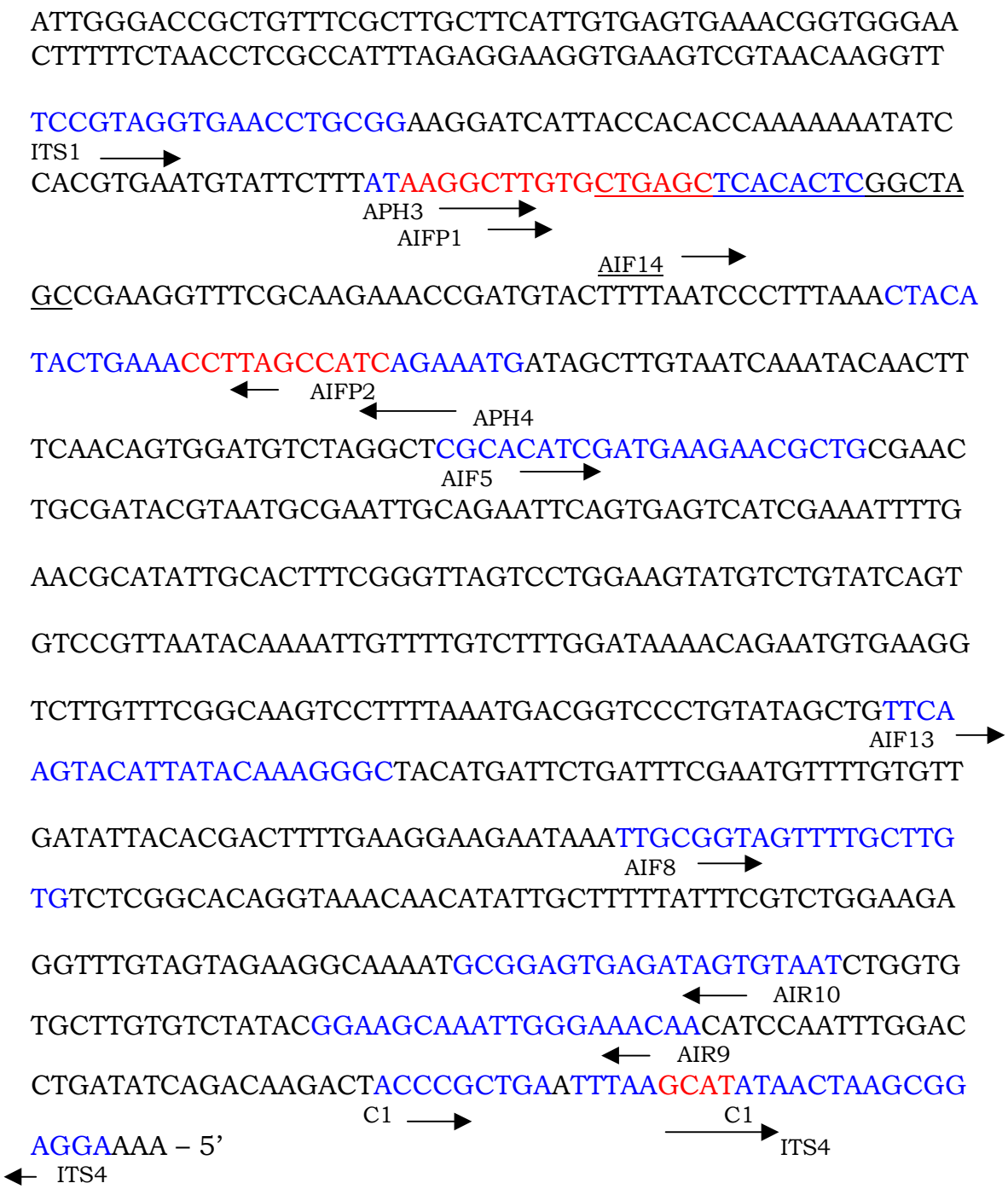
Table E 1. List of reagents and suppliers

Reagent	Supplier	Catalogue number
Amplitaq DNA polymerase	Applied Biosystems	N808-0172
DNA ladder 100 bp	Geneworks	DMW-100L
DNA lambda ladder	BioRad Laboratories	170-3635
DNAzol DNA extraction reagent	Invitrogen Life Technologies	1050-027
Dye terminator ready reaction cycle sequencing kit	Applied Biosystems	4-2080
E.Z.N.A™ Plant DNA Miniprep kit	Omega Bio-tek	D3486-01
HyperLadder IV 100 bp ladder. Quantitative DNA ladder	Astral Scientific	BioLine BIO-33029
Instagene DNA extraction kit	BioRad Laboratories	732-6030
PrepMan Reagent	Applied Biosystems	P/N 4305663
Puregene	Gentra	
IQ Supermix	BioRad Laboratories	
QIAquick PCR purification kit	Qiagen	
Qiagen DNeasy mini plant kit	Qiagen	69104

Appendix F Data for design and testing of primers for *A. invadans*

Aphanomyces invadans sequence information from internally transcribed spacer region 1, 5.8S rDNA, internally transcribed spacer region 2. 3 prime to 5 prime, and alignment of primer sequences.

Figure F 1. Sequence information for *A. invadans* and relationship of primers



Blue colour denotes primer.
Red colour indicates common areas of two different primers
Underline indicates primer

Table F 1. Sequence of primers designed for *A. invadans* in this project

Primer ID	Sequence 5 prime to 3 prime
AIF3	AGCTCACACTCGGCTAGC
AIR4	TCAACAGTGGATGTCTAGGC
AIR5	CGCACATCGATGAAGAACGCTG
AIF6	TGGCAATTGGAATGAGAACA
AIR7	AAATATGGCCCAAACATCCA
AIF8	TTGCGGTAGTTTTGCTTGT
AIR9b	GTTGTTTCCCAATTTGCTTCC
AIF12	CTGAGCTCACACTCGGCTAGC
AIF13	TTCAAGTACATTATACAAAGGGC
AIRP13	GCTAGCCGAGTGTGAGTCAG
AIRP10	ATTACACTATCTCACTCCGC
AIRP11	GCTTCCGTATAGACACAAGC
AIFP14	CTGACTCACACTCGGCTAGC
AIRP14	GCTAGCCGAGTGTGAGTCAG (Reverse compliment of AIFP14)
AIRP14	GCTAAGGTTTCAGTATGTAG
AIFP15	CTACATACTGAAACCTTAGC
AIRP15	GCTAAGGTTTCAGTATGTAG (Reverse compliment of AIFP15)
AIRP15	GCTATCATTTCTGATGGCTAAG
AIFP16	CTTAGCCATCAGAAATGATAGC
AIRP16	GCT ATC ATT TCT GAT GGC TAA G (Reverse compliment of AIFP16)
AIFP17	CCG GGC TAA TTT AGT CTG GC
AIFP18	TGA GTG AAA CGG TGG GAA
AIRP19	TGA CAT TTC GTC GAC CGG ACG C

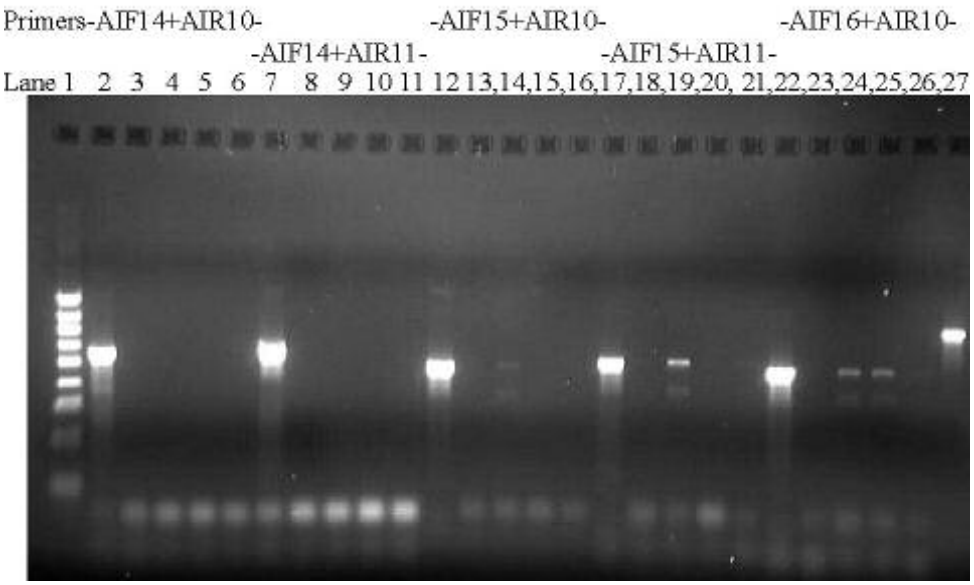


Figure F 2. Results for specificity testing of primers for *A. invadans*.

Lanes 2 to 6 = primers AIF14+AIR10; Lanes 7 to 11 = primers AIF14+AIR11; Lanes 12 to 16 = primers AIF15+AIR10; Lanes 17 to 21 = primers AIF15+AIR11; Lanes 22 to 26 = primers AIF16+AIR10; lane 27 = primers C1+D2. Lane 1 = Hyper IV molecular weight marker; Lane 2 = *A. invadans* #24 (NSW); Lane 3 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 4 = *Pythium sulcatum*. #22 (WAC 8470); Lane 5 = *Saprolegnia* sp. (45710, QLD); Lane 6 = *S. diclina* ATCC36144; Lane 7 = *A. invadans* #24 (NSW); Lane 8 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 9 = *Pythium sulcatum*. #22 (WAC 8470); Lane 10 = *Saprolegnia* sp. (45710, QLD); Lane 11 = *S. diclina* ATCC36144; Lane 12 = *A. invadans* #24 (NSW); Lane 13 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 14 = *Pythium sulcatum*. #22 (WAC 8470); Lane 15 = *Saprolegnia* sp. (45710, QLD); Lane 16 = *S. diclina* ATCC36144; Lane 17 = *A. invadans* #24 (NSW); Lane 18 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 19 = *Pythium sulcatum*. #22 (WAC 8470); Lane 20 = *Saprolegnia* sp. (45710, QLD); Lane 21 = *S. diclina* ATCC36144; Lane 22 = *A. invadans* #24 (NSW); Lane 23 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 24 = *Pythium sulcatum*. #22 (WAC 8470); Lane 25 = *Saprolegnia* sp. (45710, QLD); Lane 26 = *S. diclina* ATCC36144; Lane 27 = *A. invadans* #24 (NSW) with primers C1+D2.

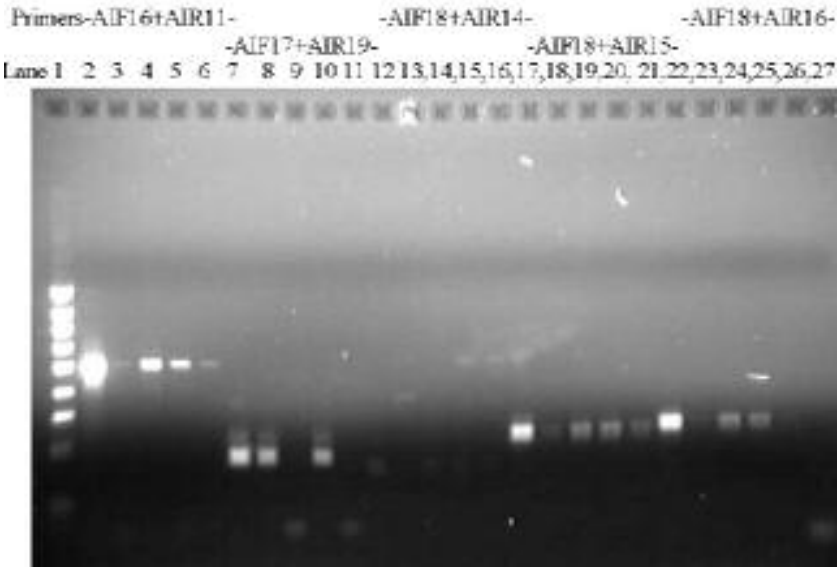


Figure F 3. Results for specificity testing of primers for *A. invadans*.

Lanes 2 to 6 = primers AIF16+AIR11; Lanes 7 to 11 = primers AIF17+AIR19; Lanes 12 to 16 = primers AIF18+AIRC14; Lanes 17 to 21 = primers AIF18+AIRC15; Lanes 22 to 26 = primers AIF18+AIRC16; Lane 1 = Hyper IV molecular weight marker; Lane 2 = *A. invadans* #24 (NSW); Lane 3 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 4 = *Pythium sulcatum*. #22 (WAC 8470); Lane 5 = *Saprolegnia* sp. (45710, QLD); Lane 6 = *S. diclina* ATCC36144; Lane 7 = *A. invadans* #24 (NSW); Lane 8 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 9 = *Pythium sulcatum*. #22 (WAC 8470); Lane 10 = *Saprolegnia* sp. (45710, QLD); Lane 11 = *S. diclina* ATCC36144; Lane 12 = *A. invadans* #24 (NSW); Lane 13 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 14 = *Pythium sulcatum*. #22 (WAC 8470); Lane 15 = *Saprolegnia* sp. (45710, QLD); Lane 16 = *S. diclina* ATCC36144; Lane 17 = *A. invadans* #24 (NSW); Lane 18 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 19 = *Pythium sulcatum*. #22 (WAC 8470); Lane 20 = *Saprolegnia* sp. (45710, QLD); Lane 21 = *S. diclina* ATCC36144; Lane 22 = *A. invadans* #24 (NSW); Lane 23 = *Saprolegnia* sp. #11 (AS-01-3195-2); Lane 24 = *Pythium sulcatum*. #22 (WAC 8470); Lane 25 = *Saprolegnia* sp. (45710, QLD); Lane 26 = *S. diclina* ATCC36144; Lane 27 = negative DNA control.

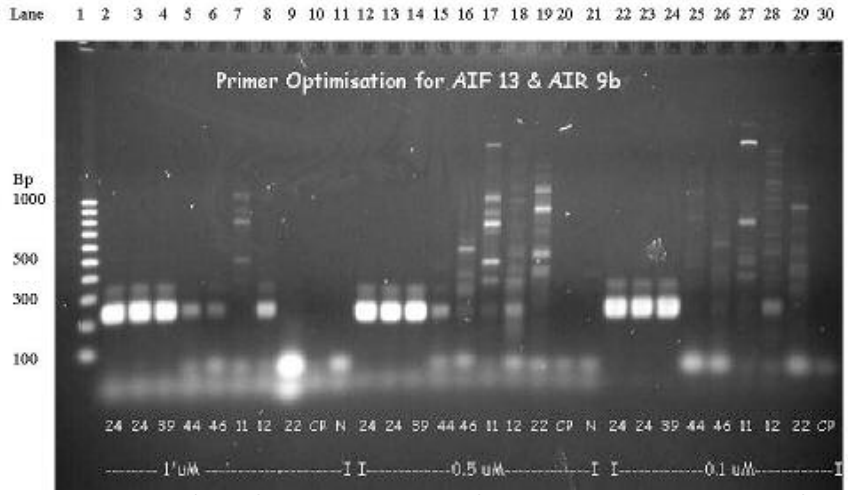


Fig F 4. Results of optimisation of primer concentration for AIF13 + AIR9b.

Lanes 2 to 11 = primer concentration 1 µM; Lanes 12 to 20 = primer concentration 0.5 µM; Lanes 21 to 30 = primer concentration 0.1 µM. Lane 1 = 100 base pair marker (Geneworks); Lane 2 = *A. invadans* A2S2 (#24); Lane 3 = *A. invadans* A2S2 (#24); Lane 4 = *A. invadans* T99G2 (#39); Lane 5 = *A. invadans* 4P (#44); Lane 6 = *A. invadans* PA8 (#46; Lane 7 = *Saprolegnia* sp. AS-01-3195-2 (#11); Lane 8 = Unidentified fungus AS-01-4189 (#12); Lane 9 = *Pythium sulcatum* WAC 8470 (#22; Lane 10 = formalin-fixed Crayfish plague tissue; Lane 11 = negative DNA control; Lane 12 = *A. invadans* A2S2 (#24); Lane 13 = *A. invadans* A2S2 (#24); Lane 14 = *A. invadans* T99G2 (#39); Lane 15 = *A. invadans* 4P (#44); Lane 16 = *A. invadans* PA8 (#46; Lane 17 = *Saprolegnia* sp. AS-01-3195-2 (#11); Lane 18 = Unidentified fungus AS-01-4189 (#12); Lane 19 = *Pythium sulcatum* WAC 8470 (#22; Lane 20 = formalin-fixed Crayfish plague tissue; Lane 21 = negative DNA control; Lane 22 = *A. invadans* A2S2 (#24); Lane 23 = *A. invadans* A2S2 (#24); Lane 24 = *A. invadans* T99G2 (#39); Lane 25 = *A. invadans* 4P (#44); Lane 26 = *A. invadans* PA8 (#46; Lane 27 = *Saprolegnia* sp. AS-01-3195-2 (#11); Lane 28 = Unidentified fungus AS-01-4189 (#12); Lane 29 = *Pythium sulcatum* WAC 8470 (#22; Lane 30 = formalin-fixed Crayfish plague tissue.

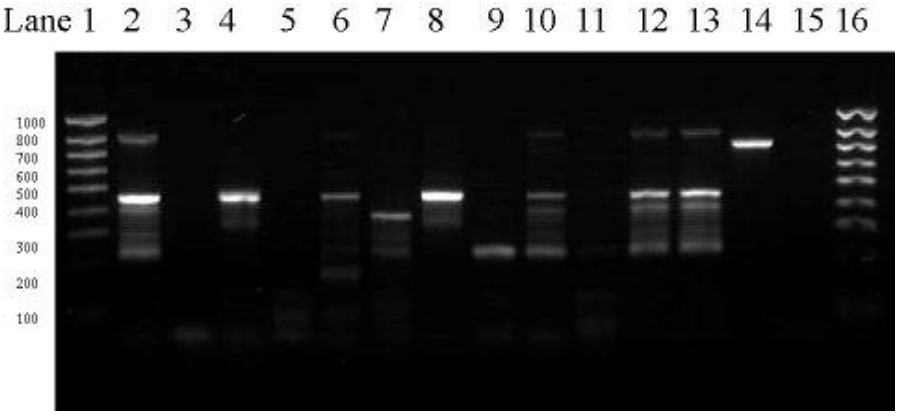


Fig F 5. Further results of specificity testing for primers AIF13 + AIR9b.

Lane 1 = Hyper IV molecular weight marker; Lane 2 = *A. invadans* #24; Lane 3 = *A. astaci*; Lane 4 = AS-01-3195 (unknown fungus); Lane 5 = AS-01-4050 (unknown fungus); Lane 6 = AS-01-4050 (*Penicillium* sp.); Lane 7 = WAC 8470 (*Pythium sulcatum*); Lane 8 = 45710 (*Saprolegnia* sp); Lane 9 = ATCC 36144 (*S. diclina*); Lane 10 = IV-d (Unknown fungus); Lane 11 – IV-r (Unknown fungus); Lane 12 = *A. invadans* #24; Lane 13 = *A. invadans* #24; Lane 14 = C1D2 universal primers; Lane 15 = DNA-negative control.

Table F 2. Results of FISH tested at 55°C hybridisation temperature

ID Code	Fungal ID	Probe	Background	Results
#24 (A2S2, NSW)	*A. invadans*	10	+	++
#24 (A2S2, NSW)	*A. invadans*	14	+	++
#24 (A2S2, NSW)	*A. invadans*	10+14	+, weak	+++
#44	4P *A. invadans*	10	weak	Lots of spore heads – faint fluorescence
#44	4P *A. invadans*	14	weak	negative
03-3416	*Saprolegnia* sp	10	+++ in clumps of hyphae	Neg on hyphae
03-3416	*Saprolegnia* sp	14	+ in clumps	+, hyphae are green, but not true fluorescence
03-3416	*Saprolegnia* sp	14+10	+ in clumps	Pale green fluorescence only. Negative in individual hyphae
03-3827-4	*Saprolegnia* sp	10	+ in clumps	+. Granules fluorescing in hyphae
03-3827-4	*Saprolegnia* sp	14	+ in clumps	+. Granules fluorescing in hyphae
03-3827-5	*Saprolegnia* sp	10	++ in clumps	negative
03-3827-5	*Saprolegnia* sp	14	+++ in clumps	+ in hyphae
03-3827-5	*Saprolegnia* sp	14+10	+ in clumps	Pale green fluorescence only
#6 01-4050	fungus	14	weak	negative
#6 01-4050	fungus	10	weak	negative
#6 01-4050	fungus	14+10	negative	Weak fluorescence in spore heads

Appendix G : Publications and Presentations

Presentations at conferences and workshops

McLetchie H, Buller N, Jones B, Fenwick S, Nicholls P, Alderman D. 2004. Rapid diagnostic PCR for Epizootic Ulcerative Syndrome. To be presented in Oct 2004 at the European Aquaculture Society Conference, Barcelona, Spain.

McLetchie H, Buller N, Jones B, Fenwick S, Nicholls P, Alderman D. 2004. A rapid diagnostic technique for *Aphanomyces invadans*. Biotechnology Forum, Department of Agriculture Western Australia, 27th of April, Perth.

Buller NB, McLetchie H, Fenwick S, Nicholls P, Jones B, Alderman D. 2003. Molecular Diagnostic Tests to Detect Epizootic Ulcerative Syndrome (*Aphanomyces invadans*) and Crayfish Plague (*Aphanomyces astaci*). Aquatic Animal Health Conference, Geelong, Victoria 8-10th Oct. 2003.

H. McLetchie, N. Buller, P. Nichols, S. Fenwick, B. Jones, D. Alderman. Rapid Diagnostic Kit for *Aphanomyces invadans* and *Aphanomyces astaci*. Combined Australian and New Zealand Society for Microbiology Conference, Auckland, New Zealand Oct 2003.

H. McLetchie, N. Buller, P. Nichols, S. Fenwick, B. Jones, D. Alderman. Rapid Diagnostic Kit for *Aphanomyces invadans* and *Aphanomyces astaci*. Presented to the Research Discussion Group, AHL, Perth WA, Sept 2003.

Buller NB. 2003. Detection of Crayfish Plague. Presented to the Yabby Producers Association, Narrogin, Western Australia, April 2003.

Conference Proceedings

Buller NB, McLetchie H, Fenwick D, Nicholls P, Jones B, Alderman D. 2003. Molecular Diagnostic Tests to Detect Epizootic Ulcerative Syndrome (*Aphanomyces invadans*) and Crayfish Plague (*Aphanomyces astaci*). *In*: Aquatic Animal Health Subprogram Scientific Conference, Geelong, Victoria 8-10th Oct. 2003.

Non-Refereed Publications

Buller NB. 2002. Improving Diagnostic Capability for Fungal Disease Detection. Health Highlights: Aquatic animal health subprogram newsletter. Vol 2 (1) Feb 2002.

Buller NB. 2002. Improving Diagnostic Capability for Fungal Disease Detection. AQWA News 2002.

Other Poster Presentations and Acknowledgements

Heather McLetchie. 2003. Development of a PCR for the detection of *Aphanomyces invadans*. Post Graduate Student poster day, Murdoch University, WA. Winner of the best technical poster.

Heather McLetchie. N. Buller, B. Jones, S. Fenwick, P. Nicholls, D. Alderman. 2002. Develop Molecular Diagnostic Tests for the Identification of Epizooztic Ulcerative Syndrome (*Aphanomyces invadans*) and Crayfish Plague (*A. astaci*). Post Graduate Student poster day, Murdoch University, WA. Winner of the best aquatic science poster.

Appendix H : Glossary and Abbreviations

AAHRI	Aquatic Animal Health Research Institute
AHL	Animal Health Laboratories
ATCC	American Type Culture Collection
AQIS	Australian Quarantine Service
BLAST	Basic Logic Alignment Search Tool
bp	base pairs
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
DAWA	Department of Agriculture Western Australia
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribose nucleic acid
DPI	Department of Primary Industries
DPI QLD = Department of Primary Industries, Queensland	
DW	Distilled water
EDTA	Ethylene-diamine-tetra acetic acid
EUS	Epizootic Ulcerative Syndrome
GP	Glucose Peptone (medium)
GP-POX	Glucose Peptone medium containing penicillin and oxolinic acid
IM	Isolation medium
ITS	Intergenic spacer regions
MG	Mycotic granulomatosis
MW	Molecular weight marker
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
QLD	Queensland
rRNA	Ribosomal ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RSD	Red Spot Disease
SDS	Sodium dodecyl sulfate
subsp.	Subspecies
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris/boric acid/EDTA buffer
TE	Tris/EDTA buffer
Tm	Melting temperature
U	Units of enzyme
UV	Ultraviolet
V	Volts
WA	Western Australia