FINAL REPORT

Further Research and Laboratory Trials for Diagnostic Tests for the Detection of *Aphanomyces invadans* (EUS) and *A. astaci* (Crayfish Plague)

Dr. Nicky Buller

June 2007

Project No. 2004/091

Australian Government
Department of Agriculture, Fisheries and Forestry

Department of Agriculture and Food

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CSIRO

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N. B. Buller

Further Research and Laboratory Trials for Diagnostic Tests for the Detection of *A. invadans* (EUS) and *A. astaci* (Crayfish Plague)

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Project Investigators

PROJECT TITLE

Further Research and Laboratory Trials for Diagnostic Tests for the Detection of *A. invadans* (EUS) and *A. astaci* (Crayfish Plague)

PROJECT NUMBER FRDC 2004/091

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2004/091	**Further research and laboratory trials for diagnostic tests for the detection of *A. invadans* (EUS) and *A. astaci* (crayfish plague)**

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OBJECTIVES:

1 Complete validation of *A. invadans* PCR on fresh tissue and wax-embedded tissue

2 Complete research to develop specific PCR for *A. astaci* (crayfish plague)

3 Complete research to develop FISH tests for *A. invadans* and *A. astaci*

4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

5 Analyse feedback from participating laboratories on PCR tests and FISH

6 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedures and FRDC Report

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

A PCR has been developed that is specific for the identification of *Aphanomyces invadans* (epizootic ulcerative syndrome) for direct detection in fish tissue and cultured hyphae but is unable to amplify DNA from formalin-fixed paraffin-embedded (FF-PE) sections. Fluorescent *in situ* hybridisation (FISH) using a peptide nucleic acid probe has been developed for use on fresh cultured hyphae and FF-PE sections.

Two primer sets for use in PCR were validated for the detection of the exotic disease, crayfish plague (*A. astaci*) for use on (FF-PE). One primer set successfully amplified DNA from FF-PE sections, but cross-reacted with *A. invadans* (which has never been reported from freshwater crayfish). The second primer set produced a larger amplicon, which was specific but unable to amplify DNA from FF-PE sections.

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% which would be catastrophic should the disease occur in Australia. It is present in North America where the native freshwater crayfish are largely resistant to the disease and can act as carriers of the fungus. 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), caused by infection with *Aphanomyces invadans*, is endemic in many fish in river systems throughout Australia. The disease causes economic losses to the freshwater finfish aquaculture industry and affects local native stocks. Both diseases are almost impossible to eradicate.

Both diseases are difficult to diagnose, especially if laboratory personnel are not sufficiently skilled in recognising or differentiating the diseases. The culture and identification of both fungal species 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 species and often requires the culture being sent to a specialist laboratory for confirmation of identification. Examination of histological samples requires expertise and although the presence of hyphae of 7-10 µm in width 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 in line with the Federal Budget Initiative "Building a National Approach to Animal and Plant Health" funding to improve disease diagnosis in aquaculture.

The research undertaken in this project sought to provide two diagnostic tests for each disease based on molecular (DNA) biological techniques, polymerase chain reaction (PCR) and fluorescent *in-situ* hybridization (FISH) using peptide-nucleic acid probes. In both techniques, identification is based on detection of a DNA sequence that is unique to the target organism. The uniqueness of the DNA sequence is demonstrated by testing specificity against other genetically similar organisms or those that may be found in the same ecological location. For detection and identification of *A. invadans* the tests were applied to fungal culture material, fresh tissue and formalin-fixed paraffin-embedded tissue. For *A. astaci* the tests were applied to formalin-fixed paraffin-embedded material.

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 developed. 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 or fungal species that are likely to occur as plate contaminants on laboratory media, such as *Aspergillus* species and *Penicillium* species. The primers, which produce an amplicon of 554 base pairs (bp), were unable to amplify the DNA from paraffin-embedded tissue. This is most likely due to the effect of the formalin fixative on the DNA, which in the process of fixing the tissue cross-links amino acids groups. This cross-linking makes it difficult for primers that produce a large amplicon (greater than 500 bp) to amplify the DNA. A different fixative may be needed for these types of samples.

Four of the thirteen primers designed to amplify a sequence from the virulence genes (chitinase and trypsin proteinase) produced strongly staining amplicons. These were validated against two primer pairs published previously (Oidtmann *et al*., 2004, 2006). Primers 525f and 640 r published in 2004 were more sensitive and had the advantage that they detected DNA from formalin-fixed paraffin-embedded sections. However, they were not as specific as the primers developed in this project. Primers published in 2006 were specific but because they produced a large amplicon they were not tested on paraffin-embedded tissue. Further work is required before a specific and sensitive PCR for *A. astaci* can be recommended for diagnostic laboratories.

Peptide nucleic acid probes (PNA) for use in the fluorescent *in situ* hybridisation (FISH) method were developed for both *A. invadans* and *A. astaci*. The PNA-FISH for *A. invadans* was specific and successfully applied to fresh cultured hyphae. Tests on aged hyphae in samples such as formalin-fixed paraffin-embedded sections yielded a reduced fluorescent signal. The PNA-FISH for *A. astaci* could only be tested on paraffin-embedded sections and the same reduced fluorescent signal occurred with this species. Further work needs to be done to optimise the PNA-FISH for both fungal species using paraffin-embedded material.

KEYWORDS: **PCR, fluorescent *in-situ* hybridization, FISH, epizootic ulcerative syndrome (EUS), Crayfish plague, *Aphanomyces astaci*, *Aphanomyces invadans***.

2. ACKNOWLEDGMENTS

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Drs Eva Jansson, Thorbjörn Hongslo and Anders Hellström from the National Veterinary Institute, Department of wildlife, fish and environment, Uppsala, SWEDEN who sent paraffin-embedded blocks of tissues from crayfish infected with *A. astaci*, especially as there seemed to be none available anywhere else in the world.

Jeff Shields, Virginia Institute of Marine Science, USA who provided paraffin-embedded material infected with epizootic ulcerative syndrome and which we used for validation of the PCR and PNA-FISH for *A. invadans*.

Drs Graeme Fraser and P.A. Gill, Department of Primary Industries NSW, Wollongbar, who sent diagnostic samples, which were invaluable as a source of fresh culture and for use in validating the tests on diagnostic samples. At the time there were no viable cultures of *A. invadans* in our laboratory or any other laboratories. No cultures existed in any of the world culture collections such as ATCC (USA) or CBS (Netherlands), which specialises in fungi.

Martin Deveney, PIRSA Aquaculture, and Allan Kessell, Gribbles Veterinary Pathology South Australia for sending paraffin-embedded sections from a case of EUS.

Dr Birgit Oidtmann, Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, for provision of cultures of *A. astaci* that were stored at the Australian Animal Health Laboratory, CSIRO, Geelong.

Nick Gudkovs, Australian Animal Health Laboratory, who extracted DNA from the *A. astaci* cultures and sent it to the AHL for use in validating the *A. astaci* primers.

DVM Satu Viljamaa-Dirks, Finnish Food Safety Authority Evira, Kuopio Research Unit, Finland who will provide crayfish plague material so that validation of the PCR and PNA-FISH for *A. astaci* can be completed.

Matt Landos, NSW, for sending diagnostic material for testing for EUS.

Appreciation to all the laboratories and staff who took part in the validation of the PCR for *A. invadans*:
Annette Thomas and Jane Oakey, Department of Primary Industries and Fisheries, Queensland; Colin Johnson from PIRSA and Rick McCoy Gribbles Pathology, Victoria; Ian Marsh and Vanessa Saunders, EMAI, NSW; Nick Gudkovs, AAHL, Geelong; Jeremy Carson and Marianne Douglas, Department of Primary Industries and Water, Tasmania; Vicki Blazer, Leetown Science Centre, USA; Suzanne Keeling, MAFF New Zealand.

Ian Marsh and Vanessa Saunders, Elizabeth Macarthur Agricultural Institute, Department of Primary Industries NSW for validating the PNA-FISH method for *A. invadans*.

Colleagues and staff of the Animal Health Laboratories (AHL), Department of Agriculture and Food Western Australia, especially staff in the Bacteriology Laboratory. Special thanks

also to Mr Tail Le from the Histopathology Laboratory for his staining of paraffin-embedded sections ready for microscopic examination or cutting of sections ready for PCR or FISH.

Colleagues at the Fish Health Unit, Department of Fisheries Western Australia (based within the AHL) for their support and assistance during this project.

During this project Dr David Alderman retired (Dec 2006). He provided knowledge to the project with regard to growing oomycete fungi, storing the fungi, the nature of crayfish disease and initially provided paraffin-embedded blocks with tissues from crayfish infected with *A. astaci* that were used in FRDC 2001/621. He visited AHL early in the first project and spent a day discussing various aspects of crayfish plague.

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 infection with *Aphanomyces invadans* and crayfish plague is caused by infection with *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 (MG) 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 (*Brevoortia tyrannus*) in the USA, (Blazer *et al.*, 2002). In Australia, *A. invadans* affects ornamental fish, freshwater and estuarine fish in particular silver perch (*Bidyanus bidyanus* Mitchell, 1838), black bream *(Acanthopagrus butcheri)* and barramundi (*Lates calcarifer*) (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 from 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 farmed) accounted for a production loss 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 using 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 from infected fish that infect other fish or crayfish, 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.

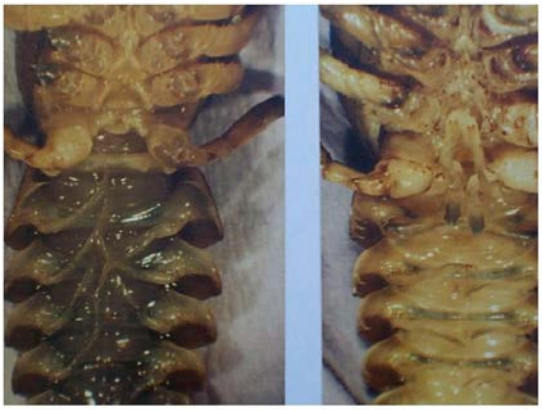

Figure 3-2. Differences in appearance between a normal crayfish, and a crayfish infected with crayfish plague.

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 2006). 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 fungal 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.

Currently, 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 author's personal observation). 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 are required for both *A. invadans* and *A. astaci* respectively, disease caused by these fungi may be misreported.

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 diagnostic laboratories have access to a thermocycler (required for PCR) 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.

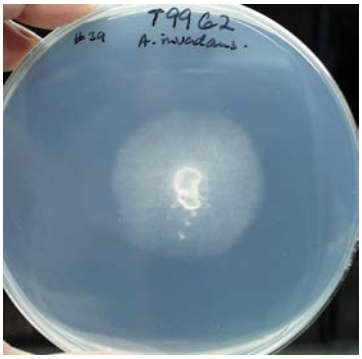

Figure 4-1. Five-day growth of *A. invadans* on IM medium.

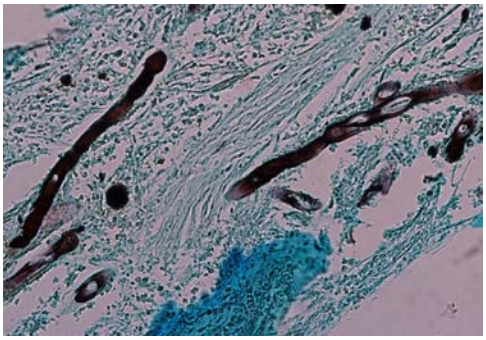

Figure 4-2. Histology section showing tissue infected with EUS (Grocott-Gomorri stain).

Photograph courtesy Dr B. Jones.

5. OBJECTIVES

1 Complete validation of *A. invadans* PCR on fresh tissue and wax-embedded tissue

2 Complete research to develop specific PCR for *A. astaci* (crayfish plague)

3 Complete research to develop FISH tests for *A. invadans* and *A. astaci*

4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

5 Analyse feedback from participating laboratories on PCR tests and FISH

6 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedure and FRDC Report

6. METHODS

6.1 Complete validation of *A. invadans* PCR on fresh tissue and wax-embedded tissue

6.1.1 Further validation of primers AIF14 + AIR10 on fish tissue and paraffin-embedded sections

The primers (AIF14 and AIR10) designed in project FRDC 2001/621 were specific and amplified DNA of *A. invadans* from culture and from fish tissue. The results from paraffin-embedded tissue were variable. Two diagnostic cases from estuarine fish (AS-00-3014 and FH-02-019) clinically appeared as EUS. *A. invadans* was not cultured, but *Saprolegnia* species and *Trichoderma* species, respectively, were cultured from each case. The paraffin-embedded sections were negative by PCR. A third case (AS-03-0417), from black bream with a large lesion typical of EUS, was culture-positive for *A. invadans*, but the PCR was negative when tested on paraffin-embedded sections. Paraffin-embedded blocks were prepared from a fungal culture of *A. invadans* and from fish tissue. The PCR on these artificially prepared paraffin-embedded sections was negative. Two DNA extraction methods were tested. These were deparaffinized using the method of Bateman *et al*., (1997) followed by the Qiagen DNeasy mini plant tissue kit with an added proteinase K digestion step. The second method followed Whittington *et al*., (1999). Both methods were tried using extracted DNA tested at neat, 1;10 and 1:100 dilutions and with and without the addition of bovine serum albumin to the PCR master mix. These regimes were done in an attempt to overcome PCR inhibitors that might have been present.

In the current project a third DNA extraction technique (Bateman *et al*., 1997; Wright and Manos, 1990) was assessed on diagnostic cases (Table 6-1). The PCR for *A. invadans* was assessed on all material received at AHL for EUS testing and compared to culture and histopathology results (Table 6-1). Primers AIF14 + AIR10, which produce a product size of 554 base pairs were compared to other primers designed for *A. invadans* in FRDC 2001/621 that produced a product of 100 base pairs in size, but were not as specific as the chosen primers. In theory primers that produce a small product size (less than 500 bp) are said to be more successful in amplification of DNA from formalin-fixed paraffin-embedded tissue (Greer *et al*., 1994).

Further assessment of specificity was undertaken using bacterial isolates that may be present on fish tissue and in the aquatic environment (Table 6-2).

6.1.1.1 Fungal isolates and diagnostic cases

Diagnostic samples received at AHL, Department of Agriculture and Food Western Australia (DAFWA) from local cases (including archived material) or from other states were used for further validation of the PCR for detection of *A. invadans* in fish tissue, cultured hyphae or formalin-fixed paraffin-embedded tissue (Table 6-1). All samples arrived at the laboratory with a preliminary clinical diagnosis documented as lesions suspected to be epizootic ulcerative syndrome. All histopathology submissions were examined and reported by the fish pathologists, Dr Brian Jones, Dr Fran Stephens or Dr John Creeper.

Table 6-1. Samples used for testing *A. invadans* PCR

AHL Case Number	Notes and Identification	Source
P-98-1514, #1 & #2	Lesion, suspect EUS	Diagnostic submission
P-98-2210	Lesion, suspect EUS	Diagnostic submission
P-98-3635 #1 , #2	Lesion, suspect EUS	Diagnostic submission
P-00-1758	Lesion, suspect EUS	Diagnostic submission
P-00-3014	Lesion, suspect EUS	Diagnostic submission
AS-01-0100	Black bream, lesion, suspect EUS	Diagnostic submission (Swan river)
AS-01-1119 #1, #2	Golden perch, ulcerative lesions	Diagnostic submission (Perth Zoo)
AS-01-1120 #1, #2	Golden perch, chronic ulcerative skin lesions	Diagnostic submission (Perth Zoo)
AS-01-1313	Aquarium fish (Blue Peacock), chronic ulcerative skin lesions	Diagnostic submission (quarantine)
AS-02-1803	Black bream, Lesion, suspect EUS	Diagnostic submission (Swan river)
AS-04-1316 #1	Aquarium fish (tropheus) with lesions	Diagnostic submission
AS-04-1316 #2	Aquarium fish with lesions	Diagnostic submission
AS-04-1373	Pygmy perch with lesions	Diagnostic submission
AS-04-2149 #1, #2, #3, #4	Paraffin blocks	Diagnostic submission, DPI QLD
AS-04-2382	Fish with lesions	Diagnostic submission (Dick Callinan, NSW)
AS-04-2487	Marron for health certification for crayfish plague	Diagnostic submission
AS-04-3599	Black bream, frozen fish tissue and PE section	Sent from PIRVIC
AS-04-3768	Fish with lesions	Diagnostic submission
AS-05-0817 fish 1	Mullet from Vasse River	Diagnostic submission
AS-05-0817 fish 2	goldfish from Vasse River	Diagnostic submission
AS-05-0817 fish 7	Nightfish from Vasse River	Diagnostic submission
AS-05-0817 fish 8	Pygmy perch from Vasse River	Diagnostic submission
AS-05-1074	Mullet with EUS-like lesion	Diagnostic submission
AS-06-1748	Black bream with EUS-like lesion	Diagnostic submission
AS-06-2255 fish #1	Imported aquarium fish for import certification for crayfish plague	Diagnostic submission
AS-06-2255 fish #2	Imported aquarium fish for import certification for crayfish plague	Diagnostic submission
AS-06-2563	Silver perch with suspect EUS	Submitted to DAFWA from Matt Landos, NSW
AS-07-0088	Fish lesion (decomposed) with suspect EUS	Submitted to DAFWA from DPI, NSW
AS-07-0189	Tissue material on culture plate and growth of fungal hyphae. Suspect EUS in east coast cod	Submitted to DAFWA from DPI, NSW, Wollongbar
AS-07-0101	Fish tissue material on culture plate. Suspect EUS	Submitted to DAFWA from DPI, NSW, Wollongbar
AS-07-1423	Paraffin embedded section. Fish with suspect EUS	Submitted to DAFWA from PIRSA, Gribbles, SA
AS-07-0438	Paraffin embedded section from EUS positive case	Sent to DAFWA from NFHL, USA

Diagnostic submission = Samples were submitted to Animal Health Laboratories, Department of Agriculture and Food Western Australia via the Fish Health Unit, Department of Fisheries WA, which resides at AHL; DPI&F QLD = Department of Primary Industries and Food Queensland; EUS = Epizootic Ulcerative Syndrome; NFHL = National Fish Health Laboratory; NSW = New South Wales; USA = United States of America.

Four cultures of *A. invadans* were sent from Japan by Professor Hatai. Unfortunately, the cultures were sent without an import permit. By the time a permit had been applied for and granted, the cultures, when released from quarantine, were no longer viable on arrival at AHL. Cultures that had been sent were NJM 0033, NJM 0204, NJM 9701 and NJM 9901.

Specificity testing was performed against bacteria that may be present on fish tissue or present as laboratory contaminants (Table 6-2).

Table 6-2. Cultures used for further specificity testing of *A. invadans* primers AIF14 + AIR10

Name	Notes
Aeromonas hydrophila	ATCC 7966
Atypical *Aeromonas salmonicida*	Australian strain AHLDA 1334
Listonella anguillarum	AHLDA 1730
Photobacterium damselae	AHLDA 1683
Streptococcus dysgalactiae	AHLDA 1616
Streptococcus iniae	AHLDA Barramundi strain
Vibrio alginolyticus	AHLDA 01-618
Vibrio cholerae	AHLDA 996
Vibrio mimicus	AHLDA 1654

ATCC = American Type Culture Collection; AHLDA = Animal Health Laboratories Department of Agriculture culture collection

6.1.1.2 DNA extraction methods for extraction from culture material

The following DNA extraction methods were validated in FRDC project 2001/621.

Preparation of culture material

For DNA extraction from culture material, the fungus was grown in a broth culture (GP broth, see appendix C.2.1) for 2-4 days. Because *Aphanomyces* grows within the top 1-2 mm of agar, culture material from a plate culture is unsuitable as the agar cannot be separated from the fungal hyphae and interferes (inhibits) with the PCR reaction.

From a 3-5 day-old plate culture on either GP or IMTAP medium (appendix C.2.1 and C.2.2), flame-sterilised forceps are used to cut out a small piece (few mm) of culture material from the growing edge of the fungal culture. The plug of fungal growth is placed into 10 ml of GP broth in a disposable MacCartney bottle. Cultures are incubated at 25°C for 4-7 days. The fungal growth is removed from the broth using flame-sterilised forceps and the liquid is allowed to drain before placing in a 1.5 ml microfuge tube. Fungal material that isn't being processed immediately can be stored at –20°C until required.

A DNA extraction from culture material using 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 is followed according to the manufacturer's instructions. These instructions have been updated since project FRDC 2001/621 and the updated instructions were followed and are presented here.

Fungal growth (5-50 mg) from a broth culture was added to 700 µl of DNAzol reagent in a 1.5 ml microfuge tube. The tissue was homogenised, applying as few strokes as possible, with a sterile disposable hand held pestle. At this stage the lysate/homogenate can be stored for 18 h at 15-30ºC or for 3 days at 2-8ºC. The solution was allowed to stand at room temperature for 5-10 min followed by centrifugation for 10 min at 16,060 x *g* (13,000 rpm) (this step is optional).

The viscous supernatant was transferred to a clean labelled tube. The DNA was precipitated by adding 400 µl of 100% ethanol, and mixed by inversion. The solution was allowed to stand at room temperature for 1 min, before centrifuging for 5 min at 3,500 x *g* (6,000 rpm). The supernatant was decanted, and the pellet washed by adding 600 µl of 75% ethanol. The pellet was re-suspended in the ethanol, and then re-pelleted by centrifuging for 3 min at 3,500 x *g* (6,000 rpm). The supernatant was decanted and the wash step repeated.

After the supernatant was decanted the pellet was air-dried so that all residual ethanol had evaporated.

The DNA was dissolved in 8 mM NaOH. If the pellet was >2 mm in diameter, 150 µl of 8mM NaOH was added. If the pellet was small, 50-100 µl of 8mM NaOH was added. As recommended by the manufacturer, heating assisted to dissolve the pellet. Alternatively, water can be used to solubilize the DNA. Genomic DNA recovery is estimated to be 70-100%.

B Qiagen DNeasy mini plant tissue kit for DNA extraction from fungal culture

The DNeasy Plant mini kit was recommended or the isolation of DNA from oomycete fungi by technical personnel at Qiagen. 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 removed from the broth using flame-sterilised forceps, and drained to remove as much moisture as possible. Removal of moisture also can be achieved by placing the fungal ball between two pieces of filter paper and pressing to remove moisture.

The fungal hyphae were ground to a fine powder in a mortar and pestle without allowing the liquid nitrogen to evaporate (if possible).

The method according to the Qiagen DNeasy mini plant tissue protocol was carried out as follows. Buffer AP1 (400 µl) and 4 µl RNase stock solution (100 mg/ml) was added to approximately 100 mg of wet weight ground material. The solution was vortexed vigorously before incubating at 65°C for 10 min with 2-4 inversions during the incubation time. Buffer AP2 (130 µl) was added to the lysate, mixed and incubated on ice for 5 min. The solution was centrifuged at a maximum speed of 16,060 x *g* (13,000 rpm) for 5 min.

The supernatant was placed into a QIAshredder spin column and centrifuged at maximum speed for 2 min. The flow through liquid was transferred to a new tube. Buffer AP3/E was added at 1.5 ml volume and mixed by pipetting. The mixture (650 µl) was added to a DNeasy spin column and centrifuged for 1 min at 6,000 x *g* (8,000 rpm). The flow through was discarded and 500 µl Buffer AW was added to the spin column, and centrifuged for 1 min at 6,000 x *g* (8,000 rpm). The flow-through was discarded and 500 µl Buffer AW was added to the spin column, and centrifuged on maximum speed of 16,060 x *g* (13,000 rpm,) for 2 min. Buffer AE was preheated to 65°C and 50 µl was added to the column, and then centrifuged for 5 min at 6,000 x *g* (8,000 rpm). The eluted DNA was collected and stored at –20°C until required.

6.1.1.3 DNA extraction from fish tissue

A DNAzol reagent for the extraction of fungal DNA from fish tissue

The DNAzol reagent used for the extraction of fungal DNA from fish tissue was used according to manufacturer's instructions as follows.

Tissue of approximately 5 mm (25-50 mg) was added to 700 µl of DNAzol reagent. The tissue was homogenised with as few strokes as possible using a disposable hand-held pestle (the lysate/homogenate can be stored for 18 h at 15-30ºC or for 3 days at 2-8ºC). The lysate was allowed to stand at room temperature for 5-10 min. The lysate was centrifuged for 10 min at 16,060 x *g* (13,000 rpm). (This step is optional).

The supernatant, which can be very viscous, was transferred to a clean, labelled tube. The DNA was precipitated by adding 400 µl of 100% ethanol, and mixed by inversion. The solution was allowed to stand at room temperature for 1 min, before centrifuging for 5 min at 3,500 x *g* (6,000 rpm). The supernatant was decanted.

The pellet was washed by adding 600 µl of 75% ethanol. The tube was gently flicked to re-suspend the pellet in the in the ethanol.

The DNA was re-pelleted by centrifugation for 3 min at 3,500 x *g* (6,000 rpm). The supernatant was decanted, and the wash step with ethanol repeated. After the final centrifugation step the supernatant was decanted. The pellet was air-dried so that all residual ethanol evaporated.

The DNA was dissolved in 8 mM NaOH. If pellet was >2 mm in diameter, 150 µl of 8mM NaOH was added to the DNA. If pellet was small, 50-100 µl of 8mM NaOH was added As recommended by the manufacturer, heating the NaOH to 50°C assisted in dissolving the pellet.
Alternatively, water can be used to solubilize the DNA. Genomic DNA recovery was estimated by the manufacturer to be 70-100%. Generally, the DNA cannot be seen if a sample is examined after electrophoresis through an agarose gel. The presence of DNA can be checked by amplification using universal primers for oomycete fungi, either ITS 1 & 2 (for amplification of the internally transcribed spacer regions), or C1 and D2, which amplify the 18S rRNA.

6.1.1.4 DNA extraction from sections cut from formalin-fixed paraffin-embedded blocks

(Bateman *et al*., 1997, Wright and Manos, 1990)

A DNA extraction from formalin-fixed, paraffin-embedded tissue (Wright and Manos, 1990)

The tissue was deparafinized using a method adapted from Wright and Manos (Wright and Manos, 1990). Three 12-micron sections per sample were cut and placed into a 1.5 ml microfuge tube. Xylene (1 ml) was added and the tube placed onto a rocking platform (IKA MS1 minishaker) at room temperature for five minutes. The tube was centrifuged at 16,060 x *g* (13,000 rpm) for three minutes to pellet the tissue. The supernatant was removed and the xylene wash and centrifugation steps repeated twice (three times in all). After the last centrifugation and removal of supernatant, 1 ml of 100 % ethanol was added and mixed by inversion. The tissue was pelleted by centrifugation as before, and the supernatant removed. The ethanol wash was repeated twice. After the final centrifugation step the supernatant was removed and the pellet dried at 50°C for 15 minutes. Parafilm was placed across the tubes and holes punched into the film to allow drying. The parafilm assisted in prevention of cross-contamination.

To the dried pellet was added 100 µl of digestion buffer [400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl, 1% sodium dodecyl sulphate] containing 10 µl proteinase K (20 mg/ml). The solution was incubated at 55°C overnight.

The tubes were boiled for five minutes to inactivate the proteinase K. The tubes were centrifuged for 1-2 minutes at 16,060 x *g* (13,000 rpm). The supernatant was removed to a new tube. The concentration and integrity of DNA was determined by electrophoresis of a 2µl aliquot on a 1% gel using Hyper IV molecular weight marker containing bands of DNA with known concentration. The DNA was stored at –20°C.

B DNA extraction from formalin-fixed, paraffin-embedded tissue using the Qiagen DNeasy mini plant tissue kit

Two 12-micron sections were cut and placed into a 0.6 ml microfuge tube. Depariffinisation was carried out according to Bateman (Bateman *et al*., 1997). The sections were dewaxed in 500 µl of xylene and mixed for 5 min. The xylene was removed by aspiration and 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 DNA was extracted using the DNeasy Qiagen Mini Plant Tissue kit.

C DNA extraction from formalin-fixed, paraffin-embedded tissue (Altschul *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 x *g* (10,000 rpm) for 1 min to pellet the tissue. Sterile water (200 µl) 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 1,060 x *g* (3,000 rpm) for 20 min. The supernatant was removed to a fresh tube. For use in the PCR, 5 µl of diluted (1:10) using ultrapure water), and undiluted, supernatant was used in the reaction.

D DNA extraction from formalin-fixed, paraffin-embedded tissue using alkaline buffer and high temperature (Shi *et al*., 2004)

Cultured fungal hyphae from case AS-07-0101 were fixed in formalin and embedded in paraffin blocks according to normal histological procedures. Three 12-micron sections were cut and placed into a 1.5 ml microfuge tube. Two tubes were prepared and 500 µl of 0.1 M NaOH (pH 12.93) was added to each tube containing the paraffin-embedded sections. One tube was heated to 100°C in a boiling waterbath and the second tube was heated to 120°C in an autoclave. Both samples were heated for 20 min. Tubes were cooled to room temperature. A 2 µl aliquot was used in the PCR reaction using primers AIF14 + AIR10. The remaining solution in each tube was further extracted and purified as follows. To each tube was added 500 µl of phenol:chloroform:isopropanol (25:24:1) (Sigma) and vortexed for 10 secs. The tubes were centrifuged at 16,060 x *g* (13,000 rpm) for 10 min. The supernatant was removed to a clean tube. One volume of chloroform was added and mixed by vortexing for 10 secs, followed by centrifugation at 16,060 x *g* (13,000 rpm) for 5 min. The upper aqueous layer was removed to a clean microfuge tube and 0.1 vol of 3M sodium acetate was added and vortexed. One vol of isopropanol was added and the tube mixed by inversion. The DNA was precipitated at –20°C overnight. The NA was pelleted by centrifugation at 16,060 x *g* (13,000 rpm) for 5 min. The supernatant was discarded and the pellet washed once with 75% ethanol. The DNA pellet was dried at 37°C for 15 min and then dissolved in 50 µl ultrapure water. Heating at 60°C assisted dissolution of DNA. DNA (2 µl) was used in the PCR reaction using primes AIF14 + AIR10.

6.1.1.5 Protocols for A. invadans PCR

The primers (Table 6-3) were developed and validated in FRDC project 2001/621.

Table 6-3. Primers AIF14+AIR10 for amplification of *A. invadans*

Primer name	Sequence 5' – 3'
AIFP14	CTG ACT CAC ACT CGG CTA GC
AIRP10	ATT ACA CTA TCT CAC TCC GC

A 25 µl PCR master mix was prepared as detailed in Table 6-4.

Table 6-4. 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
Total volume	25

The PCR reaction was performed in 0.6 ml tubes (Bio-Rad) on a Corbet FTS 320 thermocycler using the thermocycling program in Table 6-5.

Table 6-5. PCR cycling conditions for *A. invadans* PCR

Round 1	Round 2	Round 3
94°C - 5 min	95°C - 1 min	95°C - 1 min
x1	55°C - 1 min	55°C - 1 min
	72°C - 1 min	72°C - 10 min
	x 30	4°C - ∞
		x 1

Gel Electrophoresis

Amplified product (5 µl) plus 5 µl of loading buffer were loaded into wells of a 2 % agarose gel prepared with chromosomal grade agarose (Bio-Rad) and 0.5X TBE buffer. Amplified DNA was separated by electrophoresis using 100 volts for 60 minutes. A 100 base pair ladder (Geneworks) was loaded on the first and last well of the gel. 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. The expected product size for *A. invadans* was 554 bp.

6.1.2 Further validation of primers AIF14 + AIR10 compared to published primers

During the course of this project a Japanese research group published a PCR for detection of *A. invadans* (Phadee *et al*., 2004) . Primers were also published from the USA (Blazer *et al*., 2002; Vandersea *et al*., 2006). All primers were compared to the primers AIF14 and AIR10 for specificity and visual appearance of the amplicon on the gel (Table 6-6).

Table 6-6. Published primers compared to AIF14 + AIR10

Primer name	Sequence 5' – 3'	Amplicon size bp	Source
AIFP14	CTG ACT CAC ACT CGG CTA GC		FRDC 2001/621
AIRP10	ATT ACA CTA TCT CAC TCC GC	554	FRDC 2001/621
ITS 11	GCC GAA GTT TCG CAA GAA AC		Phadee *et al*., 2004
ITS 23	CGT ATA GAC ACA AGC ACA CCA	550	Phadee *et al*., 2004
AINVAD-2F	TCA TTG TGA GTG AAA CGG TG		Vandersea *et al*., 2006
AINVAD-ITSR1	GGC TAA GGT TTC AGT ATG TAG	234	Vandersea *et al*., 2006
AIFP1	AAGGCTTGTGCTGAGCTCACACTC		Blaser *et al*., 2002
AIFP2	GATGGCTAAGGTTTCAGTATGTAG	98	Blaser *et al*., 2002

The annealing temperature for the Phadee *et al*., (2004) primers was tested at 65°C as recommended in the paper. These primers were compared to the in-house primers AIF14 + AIR10 using DNA extracted from fish tissue from case AS-05-0817. DNA was extracted using the DNAzol reagent. The PCR reaction was conducted at 55°C for primers AIF14 + AIR10, and at 55°C and 65°C for primers ITS 11 + ITS 23. The master mix and annealing temperature for the AINVAD-2F and AINVAD-ITSR1 were performed at 56 °C according to the protocol (Vandersea *et al*., 2006), and primer P1 + P2 were performed at 57.5°C (Blazer *et al*., 2002).

In-house and published primers were tested for specificity against other oomycete fungi and fungi that might occur as laboratory contaminants (Table 6-7).

Table 6-7. Fungal species used for specificity testing of published primers

Isolate name	Isolate information	AHL Lab DNA ID
A. *invadans*	EUS case, NSW, 2002	#24
A. invadans	EUS case, NSW, 2007	#70 (AS-07-0101)
A. astaci strain Ti	DNA sent from AAHL	#188
A. astaci strain Pc	DNA sent from AAHL	#194
Penicillin sp.	From yabby	#228 (AS-01-4050)
Aspergillus sp.	From yabby	#347 (AS-01-4050)
Saprolegnia parasitica	From Annette Thomas, DPI QLD. 98% identification sequencing	#336
Saprolegnia declina	ATCC 36144	#348
Achlya diffusa	ATCC 16111	#349
S. salmonis	Aquarium fish	#354 (AS-03-3827)
S. *parasitica*	Aquarium fish	#355 (AS-03-3827)
A. frigidophilus	ATCC 204464	#231
Positive control	From D. Alderman, CEFAS, UK	# 194 (AS-02-1993)

6.2 Complete research to develop specific PCR for *A. astaci* (crayfish plague)

6.2.1 Testing primers designed for A. astaci in project FRDC 2001/091

In project FRDC 2001/621 13 primer pairs (Table 6-8) were developed for the detection of *A. astaci*. Specificity and sensitivity of the primers could not be assessed because of unsuitable material. White-claw crayfish stored in formalin had been sent from the United Kingdom by Dr David Alderman (CEFAS), but DNA was not detected by the *A. astaci* primers, although weak bands of DNA were obtained using universal fungal primers for the 18S rRNA. The crayfish had been stored in formalin since the 1980s and it was assumed that the formalin had cross-linked the DNA preventing amplification. To overcome this problem, the Australian Animal Health Laboratories obtained live cultures of *A. astaci* from Dr Birgit Oidtmann, Germany (Table 6-9). Cultures sent to AAHL were Di, Kv, Pc (two cultures), Ra, Ti and Yx, which represented different genetic groups from different geographical locations throughout Europe. Four genetic groups have been identified by random amplification of polymorphic DNA (Diéguez-Uribeondo, 1995; Huang, 1994) Group A (Ra) comprises *A astaci* strains isolated from the European crayfish species *Astacus astacus* and *A. leptodactylus* from crayfish introduced into Europe in the 19th century. Group B (Ti, Yx) comprises strains isolated from crayfish species *Pacifastacus leniusculus* that originated in Lake Tahoe, California, and are the more recent introductions into Sweden in the 1960s. Group C (Kv) contains a strain from *P leniusculus* originating in Pitt Lake, Canada, and imported into Sweden. Group D (Pc) comprises strains isolated from *Procambarus clarkii* located in Spain that has origins from subtropical regions of the south east of the United States of America. Group A-C strains are from crayfish of cold water origin (4-21°C) and Group D is composed of strains from warmer waters (20-26°C). DNA from these cultures was extracted at AAHL using the Qiagen DNeasy method. Culture Kv was not viable when subcultured at AAHL.

Many international laboratories were contacted in an attempt to locate paraffin-embedded blocks containing *A. astaci* infected crayfish tissue (Table 8-1). The only laboratory that had any material that they could provide was the National Veterinary Institute, Sweden, and we are very grateful for the provision of their samples. Paraffin-embedded sections from Sweden (Table 6-9) were tested by PCR and used for assessment of the PNA-FISH method developed during this project.

Table 6-8. Primers designed in project FRDC 2001/621

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

Table 6-9. *A. astaci* isolates used for *A. astaci* PCR

AHL Case No. or ID No.	Notes and Identification	Source
Ra	*A. astaci,* isolated from noble crayfish, Sweden, 1973, genetic group A	Dr Birgit Oitmann via AAHL
Di	*A. astaci,* genetic group A, (11.7 ng/µl of DNA)	Dr Birgit Oitmann via AAHL
Dc	*A. astaci,* genetic group A, (9.5 ng/µl of DNA)	Dr Birgit Oitmann via AAHL
Ti	*A. astaci,* isolated from noble crayfish, Sweden, 1970. genetic group B, (5.2 ng/µl of DNA)	Dr Birgit Oitmann via AAHL
Yx	*A. astaci,* isolated from noble crayfish, Sweden, 1973. genetic group B, (15.6 ng/µl of DNA)	Dr Birgit Oitmann via AAHL
Pc	*A. astaci,* Isolated from Red swamp crayfish, Spain 1992, genetic group D, (9.8 ng/µl of DNA)	Dr Birgit Oitmann via AAHL
07-750 #1	*hyphae in carapace, typical of *A. astaci* observed by microscopy	National Veterinary Institute, Sweden
07-750 #2	*hyphae in carapace, typical of *A. astaci* observed by microscopy	National Veterinary Institute, Sweden
07-750 #3	*no hyphae seen by microscopy	National Veterinary Institute, Sweden
AS-02-1993	Paraffin-embedded block of Crayfish plague from UK	CEFAS, UK

AHL = Animal Health Laboratories; ID = identification; No. = number; * taken from the notes that accompanied the samples

The following fungal species (Table 6-10) were used for specificity testing.

Table 6-10. Fungal species used for specificity testing

Isolate name	Isolate information	AHL Lab DNA ID
A. invadans	EUS case, NSW, 2002	#24
A. invadans	EUS case, NSW, 2007	#70 (AS-07-0101)
A. astaci strain Ti	DNA sent from AAHL	#188
A. astaci strain Pc	DNA sent from AAHL	#194
Penicillin sp.	From yabby	#228 (AS-01-4050)
Aspergillus sp.	From yabby	#347 (AS-01-4050)
Saprolegnia parasitica	From Annette Thomas, DPI QLD. 98% identification sequencing	#336
Saprolegnia declina	ATCC 36144	#348
Achlya diffusa	ATCC 16111	#349
S. salmonis	Aquarium fish	#354 (AS-03-3827)
S. *parasitica*	Aquarium fish	#355 (AS-03-3827)
A. frigidophilus	ATCC 204464	#231
Positive control	From D. Alderman, CEFAS, UK	# 194 (AS-02-1993)

6.2.2 Testing in-house primers designed for A. astaci against published primers

During the course of this project primers for *A. astaci* were published in 2004 (Oidtmann *et al*., 2004), and then revised for specificity in 2006 (Oidtmann *et al*., 2006) (Table 6-12). These primers were assessed in this project and compared for specificity and sensitivity against the best four primer pairs designed in FRDC 2001/621. These were AAF 18 + AAR 19, AAF18 + AAR 20, AAF 21 + AAR 22, AAF 21 + AAR 25 (Table 6-8). Sensitivity was performed using the same neat source of DNA and 10-fold serial dilutions that were amplified with

primers AAF 21 + AAR 25 and compared to primers 525f and 640r. The primers were tested for specificity using the cultures listed in Table 6-11.

Table 6-11. Isolates used for specificity testing of primers

Name	Case or code No
Aphanomyces invadans	AS-04-2382
A. astaci from PE tissue	AS-02-1993
A. astaci from DNA extracted from culture	Group B Ti
A. astaci from DNA extracted from culture	Group D Pc
Aspergillus niger	AS-02-4066
Penicillium sp.	AS-01-4050
Saprolegnia sp.	AS-04-2383
Saprolegnia diclina	AS-06-1928
Saprolegnia parasitica	AS-04-1735
Saprolegnia salmonis	AS-03-3827
Achlya diffusa	AS-06-1928
Aphanomyces frigidophilus	ATCC 204464

Table 6-12. Other primers for the detection of *A. astaci* published during the course of this project

Primer ID	Sequence 5' to 3'	Genome region	Product size
525f*	AAGAAGGCTAAATTGCGGTA	ITS	
640r*	CTATCCGACTCCGCATTCTG	ITS	
525 + 640			115 bp
42F[#]	GCTTGTGCTGAGGATGTTCT	ITS	
42 + 640			569

bp = base pairs; ITS – internally transcribed spacer region; * from Oidtmann *et al*., 2004; [#] from Oidtmann *et al*., 2006.

Table 6-13. Master mix used for testing *A. astaci* published primers

Reagent	Volume (µl) for single tube
Promega master mix	12.5
Primer F	1.5
Primer R	1.5
Water	7.5
DNA	2
TOTAL	25

The PCR reaction was performed in 0.6 ml tubes on a Corbet FTS 320 thermocycler using the thermocycling program as follows.

Table 6-14. PCR cycling conditions for *A. astaci* DNA extracted from tissue samples

Round 1	Round 2	Round 3
94°C - 5 min x1	94°C – 1 min 54°C – 1 min 72°C – 1 min x 45	94°C – 1 min 54°C – 1 min 72°C – 7 min x 1

Table 6-15. PCR cycling conditions for *A. astaci* DNA extracted from fungal culture

Round 1	Round 2	Round 3
94°C - 5 min x1	94°C – 30 sec 54°C – 30 sec 72°C – 30 sec x 30	94°C – 30 sec 54°C – 30 sec 72°C – 7 min x 1

Gel electrophoresis, staining in ethidium bromide and digital photography of the gel was the same as that used for the *A. invadans* PCR, Section 6.1.1.6.

6.2.3 DNA extraction methods for the detection of A. astaci in formalin-fixed, paraffin-embedded sections.

The DNA extraction methods were the DNeasy mini plant tissue kit, and the method according to Whittington (Whittington *et al*., 1999) as described in 6.1.1.5.

6.3 Complete research to develop fluorescent *in situ* hybridization (FISH) tests for *A. invadans* and *A. astaci*

6.3.1 FISH test for A. invadans

In project FRDC 2001/621, the fluorescent *in situ* hybridisation test using a primer with a fluorescein attached to the 5' end was not specific, cross-reacted with *Saprolegnia* species, and produced high background. In February 2006, at the beginning of project FRDC 2004-091, a paper was published that used a peptide nucleic acid (PNA) probe to detect *A. invadans* using cultured hyphae (Vandersea *et al*., 2006). This method was trialled in this project and used on cultured hyphae, and then developed for application to paraffin-embedded sections.

Methods and materials

6.3.1.1 Fungal cultures and paraffin-embedded sections used for PNA-FISH for A. invadans

Diagnostic samples, stored frozen hyphae, and archived paraffin-embedded samples were used for the PNA-FISH (Table 6-16).

Table 6-16. Cultures and paraffin-embedded material used for PNA-FISH for *A. invadans*

Case Number	Other Identification	Identification	Specimen Type
AS-07-0101	*A. invadans*, NSW culture	*A. invadans*	Cultured hyphae embedded into paraffin
AS-07-0101	Positive control	*A. invadans*	Paraffin-embedded section
AS-07-0438	L0157 A, USA section	*A. invadans*	Paraffin-embedded section
AS-07-1423	Clinical diagnosis of EUS	Unknown	Paraffin-embedded section from SA
WIC	#54	*A. invadans* from USA	Cultured hyphae stored frozen
AS-04-1295	#24	*A. invadans*	Cultured hyphae stored frozen
AS-04-1295	#24	*A. invadans*	Fish tissue seeded with cultured hyphae and embedded into paraffin blocks
AS-02-3995	#36c	*A. invadans*	Cultured hyphae embedded into paraffin blocks
AS-05-0817	Diagnostic case, positive EUS on histopathology	*A. invadans*	Formalin-fixed paraffin-embedded section
AS-04-2149	Diagnostic case, positive EUS on histopathology	*A. invadans*	Formalin-fixed paraffin-embedded section
AS-07-821 Pc2	From AAHL	*A. astaci*	Cultured hyphae embedded into paraffin and used to test specificity
AS-07-750 #2	Swedish section	*A. astaci*	Paraffin-embedded section used to test specificity
AS-01-3195	#9	*Saprolegnia* species	Used for specificity testing
ATCC 36144	#50	*S. diclina*	Fresh cultured hyphae
ATCC 16111	#51	*Achlya diffusa*	Cultured hyphae stored frozen
AS-03-3827-4	#57	*S. salmonis*	Fresh cultured hyphae
AS-03-3827-5	#58	*S. parasitica*	Fresh cultured hyphae
AS-04-1735	#59	*S. parasitica/salmonis*	Fresh cultured hyphae
AS-03-3416	#60	Unknown fungus	Cultured hyphae stored frozen
AS-05-0817	EU-Uni 1 probe	Positive control	Paraffin-embedded section used to test specificity
AS-05-0817	No probe	Negative control	Paraffin-embedded section used to test specificity
AS-03-1316	Ainv-FLU3 probe	Negative control	Fish tissue

EUS = epizootic ulcerative syndrome; NSW = New South Wales; SA = South Australia

6.3.1.2 PNA probes for A. invadans

The peptide nucleic acid probe for *A. invadans*, plus universal positive and universal negative PNA probes (Table 6-17) as described by Vandersea *et al* (2006) were sourced from Applied Biosystems.

For the FISH assay, controls included a positive (EuUni-1), negative (PpiscFLU-1), and a no-probe control. The universal positive control probe EuUni-1 hybridizes to the SSU rRNA of all eukaryotes and is used to ensure that the RNA has not degraded and that the probe has penetrated the cell.

Table 6-17. PNA probes used for *A. invadans*

Probe name	Target	Sequence 5' to 3'
Ainv-FLU3	*A. invadans*	FLU-gTA CTg ACA TTT CgT
EuUni-1	Universal positive	FLU-ACC AgA CTT gCC CTC C
PpiscFLU	Universal negative	gAA AgT gAT ATg gTA

FLU = fluorescein conjugate

6.3.2 FISH test for A. astaci

6.3.2.1 Fungal cultures and paraffin-embedded sections used for PNA-FISH for A. astaci

Live cultures could not be used in the development and validation of the PNA-FISH for *A. astaci* because the fungus is exotic to Australia. Cultures were held at AAHL but these were non-viable when tested by subculture from cultures obtained from Germany and stored on sloped PG1 medium under oil in 10 ml McCartney containers. The non-viable culture material was cut from the medium slope, fixed in formalin and then stored in 95% ethanol and transported to AHL. At AHL the culture material was embedded into paraffin blocks from which 12-micron sections were cut for use in the FISH method.

Three paraffin-embedded blocks containing crayfish tissue infected with *A. astaci* were imported from the National Veterinary Institute, Sweden. Tissue from the infected crayfish had been fixed in "T-röd" (Kemetyl) followed by further fixation in 70% ethanol as requested by the Australian Quarantine service. The fixed material was embedded in paraffin.

Details of cultures and paraffin-embedded material used in the development and validation of the PNA-FISH for *A. astaci* are listed in Table 6-18.

Table 6-18. Cultures and paraffin-embedded sections used in development and validation of PNA-FISH for *A. astaci*

AHL Case Number	Other Identification	Notes and Identification
07-750 #1	Swedish paraffin-embedded (PE) section	Identified in Sweden as *A. astaci*. Hyphae seen in smears, weak positive by PCR
07-750 #2	Swedish PE section	Identified in Sweden as *A. astaci*. Hyphae seen in smears, strong positive by PCR
07-750 #3	Swedish PE section	No hyphae seen on microscopy
07-821 Ra	Non-viable culture material from AAHL	*A. astaci,* isolated from noble crayfish, Sweden, 1973, genetic group A
07-821 Dc	Non-viable culture material from AAHL	*A. astaci,* genetic group A
07-821 Di	Non-viable culture material from AAHL	*A. astaci,* genetic group A
07-821 Ti	Non-viable culture material from AAHL	*A. astaci,* isolated from noble crayfish, Sweden, 1970. genetic group B
07-821 Yx	Non-viable culture material from AAHL	*A. astaci,* isolated from noble crayfish, Sweden, 1973. genetic group B
07-821 Pc	Non-viable culture material from AAHL	*A. astaci,* Isolated from Red swamp crayfish, Spain 1992, genetic group D
07-101	*A. invadans*, NSW culture	Negative control for *A. astaci*
#9	*Saprolegnia* sp.	Used for specificity testing
#48	*Aspergillus* sp.	Used for specificity testing
#50	*A. invadans*	Used for specificity testing
#51	*Achlya diffusa*	Used for specificity testing
#59	*S. salmonis/parasitica*	Used for specificity testing
#11	*Saprolegnia* sp.	Used for specificity testing
#51	*Achlya diffusa*	Negative control
PC2	*A. astaci*	Positive control

6.3.2.2 PNA probe for A. astaci

A PNA probe (Table 6-19) was designed using the first 15 base pairs of the reverse complement of primer 640r (Table 6-12) and sourced from Panagene, Korea (PNA probes were no longer being offered by Applied Biosystems). The universal positive probe (EuUni) and the universal negative probe (PpiscFLU), as described for the PNA-FISH method for *A. invadans*, was used in the PNA-FISH method for *A. astaci*.

Table 6-19. PNA-FISH probe designed for the detection of *A. astaci* in tissue and culture material

Probe name	Target	Sequence
Aastaci-PNAFLU	*A. astaci*	5'-FLU- CAgAATgCggAgTCg –3'

FLU = fluorescein conjugate; PNA = peptide nucleic acid probe

The method for the PNA-FISH probe is the same as described in 6.3.3 for *A. invadans*, with the exception that the PNA probe, Aastaci-PNAFLU is used in the place of Ain-PNAFLUA.

6.3.3 Method for PNA-FISH

The PNA-FISH was performed on fungal material from culture, and sections on silane glass slides, or sections that had been cut and placed into a 1.5 ml microfuge tube. Paraffin-embedded material was deparaffinised before undergoing the PNA-FISH method, as described below.

Deparaffinisation of sections on microscope slides

Paraffin-embedded sections (6 microns thick) were cut on a microtome and placed onto silane coated slides. Slides were placed onto an automatic slide stainer (Shandon *Linistain GLX*, model 24 3603) for automatic heating, xylene washes (x3), absolute alcohol washes (x 3) and then removed. Two washes in 70% ethanol were done manually in wilson jars for five minutes at room temperature followed by a final wash in ultrapure or deionized water. Slides were dried at 60°C for 10 minutes.

If paraffin-embedded sections on microscope slides were handled manually for the above steps the protocol was as follows.

Slides were placed into a 60°C incubator and heated for 15 minutes. They were placed into xylene washes (x3) in wilson jars for 5 minutes per wash. Three washes in absolute alcohol were performed for five minutes each at room temperature. Two washes in 70% ethanol were done manually in wilson jars for five minutes at room temperature followed by a final wash in ultrapure or deionized water. Slides were dried at 60°C for 10 minutes.

Deparaffinisation of sections in microfuge tubes

Three 6-micron sections were cut and placed into a 1.5 ml microfuge tube. Xylene (1 ml) was added to the tube and placed on a rocking platform for five minutes at room temperature. The tissue was pelleted by centrifugation at 16,060 x *g* (13,000 rpm) for 3 minutes. The supernatant was removed and the process repeated twice. After the last centrifugation step the supernatant was removed and 1 ml of absolute ethanol was added to the tube. The tube was placed on a rocking platform for five minutes. The tissue was pelleted by centrifugation at 16,060 x *g* (13,000 rpm) and the supernatant removed. The ethanol washes were repeated twice. After the final centrifugation step 1 ml of 70 % alcohol was added to the tube, followed by incubation at room temperature on a rocking platform for five minutes. The process was

repeated once. A final wash was performed in ultrapure or deionized water. The pellet was dried at 60°C for 10 minutes.

Preparation using cultured hyphae

For the PNA-FISH using culture material the method of Vandersea *et al*., (2006) was followed with the exception that the test was carried out in microfuge tubes rather than the 24-well microtitre trays. Fungal hyphae were removed from a broth culture and placed into a 1.5 ml microfuge tube and rinsed with 1 ml of ultrapure or deionized water with agitation for five minutes at room temperature. The fungal material was pelleted by centrifugation at 16,060 x *g* (13,000 rpm) for three minutes.

PNA-FISH method

Fixative buffer (1 ml) was added to all samples and incubated at room temperature for 30 mins. The tissue was pelleted by centrifugation at 16,060 x *g* (13,000 rpm) for 3 min and the fixation buffer removed. Hybridisation buffer (0.5 ml) was added and the tubes placed onto a rocking platform for 15 minutes at room temperature. The hyphae or tissue was pelleted by centrifugation as before and the buffer removed. From this point onwards in the procedure, the light was kept as low as possible to prevent loss of fluorescence of the probe. 0.5 ml of hybridisation buffer containing 0.5 µl of probe (20 mM) (see Appendix C) was added to each tube and incubated in the dark at 60°C for one hour. The appropriate probe was added to either the positive control or negative control. After incubation the tubes are centrifuged at 16,060 x *g* (13,000 rpm) for three minutes at room temperature. The supernatant was removed and the pellet rinsed twice in 1 ml of 5X SET buffer (see Appendix C) (heated to 60°C). After the final centrifugation step the pellet of hyphae was removed from the tube and placed onto a microscope slide. A coverslip was placed over the hyphae and gentle pressure applied to spread the hyphae in a thin layer. The use of a fluoro retardant is recommended so as to prolong the fluorescence - particularly needed when photographs were taken, as the loss of fluorescence was dramatic during photography.

The slides were kept in the dark and examined using a fluorescence microscope (Olympus BX50) with an excitation filter of 450 nm and an emission filter of 510 nm suitable for the detection of fluorescein isothiocyanate. The results were photographed using a digital camera attached to the microscope and downloaded to a computer. The results for the test samples were compared to the positive and negative control.

6.4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

6.4.1 Validation trial of the PCR developed for the detection of A. invadans

Nine laboratories agreed to participate in validation of the PCR test for *A. invadans* (Table 6-20). Kits were sent to a majority of laboratories in October 2004. Further kits were sent out in 2006 and 2007.

Laboratories were sent a kit that contained reagents, fungal or DNA material, and instructions (Appendix G.2). Fungal hyphae (20-25 mg) included in the kit had been stored frozen at –20°C for more than a week. Ten coded samples were sent comprising two strains of *A. invadans* and one each of *A. frigidophilus*, *Aphanomyces* species, *Saprolegnia australis*, *S. declina*, *S. parasitica*, *Aspergillus niger*, *Penicillium* species, and *Pythium* species (plant oomycete). A positive control of *A. invadans* was also included in the kit. The kit contained

all reagents required to perform the PCR; master mix reagent (Promega), water, primers, DNAzol DNA extraction reagent, and a micropestle. Instruction sheet and result sheets were included with the kit. All kits were shipped on dry ice, and laboratories notified AHL on receipt of their kits.

MAFF, NZ was unable to import fungal hyphae (non-viable) due to import restrictions, therefore their kit was sent in 2006, and contained DNA only. The DNA extractions were performed at AHL.

The kit was sent to DPIW Tasmania in 2006.

Table 6-20. List of laboratories who agreed to participate in the validation of the *A. invadans* PCR

Lab	State	Country	Kit Sent
AAHRI		Thailand	2004
AAHL	Victoria	Australia	2004
CEFAS		United Kingdom	2004
DPI & F	Queensland	Australia	2004
DPIW	Tasmania	Australia	2006
Gribbles via PIRSA	Victoria/SA	Australia	2004
NFRL		USA	2004
MAFF		New Zealand	2006
DPI NSW	NSW	Australia	2007

AHRI = Aquatic Animal Health Research Institute; AAHL = Australian Animal Health Laboratory; NFRL = National Fish Reference Laboratory; DPI & F = Department of Primary Industries and Fisheries; DPIW = Department of Primary Industries and Water; PIRSA = Department of Primary Industries and Resources of South Australia

6.4.2 Validation trial of the PNA-FISH developed for the detection of A. invadans

One Australian laboratory (DPI NSW) was invited to participate in testing the PNA-FISH method for the detection of *A. invadans* from culture material and paraffin-embedded tissue. The kit included all buffers (fixation buffer, hybridisation buffer), and probes (probe for *A. invadans*, universal positive probe, universal negative probe). Samples that were sent included three paraffin-embedded sections in tubes, a paraffin-embedded positive control, a paraffin-embedded negative control, five culture samples, a culture positive control and a culture negative control (Table 6-21). The kit details and instructions are attached in Appendix G.2.

Table 6-21. Samples included in PNA-FISH validation kit

Tube	Sample type	Sample Identification	Fungal identification
1	Paraffin-embedded section in microfuge tube	07-821 Yx	*A. astaci*
2	Paraffin-embedded section in microfuge tube	07-189	*A. invadans* from NSW, 2007
3	Paraffin-embedded section in microfuge tube	07-101	*A. invadans* from NSW, 2007
4	Paraffin-embedded section in microfuge tube, universal positive control	04-1316	EUS diagnostic sample
5	Paraffin-embedded section in microfuge tube. No probe control	04-1316	EUS diagnostic sample
6	Culture	07-101	*A. invadans* from NSW, 2007
7	Culture	07-189	*A. invadans* from NSW, 2007
8	Culture	#50	*Saprolegnia diclina*
9	Culture	#59	*Saprolegnia parasitica/salmonis*
10	Culture	#51	*Achlya diffusa*
11	Culture	07-101	*A. invadans*
12	Culture	07-101	*A. invadans*

6.5 Analyse feedback from participating laboratories on PCR tests and FISH

The results returned from the participating laboratories were assessed for expected positive and negative results of controls and for test samples. The gel photos were examined to determine the absence of non-specific bands in test samples, and the absence of amplification product at 554 bp in non-*Aphanomyces invadans* samples. The different types of thermocycler were recorded and noted for accuracy of test results particularly to test the specificity of the PCR primers. Comments and feedback from the participating laboratories was encouraged and these comments were assessed to determine if any laboratory had problems with any aspect of the test or had any recommendations to make.

6.6 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedure and FRDC Report

A method for the detection of *Aphanomyces invadans* by PCR from fish tissue and culture material was written according to SCAHLS and NAAH-TWG guidelines.

7. RESULTS

7.1 Complete validation of FRDC 2001/621 *A. invadans* PCR using fresh tissue and wax-embedded tissue

7.1.1 Further validation of primers AIF14 + AIR10 using fish tissue and paraffin-embedded sections

7.1.1.1 Further validation using fish tissue

Primers AIF14 + AIR10 (designed and validated in project FRDC 2001/621) were further tested using 25 diagnostic cases (comprising 36 samples) presented to AHL during the course of the current project (Table 7-1). In all submission, fish had lesions with a preliminary diagnosis of EUS.

Table 7-1. Results of testing FRDC 2001/621 *A. invadans* primers using diagnostic cases, cultured hyphae, fish tissue and/or paraffin-embedded sections

AHL Case Number	Histo result	Culture result	PCR on fresh tissue	DNA extraction method for PE	PCR result on PE section	Final Diagnosis
P-98-1514 #1	Pos	Neg	TNA	DNeasy	Negative	EUS
P-98-1514 #2	Pos	Neg	TNA	RW method	Negative	EUS
P-98-2210	Pos	NA	TNA	DNeasy	Negative	EUS
P-98-2210	Pos	NA	TNA	RW method	Negative	EUS
P-98-3635 #1	Pos	NA	TNA	DNeasy	Negative	EUS
P-98-3635 #1	Pos	NA	TNA	RW method	Negative	EUS
P-98-3635 #2	Pos	NA	TNA	DNeasy	Negative	EUS
P-98-3635 #2	Pos	NA	TNA	RW method	Negative	EUS
P-00-1758	Pos	NA	TNA	DNeasy	Negative	EUS
P-00-1758	Pos	NA	TNA	RW method	Negative	EUS
P-00-3014 #1	Pos	Pos – AI	TNA	DNeasy	Negative	EUS
P-00-3014 #1	Pos	Pos – AI	TNA	RW method	Negative	EUS
AS-01-0100	Pos	NA	TNA	RW method	Negative	EUS
AS-01-1119 #1	Pos	NA	TNA	DNeasy	Negative	EUS
AS-01-1119 #1	Pos	NA	TNA	RW method	Negative	EUS
AS-01-1119 #2	Pos	NA	TNA	RW method	Negative	EUS
AS-01-1120 #1	Pos	NA	TNA	DNeasy	Negative	EUS
AS-01-1120 #1	Pos	NA	TNA	RW method	Negative	EUS
AS-01-1120 #2	Pos	NA	TNA	DNeasy	Negative	EUS
AS-01-1120 #2	Pos	NA	TNA	RW method	Negative	EUS
AS-01-1313 #2	Pos	NA	TNA	DNeasy	Negative	EUS
AS-01-1313 #2	Pos	NA	TNA	RW method	Negative	EUS
AS-02-1803	Pos	NA	TNA	DNeasy	Negative	EUS
AS-02-1803	Pos	NA	TNA	RW method	Negative	EUS
AS-04-1316 #1	Neg	Neg	ND	DNeasy	Negative	*Cryptobia*-like flagellate
AS-04-1316 #2	Neg	Neg	ND	RW method	Negative	*Cryptobia*-like flagellate
AS-04-1373	Neg	Neg	ND	ND	ND	Microsporidiosis
AS-04-2149 #1	Pos	Neg	NS	DNeasy	Negative	EUS
AS-04-2149 #1	Pos	Neg	NS	RW method	Negative	EUS
AS-04-2149 #2	Pos	Neg		DNeasy	Negative	EUS
AS-04-2149 #2	Pos	Neg		RW method	Negative	EUS
AS-04-2149 #3	Pos	Neg		DNeasy	Negative	EUS
AS-04-2149 #3	Pos	Neg		RW method	Negative	EUS
AS-04-2149 #4	Pos	Neg		DNeasy	Negative	EUS
AS-04-2149 #4	Pos	Neg		RW method	Negative	EUS

Table 7-1, continued

AHL Case Number	Histo result	Culture result	PCR on fresh tissue	DNA extraction method for PE	PCR result on PE section	Final Diagnosis
AS-02-2382 #24	Pos	Yes	TNA	RW method	Negative	EUS
AS-04-3599#1	Pos	Neg	Neg	DNeasy	Negative	EUS
AS-04-3599#1	Pos	Neg	Neg	RW method	Negative	EUS
AS-04-3599#2	Neg	Neg	Neg	RW method	Negative	
AS-05-0817 fish #1	Pos	Neg	Pos*	DNeasy	Negative	EUS
AS-05-0817 fish #3	Pos	Neg	Pos*	DNeasy	Negative	EUS
AS-05-0817 fish #6	Pos	Neg	Pos*	DNeasy	Negative	EUS
AS-05-0817 fish #2, 4, 5, 7-12	Neg	Neg	Neg*	DNeasy	Negative	Not EUS
AS-05-1074	Neg	Neg	Neg	Not done	Negative	Myxosporidia
AS-06-1748	Neg	Neg	Neg*	Not done	Not done	Not EUS
AS-06-2255 fish #1	Neg	Neg	Neg*	Not done	Not done	Not EUS
AS-06-2255 fish #2	Neg	Neg	Neg*	Not done	Not done	Not EUS
AS-06-2563	Neg	Neg	Neg*	Not done	Not done	Not EUS
AS-07-0088		Neg	Neg*	N/A	No material	EUS
AS-07-0189	N/A	Pos	Pos*	N/A	No material	EUS
AS-07-0101	N/A	Pos	Pos*	N/A	No material	EUS
AS-07-1423	Pos	Not sent	No fresh tissue	DNeasy	neg	EUS
AS-07-0438	Pos	Not sent	Not sent	DNeasy	neg	EUS

* = DNA extraction method using DNAzol reagent; AI = *A. invadans*; NA = result not available; NS = sample not sent; RW = DNA extraction method (Whittington *et al*., 1999), section 6.1.1.5; TNA = test not available

All except three samples were examined histologically, which is the gold standard test for a diagnosis of EUS. No material was sent for the three samples that did not have paraffin-embedded sections examined. Of the 25 suspect cases, seven were negative for EUS and included final diagnoses of *Cryptobia*-like flagellate, Microsporidiosis, Myxosporidia, or not EUS.

Of the total 25 cases, 16 cases (24 samples) had fungal elements with morphology consistent for *A. invadans* and were diagnosed as EUS. Ten cases (13 samples) were from cases submitted prior to the *A. invadans* PCR being available. Of two cases (AS-04-3599, AS-05-0817) positive for histopathology and where fresh fish tissue was received, one case was positive for *A. invadans* PCR performed directly on fish tissue. Case AS-05-0817 comprised samples from 12 fish and three of these were positive for histopathology and for PCR performed directly on fish tissue. No fungus was cultured from these samples. Case AS-04-3599 was positive for fungal elements by histological examination but fungus was not cultured and no fungus was detected by PCR on the tissue.

DNA of *A. invadans* extracted from fresh fish tissue or cultured hyphae was successfully amplified using primers AIF14 + AIR10 with a DNA extraction protocol that used the DNAzol reagent for extraction from fish tissue and the DNAzol or Qiagen DNeasy mini plant tissue kit for use on hyphae grown in broth culture. Fungal growth obtained directly from an agar plate is not recommended because the nature of the fungus is that it grows within the top layer of agar and this agar interferes with the PCR reaction (FRDC 2001/621). Fish tissue (AS-07-0101) submitted from NSW was identified as *A. invadans* using PCR direct on fish

tissue and by culture. A sample of badly decomposed tissue (AS-07-0088) was PCR-negative and culture negative (Figure 7-1).

Results from PCR testing agreed with clinical diagnosis and microscopic examination of histology sections. DNA extraction methods using the DNAzol reagent or the Qiagen DNeasy mini plant tissue kit were successful in amplifying DNA from tissue and culture material and much of this work was validated in FRDC 2001/621, but additional case work done in the current project added to the validation data.

A. invadans was cultured from two cases (AS-07-0101 and AS-07-0189) and the identity was checked by 16S rRNA sequencing, which was carried out by PathWest Laboratories, Perth, WA. Results confirmed presence of *A. invadans*.

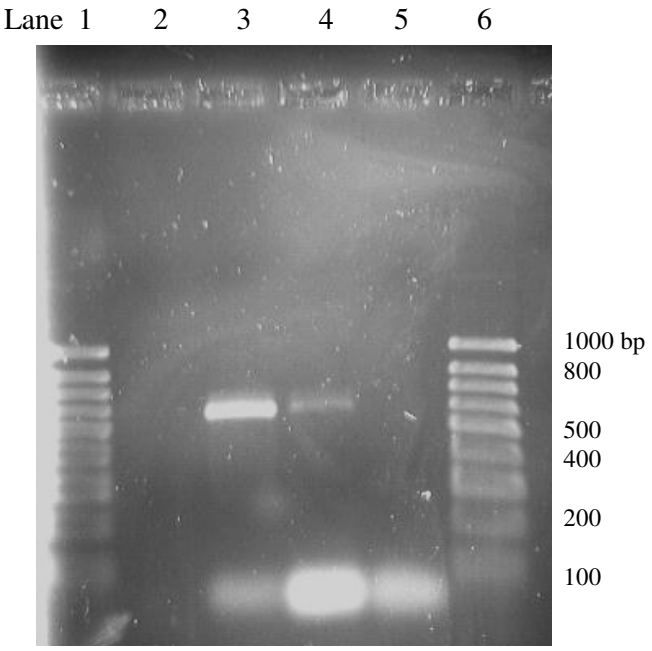

Figure 7-1. PCR results for direct detection using fish tissue

Lane 1 = HyperIV molecular weight marker; Lane 2 = AS-07-0088; Lane 3 = AS-07-0101; Lane 4 = positive control; Lane 5 = negative control; Lane 6 = HyperIV molecular weight marker.

7.1.1.2 Further validation using formalin-fixed paraffin-embedded sections

Samples (n=25) from 16 diagnostic cases that were submitted to AHL and were positive for fungal elements by histopathology were tested by PCR on formalin-fixed paraffin-embedded tissue. The results are presented in Table 7-1. DNA was not amplified from paraffin-embedded tissue using four different DNA extraction protocols. Different DNA extraction protocols using the Qiagen DNeasy mini plant tissue kit, manual methods of Wright and Manos (1990), Bateman *et al*., (1997), Whittington *et al*., (1999), or high heat with an alkaline retrieval buffer did not produce amplified product from paraffin-embedded tissue.

A case (AS-07-1423) comprising paraffin-embedded material and stained sections was received from South Australia from PIRSA via Gribbles laboratory. Results from histological examination of the stained sections were consistent with a diagnosis of EUS, but PCR on paraffin-embedded sections was negative.

Two other primer pairs (AIF14 + AIR15 and AIF14 + AIR16) designed in the course of project FRDC 2001/621 and which produced a small amplicon (approximately 100 bp in size) were tested on formalin-fixed paraffin-embedded material for cases AS-07-0438 and AS-07-1423. The DNA was extracted by the Qiagen DNeasy mini plant tissue kit and the PCR tests yielded negative results.

7.1.1.3 Further specificity testing of A. invadans primers AIF14 + AIR10

Primers AIF14 + AIR10 that were designed during project FRDC 2001/621 were found to be specific when tested against other oomycete fungi and from true fungi, but had not been tested for specificity against bacteria that could be present on fish tissue or as secondary invaders in a tissue lesion. The primers were shown to be specific yielding negative results when these bacteria and the *Trichoderma* fungus were tested (Table 7-2). A photograph of the gel results is presented in Figure F-1, Appendix F.

Table 7-2. Further specificity testing of *A. invadans* primers AIF14 + AIR10

Name	Results AIF14 + AIR10
Aeromonas hydrophila	Negative
Aeromonas salmonicida (atypical strain)	Negative
Listonella anguillarum	Negative
Photobacterium damselae subsp. *damselae*	Negative
Streptococcus dysgalactiae	Negative
Streptococcus iniae	Negative
Trichoderma sp	Negative
Vibrio alginolyticus	Negative
Vibrio cholerae	Negative
Vibrio mimicus	Negative

7.1.2 Further validation of primers AIF14 + AIR10 compared to published primers

Three sets of primers (ITS 11 + ITS 23, 2F + R1, P1 + P2) were published during the course of this project and validated against primers AIF14 + AIR10 developed in FRDC 2001/621. The results for the performance of the primers compared to each other for specificity against other oomycete fungi and true fungi are presented in Table 7-3.

Primers AIF14 + AIR10 produced an amplicon at 554 bp; primers ITS 11 + ITS 23 produced an amplicon at 550 bp; primers 2F + R1 produced an amplicon at 234 bp and primers P1 + P2 produced an amplicon at 98 bp. All primer sets were tested for specificity and it was demonstrated that they did not amplify non-specific products from the six oomycete fungi (*Saprolegnia* and *Achlya* species) or the two true fungi (*Penicillium* sp and *Aspergillus* sp.) that were tested, the latter being possible plate contaminants.

Table 7-3. Results using other previously published primers for the detection of *A. invadans*

		Result for primers			
Isolate name	**AHL Lab DNA ID**	**AIF14+AI R10[1]**	**ITS 11 + ITS 23[2]**	**2F + R1[3]**	**P1 + P2[4]**
A. invadans, NSW 2002	#24	Pos* ++++	Pos* ++++	Pos ++	Pos +
A. invadans, NSW 2007	#70	Pos* ++++	Pos* ++++	Pos ++	Pos +
A. astaci strain Ti	#188	Neg	Neg	Neg	Neg
A. astaci strain Pc	#194	Neg	Neg	Neg	Neg
Penicillin sp.	#228	Neg	Neg	Neg	Neg
Aspergillus sp.	#347	Neg	Neg	Neg	Neg
Saprolegnia parasitica, QLD	#336	Neg	Neg	Neg	Neg
Saprolegnia declina ATCC 36144	#348	Neg	Neg	Neg	Neg
Achlya diffusa, ATCC 16111	#349	Neg	Neg	Neg	Neg
S. salmonis, AS-03-3827	#354	Neg	Neg	Neg	Neg
S. parasitica, AS-03-3827	#355	Neg	Neg	Neg	Neg
A. frigidophilus, ATCC 204464	#231	Neg	Neg	Neg	Neg

* = Annealing temperature of 55°C; [1] = primers designed in project 2001/621; [2] = Phadee *et al*., 2004; [3] = Vandersea *et al*., 2006; [4] = Blaser *et al*., 2002; + = indicates the amount of amplified product seen on the gel. + = lightly staining, +++ = strongly staining; AHL Lab ID = Animal Health Laboratory identification number given to the extracted DNA; neg = negative result.

Primers ITS 11 + ITS 23 were tested for specificity against the same range of bacteria as tested for primers AIF14 + AIR10 (Table 7-2). Primers ITS 11 + ITS 23 were specific as non-specific product was not amplified from these bacteria (Figure F-1, Appendix F).

The amount of amplicon generated by all primers from two isolates of *A. invadans* (AS-04-2382, 1 & 2) was approximated using a molecular weight ladder, Hyper IV (GeneRuler™, Bioline, MBI Fermentas). This molecular weight ladder contains known concentrations of DNA in each of the bands. The largest band of 1000 bp contains 100 ng/band, whereas the 200 bp band contains 20 ng/band. The 100 bp band contains less DNA and is often not seen on the gel unless the light from the UV transilluminator is turned to maximum.

Primers ITS 11 + ITS 23 were tested at the recommended annealing temperature of 65°C, (Phadee *et al*., 2004). Amplicon generated from *A. invadans* AS-04-2382-1, (Figure 7-2, Lane 2) was approximately 50-60 ng/band. Even less product was amplified for AS-04-2382-2, Lane 3 (Figure 7-2). Thus the amplicon generated from *A. invadans* AS-04-2382-2 equates to approximately 20 ng/band in the gel.

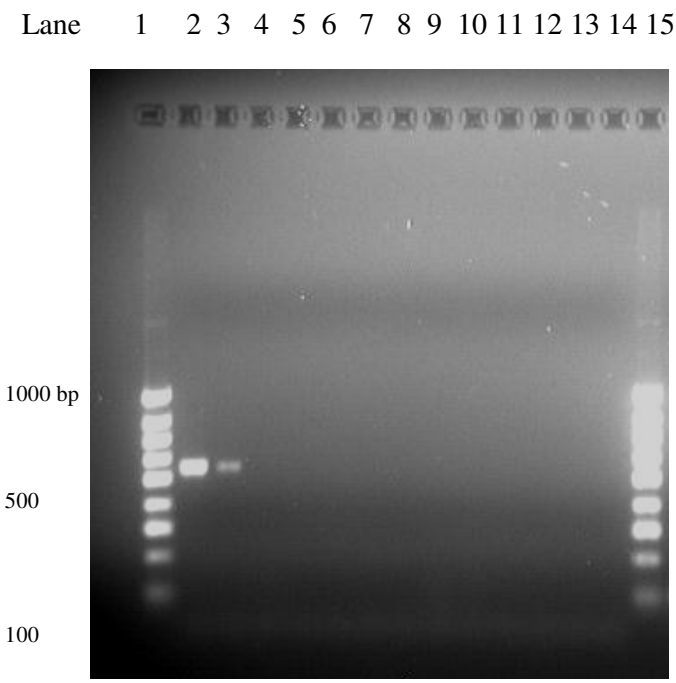

Figure 7-2. Primers ITS 11 + ITS 23 using the recommended annealing temperature of 65°C

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-04-2382; Lane 4 = *Aphanomyces* sp; Lane 5 = *A. astaci* Group B – Ti; Lane 6 = *A. astaci* Group D – Pc; Lane 7 = *Penicillium* sp. AS-01-4050; Lane 8 = *Aspergillus niger* AS-02-4066; Lane 9 = *Saprolegnia* sp. AS-04-2383; Lane 10 = *S. diclina* ATCC 36144; Lane 11 = *Achlya diffusa* ATCC 16111; Lane 12 = *S. salmonis* AS-03-3827; Lane 12 = *A. frigidophilus* ATCC 204464; Lane 13 = *Aphanomyces* sp; Lane 14 = DNA-free control; Lane 15 = Hyper IV molecular weight ladder

Primer pairs AIF14 + AIR10 and ITS 11 + ITS 23 produced equally strong staining amplicon (recorded as ++++, Table 7-3) when the annealing temperature was 55°C (Figures 7-3 and 7-4), which is the annealing temperature recommended for primers AIF14 + AIR10. Some smearing above the amplified product was seen at an annealing temperature of 55°C for primers ITS 11 + ITS 23 (Figure 7-4). Both primers sets produced amplicons greater than 100 ng/band.

Amplified product obtained from primers 2F + R1 (recorded as ++, Table 7-3) stained weaker than product obtained with primers AIF14 + AIR10 (Figure 7-3). According to the Hyper IV molecular weight ladder this would equate to 70-100 ng/band, whereas the amplicon generated from primers AIF14 + AIR10 was considerably greater than 100 ng/band (Figure 7-3). An annealing temperature of 55 °C was used which is the recommended temperature for primers AIF14 + AIR10. The recommended temperature for primers 2F + R1 is 56°C (Vandersea *et al*., 2006).

An even weaker-staining amplicon was obtained for primers P1 + P2 (Figure 7-4) (recorded as +, Table 7-3). According to the Hyper IV ladder, the molecular weight of the amplicon for primers 2F + R1 was 90-100 ng/band, whereas product for primers AIF14 + AIR10 was greater than 100 ng/band.

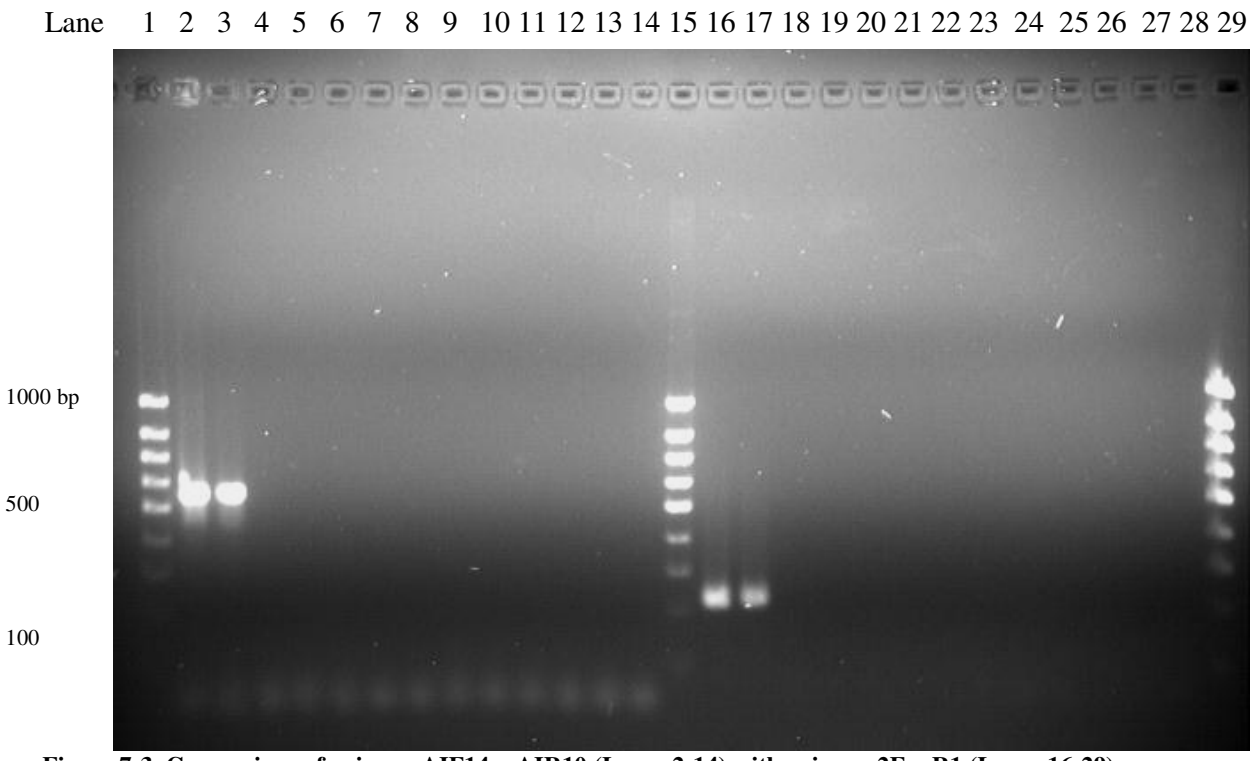

Figure 7-3. Comparison of primers AIF14 + AIR10 (Lanes 2-14) with primers 2F + R1 (Lanes 16-29)

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-04-2382; Lane 4 = *Aphanomyces* sp; Lane 5 = *A. astaci* Group B – Ti; Lane 6 = *A. astaci* Group D – Pc; Lane 7 = *Penicillium* sp. AS-01-4050; Lane 8 = *Aspergillus niger* AS-02-4066; Lane 9 = *Saprolegnia* sp. AS-04-2383; Lane 10 = *S. diclina* ATCC 36144; Lane 11 = *Achlya diffusa* ATCC 16111; Lane 12 = *S. salmonis* AS-03-3827; Lane 12 = *A. frigidophilus* ATCC 204464; Lane 13 = *Aphanomyces* sp; Lane 14 = DNA-free control; Lane 15 = Hyper IV molecular weight ladder; Lane 16 = *A. invadans* AS-04-2382-1; Lane 17 = *A. invadans* AS-04-2382; Lane 18 = *Aphanomyces* sp; Lane 19 = *A. astaci* Group B – Ti; Lane 20 = *A. astaci* Group D – Pc; Lane 21 = *Penicillium* sp. AS-01-4050; Lane 22 = *Aspergillus niger* AS-02-4066; Lane 23 = *Saprolegnia* sp. AS-04-2383; Lane 24 = *S. diclina* ATCC 36144; Lane 25 = *Achlya diffusa* ATCC 16111; Lane 26 = *S. salmonis* AS-03-3827; Lane 27 = *A. frigidophilus* ATCC 204464; Lane 28 = DNA-free control; Lane 29 = Hyper IV molecular weight ladder.

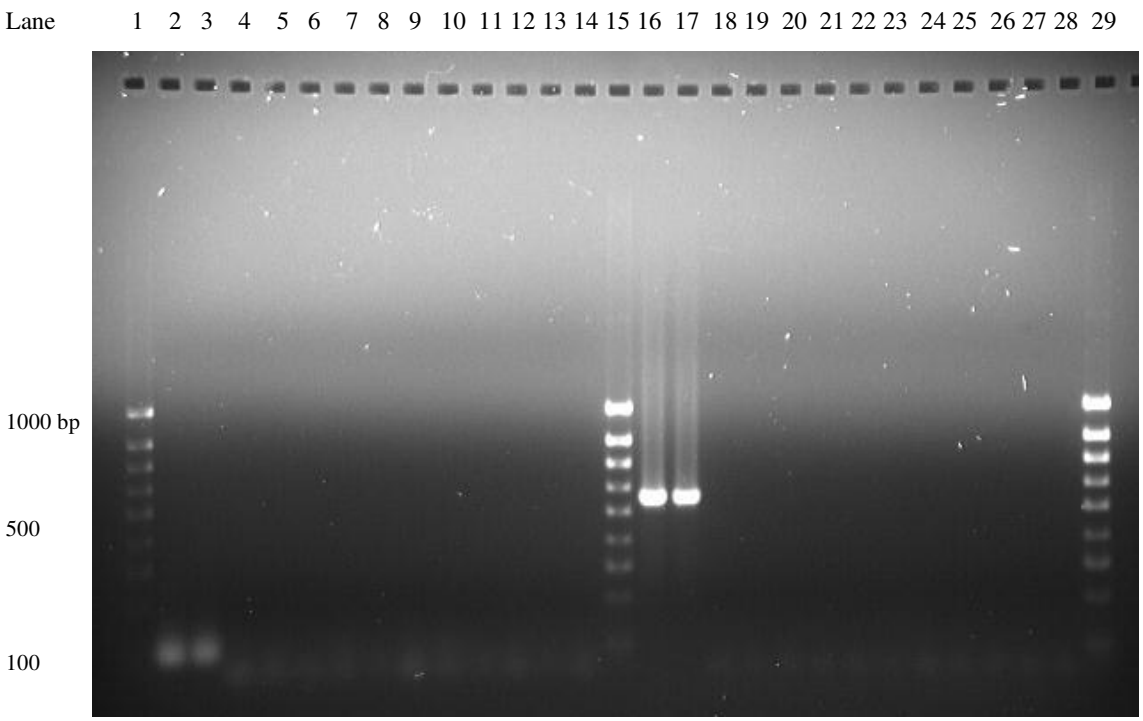

Figure 7-4. Comparison of primers P1 + P2 with primers ITS 11 + ITS 23

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-04-2382; Lane 4 = *Aphanomyces* sp; Lane 5 = *A. astaci* Group B – Ti; Lane 6 = *A. astaci* Group D – Pc; Lane 7 = *Penicillium* sp. AS-01-4050; Lane 8 = *Aspergillus niger* AS-02-4066; Lane 9 = *Saprolegnia* sp. AS-04-2383; Lane 10 = *S. diclina* ATCC 36144; Lane 11 = *Achlya diffusa* ATCC 16111; Lane 12 = *S. salmonis* AS-03-3827; Lane 12 = *A. frigidophilus* ATCC 204464; Lane 13 = *Aphanomyces* sp; Lane 14 = DNA-free control; Lane 15 = Hyper IV molecular weight ladder; Lane 16 = *A. invadans* AS-04-2382-1; Lane 17 = *A. invadans* AS-04-2382; Lane 18 = *Aphanomyces* sp; Lane 19 = *A. astaci* Group B – Ti; Lane 20 = *A. astaci* Group D – Pc; Lane 21 = *Penicillium* sp. AS-01-4050; Lane 22 = *Aspergillus niger* AS-02-4066; Lane 23 = *Saprolegnia* sp. AS-04-2383; Lane 24 = *S. diclina* ATCC 36144; Lane 25 = *Achlya diffusa* ATCC 16111; Lane 26 = *S. salmonis* AS-03-3827; Lane 27 = *A. frigidophilus* ATCC 204464; Lane 28 = DNA-free control; Lane 29 = Hyper IV molecular weight ladder.

All primers were tested at 57.5°C, which is the recommended temperature for primers P1 + P2 (Blazer *et al*., 2002). Primers ITS 11 + ITS 23 produced a strongly staining amplicon equivalent to the amplicon produced at 55 °C (results not shown). Primers P1 + P2 produced a stronger staining amplicon (approximately 100 ng/band) at their recommended temperature of 57.5 °C compared with an amplicon of 90-100 ng/band at 55°C. However, at 57.5°C a weakly staining amplicon (approximately 20 ng/band) was seen with *Penicillium* sp. (results not shown).

The primers 2F + R1 were tested at 55°C (Figure 7-3), but were also tested at a temperature of 57.5°C (Figure 7-5). At this temperature non-specific product was amplified from *A. astaci* Group D Pc, *Penicillium* sp., *Aspergillus niger*, *Saprolegnia* sp., and *S. salmonis* (Figure 7-5).

Primers AIF14 + AIR10 were also tested at 57.5°C (Figure 7-5) in contrast to their recommended annealing temperature of 56°C, but the primers still produced amplicon estimated greater than 100 ng/band for *A. invadans*, and did not produce any non-specific product from the other fungal species tested.

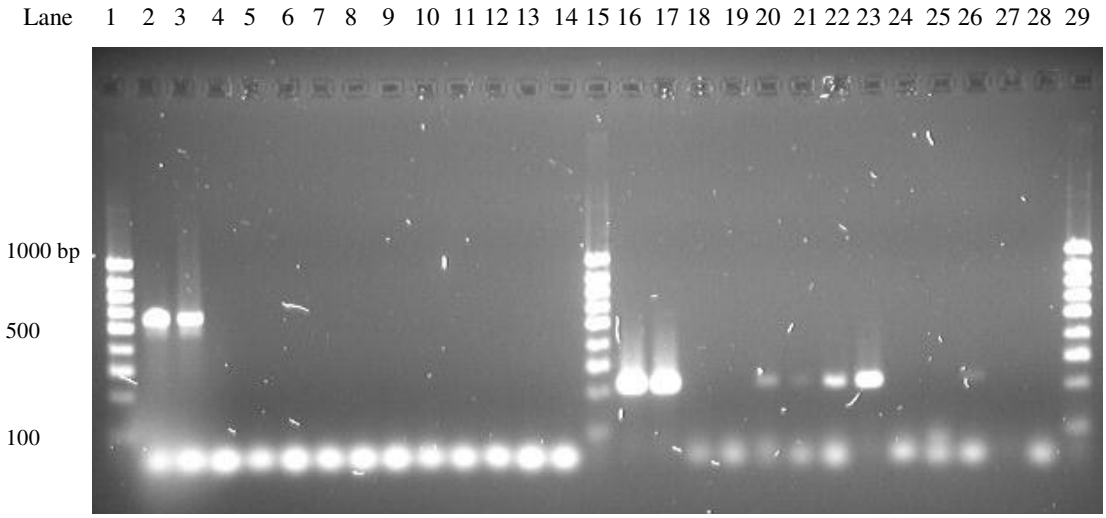

Figure 7-5. Comparison of primers AIF14 + AIR 10 (Lanes 2-14) with primers 2F + R1 (Lanes 16-28)

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-04-2382; Lane 4 = *Aphanomyces* sp; Lane 5 = *A. astaci* Group B – Ti; Lane 6 = *A. astaci* Group D – Pc; Lane 7 = *Penicillium* sp. AS-01-4050; Lane 8 = *Aspergillus niger* AS-02-4066; Lane 9 = *Saprolegnia* sp. AS-04-2383; Lane 10 = *S. diclina* ATCC 36144; Lane 11 = *Achlya diffusa* ATCC 16111; Lane 12 = *S. salmonis* AS-03-3827; Lane 12 = *A. frigidophilus* ATCC 204464; Lane 13 = *Aphanomyces* sp; Lane 14 = DNA-free control; Lane 15 = Hyper IV molecular weight ladder; Lane 16 = *A. invadans* AS-04-2382-1; Lane 17 = *A. invadans* AS-04-2382; Lane 18 = *Aphanomyces* sp; Lane 19 = *A. astaci* Group B – Ti; Lane 20 = *A. astaci* Group D – Pc; Lane 21 = *Penicillium* sp. AS-01-4050; Lane 22 = *Aspergillus niger* AS-02-4066; Lane 23 = *Saprolegnia* sp. AS-04-2383; Lane 24 = *S. diclina* ATCC 36144; Lane 25 = *Achlya diffusa* ATCC 16111; Lane 26 = *S. salmonis* AS-03-3827; Lane 27 = *A. frigidophilus* ATCC 204464; Lane 28 = DNA-free control; Lane 29 = Hyper IV molecular weight ladder.

Case AS-05-0817 was tested using primers AIF14 + AIR10 and primers ITS 11 + ITS 23 at an annealing temperature of 55°C, and primers ITS 11 + ITS 23 at their recommended annealing temperature of 65°C. DNA was extracted from fish tissue from case AS-05-0817 using the DNAzol extraction reagent. Details of fish tested in case AS-05-0817 are given in Table 6-1.

Primers AIF14 + AIR10 amplified DNA from *A. invadans* from the fish tissue samples 1(a), 1(b), 2, and fish 6 at an annealing temperature of 55°C (Figure 7-6, gel 1anes 1-15). At an annealing temperature of 55°C (10°C below the recommended temperature) primers ITS 11 + ITS 23 amplified *A. invadans* DNA extracted directly from fish tissue for the same fish tissue samples (Figure 6-7, gel 2 Lanes 17-30). However, variable results for primers ITS 11 + ITS 23 were obtained when used at their recommended annealing temperature of 65°C (Figure 7-6, gel 2 Lanes 31-47). DNA from *A. invadans* extracted directly from fish tissue was only amplified from fish tissue 1(a). A repeated sample of fish 1(a) (Lane 33) was negative. Fish tissue 1(b) produced a very weak amplicon of less than 20 ng/band and no band was amplified from fish tissue 6, previously positive at 55 °C annealing temperature. A repeat amplification with primers ITS 11 + ITS 23 at 65°C annealing temperature using the same DNA samples was negative for all samples re-tested (results not shown). These results indicate that these primers may give unreliable test results.

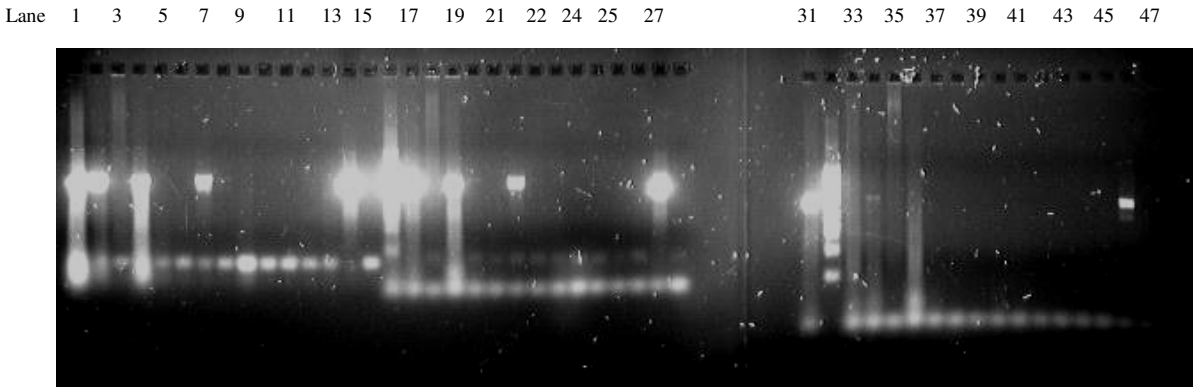

Figure 7-6. Comparison of primers AIF14 + AIR10 tested at 55°C (Lanes 1-15, gel 1) with primers ITS 11 + ITS 23 tested at 55°C (Lanes 17-30, gel 1) and at 65°C (Lanes 31, 33-47, gel 2) using samples from case AS-05-0817

Gel 1 primers AIF14 + AIR10 at annealing temperature of 55°C. Lane 1 = AS-05-0817, fish 1(a); Lane 2 = fish 1(b); Lane 3 = fish 2; Lane 4 = fish 3; Lane 5 = fish 4; Lane 6 = fish 5; Lane 7 = fish 6; Lane 8 = fish 7; Lane 9 = fish 8; Lane 10 = fish 9; Lane 11 = fish 10; Lane 12 = fish 11; Lane 13 = fish 12; Lane 14 = DNA positive control; Lane 15 = DNA-free control; Lane 16 = Hyper IV molecular weight ladder. **Gel 1 primers ITS 11 + ITS 23 at annealing temperature of 55°C**. Lane 17 = AS-05-0817, fish 1(b); Lane 18 = fish 2; Lane 19 = fish 3; Lane 20 = fish 4; Lane 21 = fish 5; Lane 22 = fish 6; Lane 23 = fish 7; Lane 24 = fish 8; Lane 25 = fish 9; Lane 26 = fish 10; Lane 27 = fish 11; Lane 28 = fish 12; Lane 29 = DNA positive control; Lane 30 = DNA-free control. **Gel 2 primers ITS 11 + ITS 23 at annealing temperature of 65°C.** Lane 31 = AS-05-0817, fish 1(a); Lane 32 = Hyper IV molecular weight ladder; Lane 33 = fish 1(a); Lane 34 = fish 1(b); Lane 35 = fish 2; Lane 36 = fish 3; Lane 37 = fish 4; Lane 38 = fish 5; Lane 39 = fish 6; Lane 40 = fish 7; Lane 41 = fish 8; Lane 42 = fish 9; Lane 43 = fish 10; Lane 44 = fish 11; Lane 45 = fish 12; Lane 46 = DNA positive control; Lane 47 = DNA-free control.

Overall, the primer set AIF14 + AIR10 out-performed the primer sets (ITS 11 + ITS 23, 2F + R1, P1 + P2) published during the course of this project. Primers AIF14 + AIR10 maintained specificity at the temperatures of 55°C and 57.5°C, whereas primers 2F + R1 produced non-specific product when tested at 57.5°C. Their recommended temperature is 56°C. This means that a 1.5°C increase in temperature would lead to amplification of non-specific products indicating that these primers work within a very narrow temperature range for specificity. Primers P1 + P2 also worked within a very narrow temperature range. At 1.5oC below their recommended temperature range, considerably less amplicon was produced than at the recommended temperature, whereas primers AIF14 + AIR10 produced the same amount of product at these two temperatures.

7.2 Complete research to develop specific PCR for *A. astaci* (crayfish plague)

7.2.1 Testing primers designed for A. astaci in project FRDC 2001/621

The DNA extracted from *A. astaci* cultures held at AAHL was tested using universal fungal primers C1D2 to check the presence of fungal DNA. All samples tested positive producing an amplicon of approximately 850 bp indicating fungal DNA was present in the samples.

The 13 primer sets (Table 6-8) designed in FRDC 2001/621 to detect *A. astaci* were tested against the *A. astaci* (strain Pc) DNA sent from AAHL. Ten primers produced a strong band of amplified DNA, whereas one primer pair amplified a moderate band of DNA, and two primer pairs produced a weak amplicon. Four primer pairs that produced a strong band of

amplified DNA of less than 300 base pairs were tested using all five types of *A. astaci* DNA. These were AAF 18 + AAR 19 (204 bp), AAF 18 + AAR 20 (204 bp), AAF 21 + AAR22 (212 bp), AAF 21 + AAR25 (162 bp). After optimisation, primer pair AAF 21 + AAR 25 with a product size of 162 bp, gave the best results. Results of specificity testing are included in Section 7.2.2.

7.2.2 Testing in-house primers designed for A. astaci compared to previously published primers

The four primer pairs (AAF 18 + AAR 19, AAF 18 + AAR 20, AAF 21 + AAR22, AAF 21 + AAR25) were tested in parallel with the published primers 525f+640r (Oidtmann *et al*., 2004). The DNA that was tested was extracted from formalin-fixed, paraffin-embedded sections sent from the UK during project FRDC 2001/621. DNA was extracted by the DNeasy mini plant tissue kit (Qiagen) and the method according to Whittington *et al*., (19991). DNA from paraffin-embedded material was not amplified by the primers designed in-house (Figure7-7).

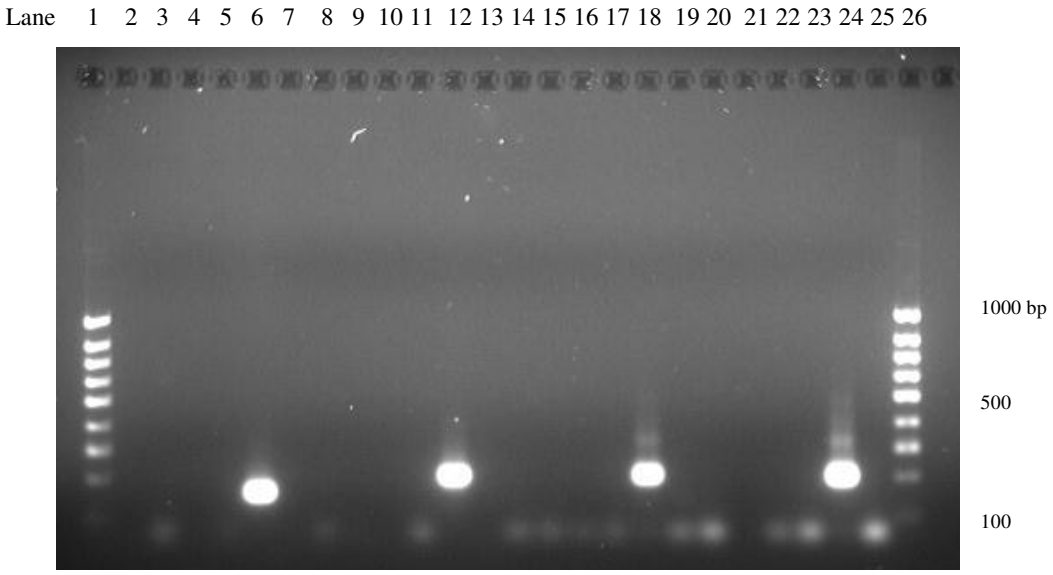

Figure 7-7. Results for detection of *A. astaci* from paraffin-embedded tissue using primers designed as part of this project. Lanes 2-7 = primers AAF + AAR25; Lanes 8-13 = primers AAF 21 + AAR 22; Lanes 14-19 = primers AAF 18 + AAR 20; Lanes 20-25 = primers AAF 18 + AAR 19

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = AS—0-1993-1 (DNeasy extraction); Lane 3 = AS—0-1993-2 (DNeasy extraction); Lane 4 = AS—0-1993-1 (Whittington extraction); Lane 5 = AS—0-1993-2 (Whittington extraction); Lane 6 = DNA positive control; Lane 7 = DNA-free control; Lane 8 = AS—0-1993-1 (DNeasy extraction); Lane 9 = AS—0-1993-2 (DNeasy extraction); Lane 10 = AS—0-1993-1 (Whittington extraction); Lane 11 = AS—0-1993-2 (Whittington extraction); Lane 12 = DNA positive control; Lane 13 = DNA-free control; Lane 14 = AS—0-1993-1 (DNeasy extraction); Lane 15 = AS—0-1993-2 (DNeasy extraction); Lane 16 = AS—0-1993-1 (Whittington extraction); Lane 17 = AS—0-1993-2 (Whittington extraction); Lane 18 = DNA positive control; Lane 19 = DNA-free control; Lane 20 = AS—0-1993-1 (DNeasy extraction); Lane 21 = AS—0-1993-2 (DNeasy extraction); Lane 22 = AS—0-1993-1 (Whittington extraction); Lane 23 = AS—0-1993-2 (Whittington extraction); Lane 24 = DNA positive control; Lane 25 = DNA-free control; Lane 26 = Hyper IV molecular weight ladder.

The same samples were tested using published primers 525f + 640r (Oidtmann *et al*., 2004). DNA was amplified from paraffin-embedded tissue using these primers (Figure 7-8).

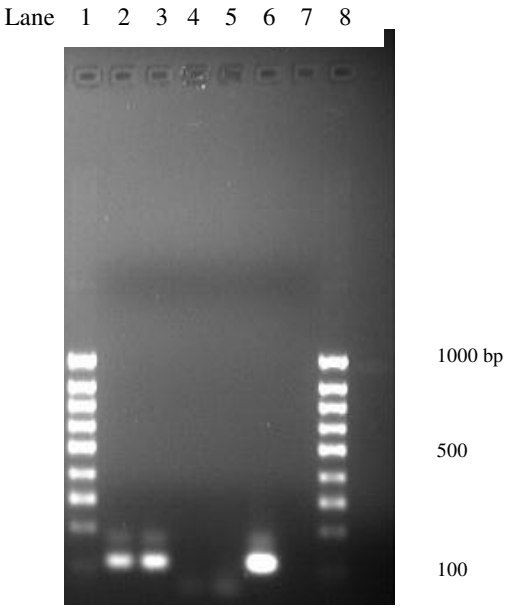

Figure 7-8. Results for detection of *A. astaci* from paraffin-embedded tissue using primers 525f + 640r

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = AS—0-1993-1 (DNeasy extraction); Lane 3 = AS—0-1993-2 (DNeasy extraction); Lane 4 = AS—0-1993-1 (Whittington extraction); Lane 5 = AS—0-1993-2 (Whittington extraction); Lane 6 = DNA positive control; Lane 7 = DNA-free control; Lane 8 = Hyper IV molecular weight ladder.

Primers 525f + 640r were tested for specificity against other species of oomycete and true fungi. Product of the expected size of 115 bp was amplified from two stains of *A. astaci*, but weak product was amplified from *A. invadans* (Figure 7-9). No product was produced from *A. frigidophilus*, however this was reported to give cross-reaction when tested in further studies (Oidtmann *et al*., 2006).

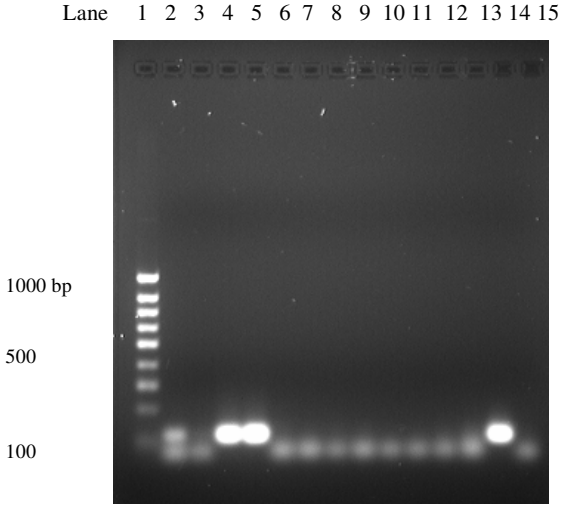

Figure 7-9. Results of specificity testing of primers 525f + 640r against oomycete and true fungi

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-07-0101; Lane 4 = *A. astaci* Group B – Ti; Lane 5 = *A. astaci* Group D – Pc; Lane 6 = *Penicillium* sp. AS-01-4050; Lane 7 = *Aspergillus niger* AS-02-4066; Lane 8 = *Saprolegnia* sp. AS-04-2383; Lane 9 = *S. diclina* ATCC 36144; Lane 10 = *Achlya diffusa* ATCC 16111; Lane 11 = *S. salmonis* AS-03-3827; Lane 12 = *S. parasitica* AS-03-3827; Lane 13 = *A. frigidophilus* ATCC 204464; Lane 14 = DNA positive control; Lane 15 = DNA-free control.

Primers 525f + 640r were tested for specificity against bacteria that may be present as contaminants (Figure7-10).

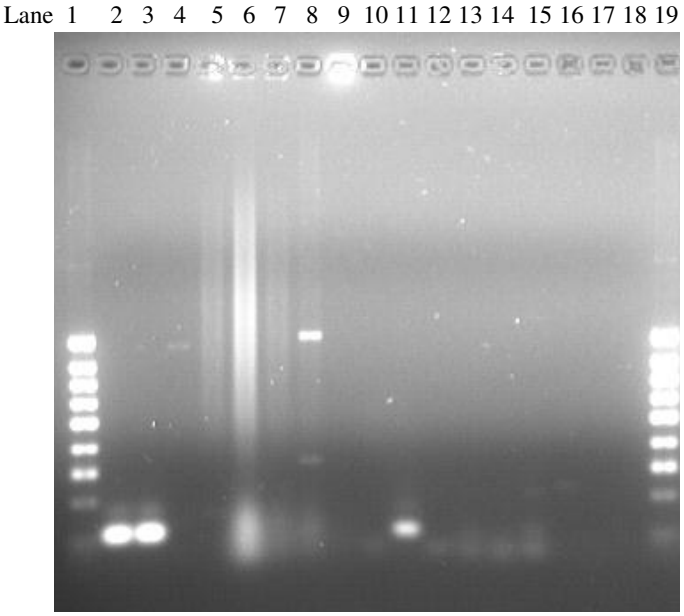

Figure 7-10. Results of specificity testing for primers 525f + 640r against bacterial species

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A astaci* Ti; Lane 3 = *A. astaci* Pc; Lane 4 = *Vibrio alginolyticus*; Lane 5 = *V. cholerae*; Lane 6 = *V. mimicus*; Lane 7 = *Aeromonas salmonicida*; Lane 8 = *A. hydrophila*; Lane 9 = *Streptococcus iniae*; Lane 10 = unknown fungus; Lane 11 = *A. invadans*; Lane 12 = *Aspergillus niger*; Lane 13 = *S. diclina*; Lane 14 = *A. diffusa*; Lane 15 = *A. invadans*; Lane 16 = *S. salmonis*; Lane 17 = *S. parasitica*; Lane 18 = DNA-free control; Lane 19 = Hyper IV molecular weight ladder.

Some non-specific bands at different molecular weights were amplified for *Vibrio alginolyticus*, *A. hydrophila* and smearing was seen through the lane for *Vibrio mimicus*. The weak bands amplified for *V. alginolyticus* and *A. hydrophila* were at high molecular weight, but the smearing for *V. mimicus* was seen the entire length of the lane.

The same bacteria were used for specificity testing of primers AAF 21 + AAR 25. These primers were more specific than primers 525f + 640r as no non-specific bands were seen, except some smearing in the lane for *V. mimicus* (results not shown).

The other primer pairs designed in the course of this project were also tested for specificity. Primers AAF 21 + AAR 22 produced non-specific bands at different molecular weights for *A. salmonicida*, *A. hydrophila*, *A. invadans*. Smears were seen in the lanes for *V. alginolyticus*, *V. cholerae* and *V. mimicus* (results not shown). No amplicon was seen at the expected molecular weight of 212 bp. Primer pairs AAF 18 + AAR 20 produced non-specific product at molecular weights different to the expected product size of 204 bp for *V. alginolyticus*, *V. mimicus*, *A. salmonicida* and *A. hydrophila*. A smear was seen in the lane for V. cholerae (results not shown). Primer pair AAF 18 + AAR 19 produced an amplicon of 204 bp for *A. astaci* stain Pc, but no amplicon was produced for *A. astaci* strain Ti. No non-specific products were seen for any of the bacteria tested. Smearing of DNA was seen in the lanes for *V. cholerae*, *V. mimicus* and *A. salmonicida* (results not shown).

Primers 525f + 640r and AAF 21 + AAR 25 were tested for sensitivity using 10-fold serial dilutions of *A. astaci* strain Yx. Weak amplicon for primers 525f + 640r could be seen visually at a dilution of 1:100,000, which equated to $15.6x10^{-5}$ ng/µl of DNA. An amplicon for primers AAF 21+ AAR 25 was seen for 1:1,000 a detection limit of $15.6x10^{-2}$ ng/µl of DNA (Figure 7- 11). The test was repeated for primers AAF 21 + AAR 25 and the detection limit increased to 1:10,000 (15.6×10^{-4} ng/µl) (Figure 7-12). Primers 525f + 640r were ten-fold more sensitive than primers AAF 21 + AAR 25.

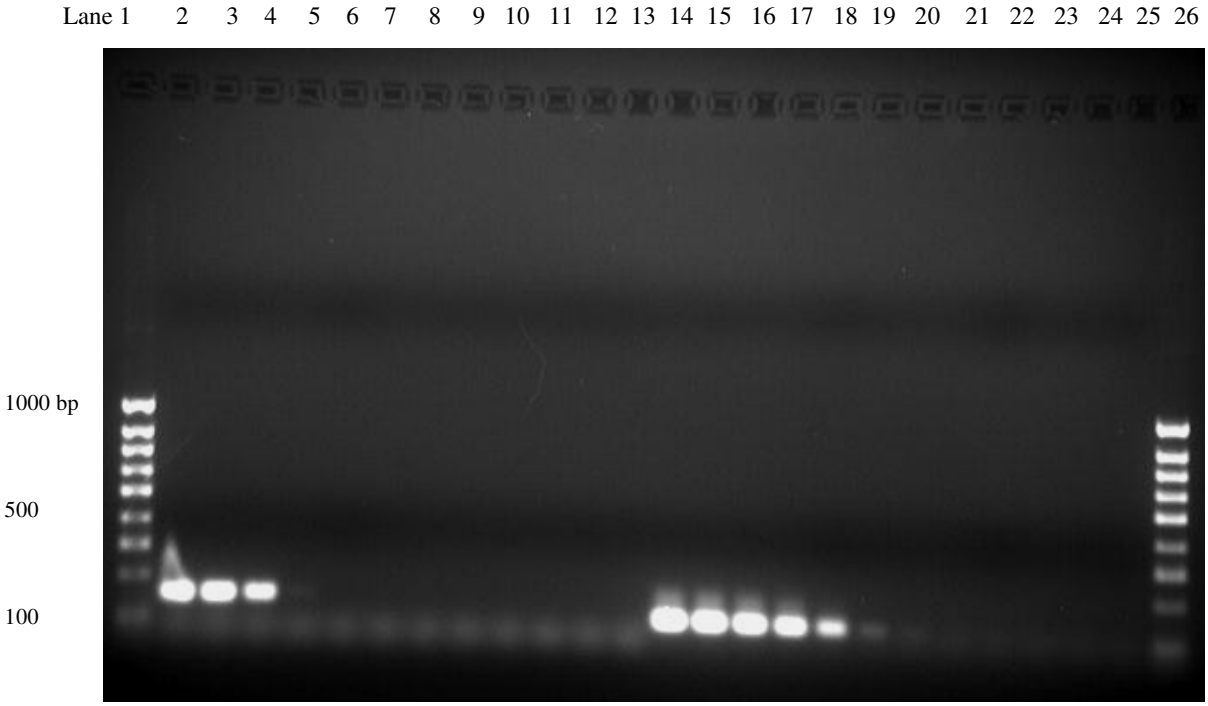

Figure 7-11. Comparison of sensitivity of detection using primers AAF21 + AAR25 and 525f + 640r. Lanes 2-13 = primers AAF 21 + AAR 25, and lanes 14-25 = primers 525f + 640r

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = neat DNA; Lane 3 = 1:10; Lane 4 = $1:10^2$ Lane 5 = $1:10^3$; Lane 6 = $1:10^4$; Lane 7 = $1:10^5$; Lane 8 = $10^{6;}$ Lane 9 = $1:10^{7;}$ Lane 10 = $1:10^{8;}$ Lane 11 = $1:10^9$; Lane 12 = $1:10^{10}$; Lane 13 = DNA-free control; Lane 14 = neat DNA; Lane 15 = 1:10; Lane 16 = $1:10^2$ Lane 17 = $1:10^3$; Lane 18 = $1:10^4$; Lane 19 = $1:10^5$; Lane 20 = $10^{6;}$ Lane 21 = $1:10^{7;}$ Lane 22 = $1:10^{8;}$ Lane 23 = $1:10^9$; Lane 24 = $1:10^{10}$; Lane 25 = DNA-free control; Lane 26 = Hyper IV molecular weight ladder.

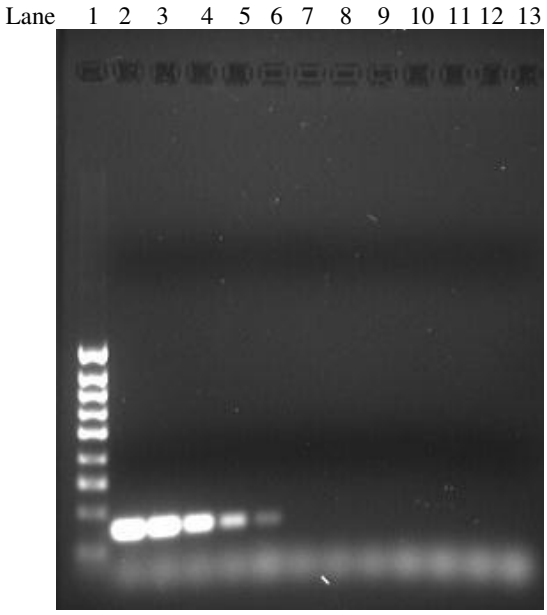

Figure 7-12. Repeat of sensitivity testing for primers AAF 21 + AAR 25

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = neat DNA; Lane 3 = 1:10; Lane 4 = $1:10^2$ Lane 5 = $1:10^3$; Lane 6 = $1:10^4$; Lane 7 = $1:10^5$; Lane 8 = $10^{6;}$ Lane 9 = $1:10^{7;}$ Lane 10 = $1:10^{8;}$ Lane 11 = $1:10^9$; Lane 12 = $1:10^{10}$; Lane 13 = DNA-free control.

Table 7-4. Result of testing in-house primers designed for *A. astaci* compared to published primers 525f + 640r (Oidtmann *et al* 2004)

Case Number	Result for primers 21+25 (162 bp)	Result for primers 18+19 (204 bp)	Result for primers 18+20 (202 bp)	Result for primers 21+22 (212 bp)	Result for primers 525+640 (115 bp)
AS-02-1993 – paraffin-embedded block of Crayfish plague from UK	negative	negative	negative	negative	positive
A. astaci group B	positive	negative	positive	positive	positive
A. astaci group D	positive	positive	positive	positive	positive
#24, *A invadans* culture, NSW	negative	negative	negative	negative	positive
Other oomycetes	negative	negative	some non-specific results	some non-specific results	negative

Primers 21+25, 18+19, 18+20, 21+22 were designed as part of this project; primers 525+640 from Oidtmann *et al*., 2004.

The Oidtmann primers designed in 2004 were not specific and produced a weak amplicon from *A. invadans* and *A. frigidophilus* (Oidtmann *et al*., 2006). The forward primer was redesigned and the new primer pair (42f + 640r) produced an amplicon of 569 bp. These primers were validated against the primers developed in this project (AAF 21 + AAR 25).

The primers (42f + 640r) were tested for specificity against the same fungi as previously. No non-specific bands were seen at the same molecular weight as the amplicon from *A. astaci* (569 bp). However, a number of bands at other molecular weights were seen (Figure 7-13). No DNA was amplified in the positive control, but it was later found that this DNA was unsuitable due to the presence of agar.

Further validation of primers (42f + 640r) is required for sensitivity comparisons with primers 525f + 640r, the in-house primers AAF21 + AAR 25, and also for detection of DNA from formalin-fixed paraffin-embedded material.

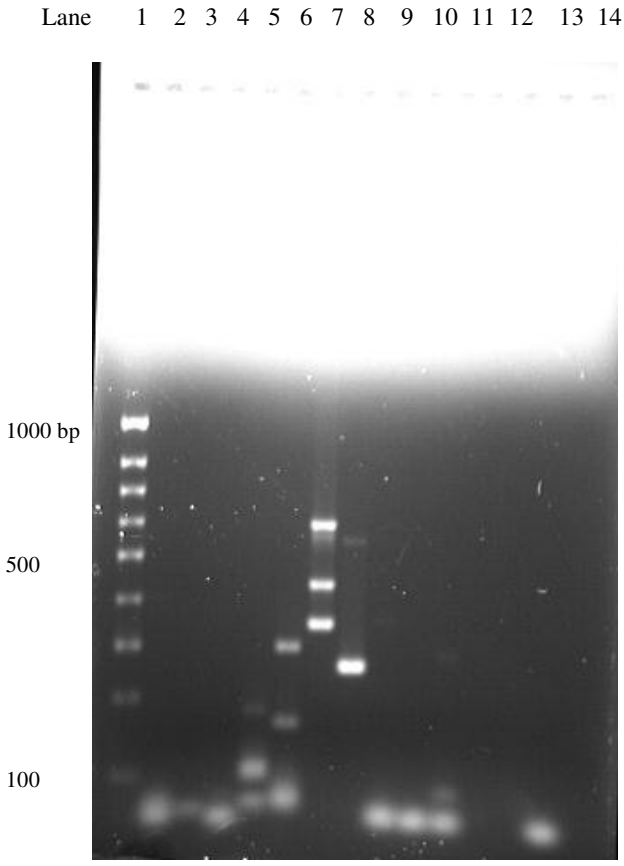

Figure 7-13. Results of specificity testing using primers 42f + 640r

Lane 1 = Hyper IV molecular weight ladder; Lane 2 = *A. invadans* AS-04-2382-1; Lane 3 = *A. invadans* AS-07-0101; Lane 4 = *A. astaci* Group B – Ti; Lane 5 = *A. astaci* Group D – Pc; Lane 6 = *Penicillium* sp. AS-01-4050; Lane 7 = *Aspergillus niger* AS-02-4066; Lane 8 = *Saprolegnia* sp. AS-04-2383; Lane 9 = *S. diclina* ATCC 36144; Lane 10 = *Achlya diffusa* ATCC 16111; Lane 11 = *S. salmonis* AS-03-3827; Lane 12 = *S. parasitica* AS-03-3827; Lane 13 = *A. frigidophilus* ATCC 204464; Lane 14 = DNA positive control; Lane 15 = DNA-free control.

7.3 Complete research to develop FISH tests for *A. invadans* and *A. astaci*

7.3.1 Fluorescent in situ hybridisation using peptide nucleic acid probes for detection of A. invadans

In project 2001/641 fluorescent *in situ* hybridisation (FISH) using a fluorescein molecule attached to the 5' end of the PCR primer (for *A. invadans* amplification) produced cross-reaction with *Saprolegnia* species. In the current project a peptide nucleic acid (PNA) probe (DNA mimic) with a fluorescein molecule attached to the 5 prime end was tested according to the method of Vandersea *et al.*, (2006). The probe (Ainv-FLU3) was specific for *A. invadans* and did not cross-react with *Saprolegnia* species. The probe was tested on fresh culture, frozen hyphae and paraffin-embedded material from either diagnostic cases or cultured hyphae seeded into paraffin blocks.

The PNA probes produced a bright apple-green fluorescence using the FISH technique for *A. invadans* from fresh cultured hyphae (Figure 7-14).

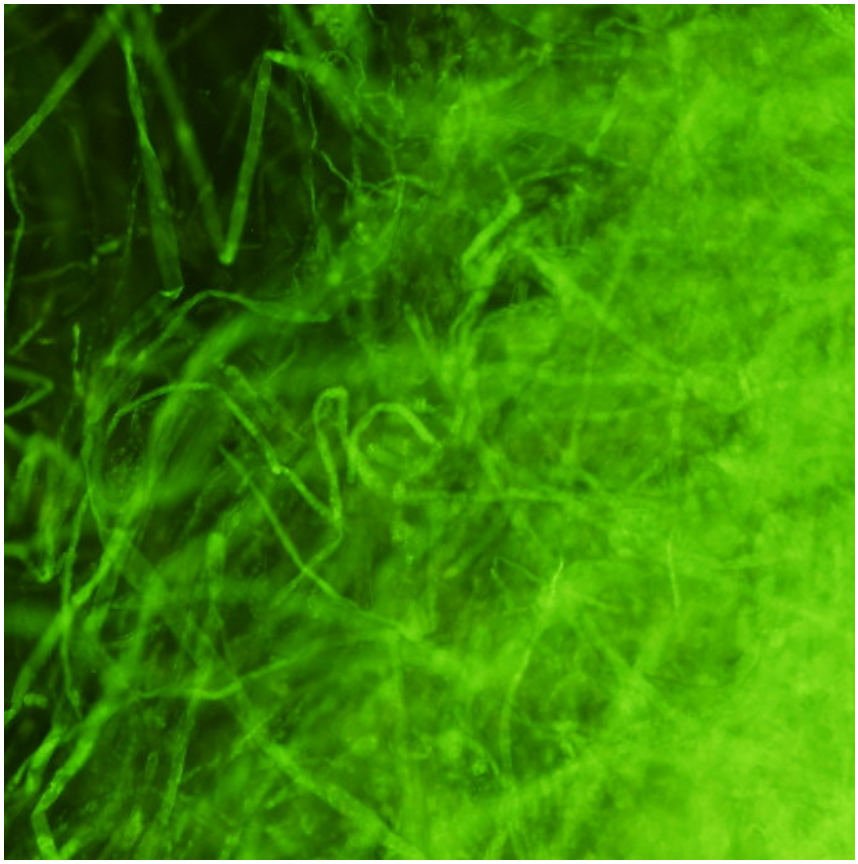

Figure 7-14. Results of PNA-FISH using fresh cultured hyphae of *A. invadans* from case AS-07-0189 showing bright apple-green fluorescence

Fluorescence obtained from *A. invadans* in paraffin-embedded sections was variable. PNA-FISH on the same case as Figure 7-14 did not produce as bright or as consistent fluorescence as fresh cultured fungus. In Figure 7-15 where FISH was performed on cultured hyphae embedded in paraffin blocks, brightly staining hyphae were seen (Figure 7-15) but were not as numerous or as deeply stained as hyphae used directly from an actively growing culture.

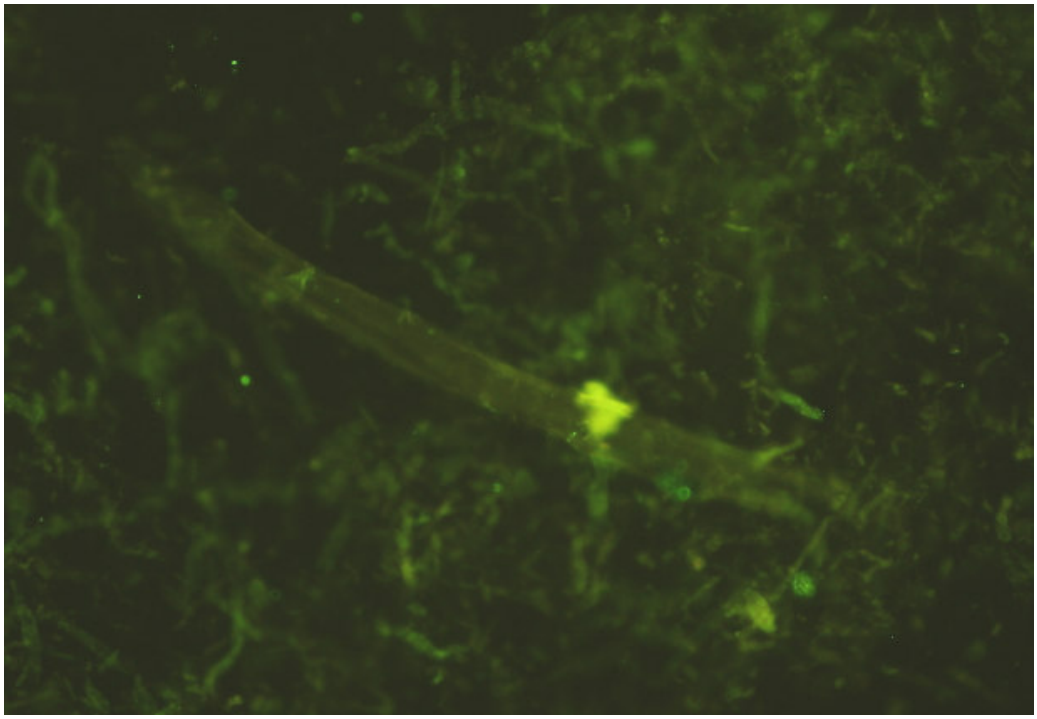

Figure 7-15. Results of PNA-FISH from case AS-07-0189 using hyphae of *A. invadans* embedded into a paraffin block showing reduced fluorescence

Case AS-07-0101 produced some brightly fluorescent hyphae when embedded into paraffin blocks but the number of hyphae that fluoresced was reduced (Figure 7-16).

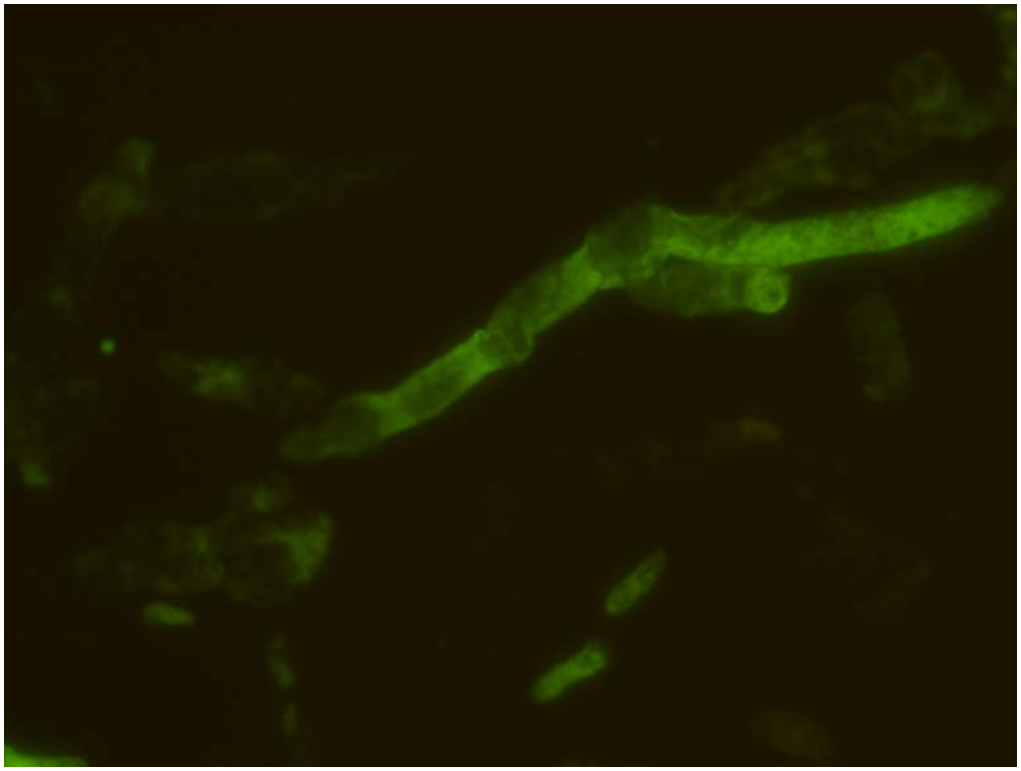

Figure 7-16. Results of PNA-FISH from Case AS-07-0101 using cultured hyphae embedded into paraffin blocks

Some paraffin-embedded cases were difficult to interpret. Case AS-07-1423 from South Australia was diagnosed as EUS according to histopathology, but results from the PNA-FISH had a high background compared to the fungal hyphae (Figure 7-17). Only paraffin-embedded material was received and this was negative by PCR, although the primers for *A. invadans* are unable to amplify DNA from paraffin-embedded material and thus the PCR test was invalid.

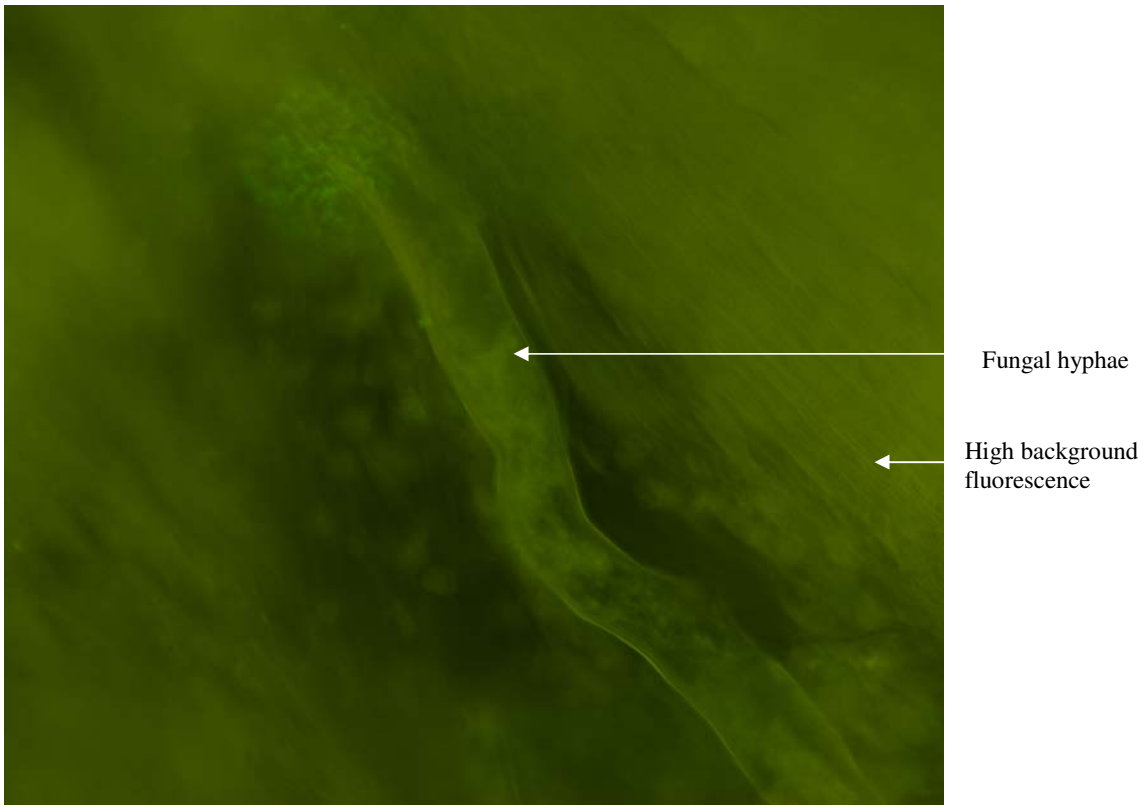

Figure 7-17. Example of equivocal result showing high background fluorescence using paraffin-embedded tissue from case AS-07-1423

Some hyphae from paraffin-embedded material showed poor fluorescence or only spots of fluorescence within the hyphae (Figure 7-18).

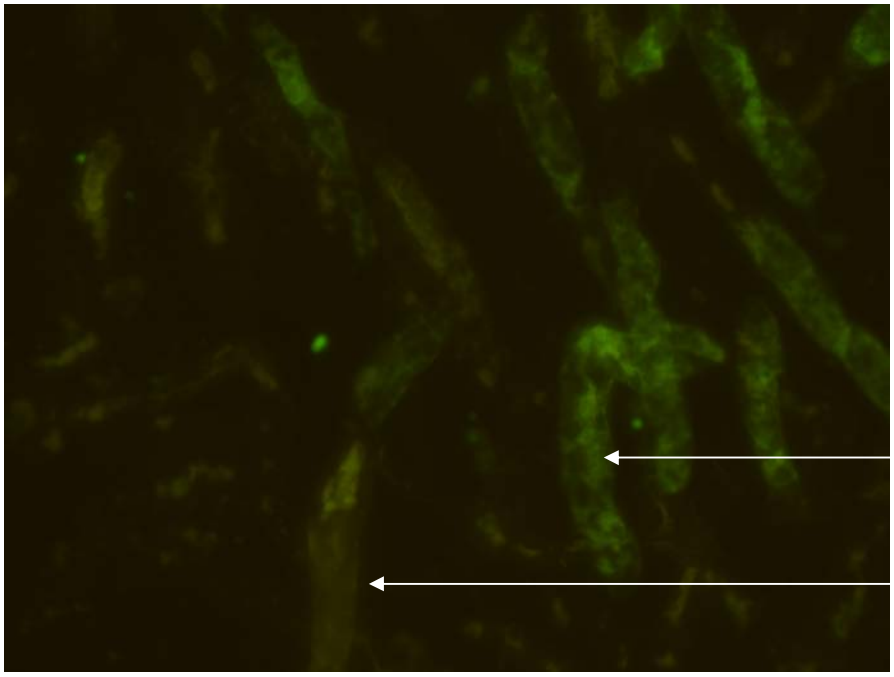

Figure 7-18. Example of variation in fluorescent staining seen within the hyphae

Results of specificity testing showed that the PNA probe was specific. Bright apple-green fluorescence was not seen from other oomycete or true fungi. *Saprolegnia salmonis* produced a yellow fluorescence (Figure 7-19) that could be distinguished from the bright green fluorescence obtained from fresh cultured hyphae from *A. invadans*.

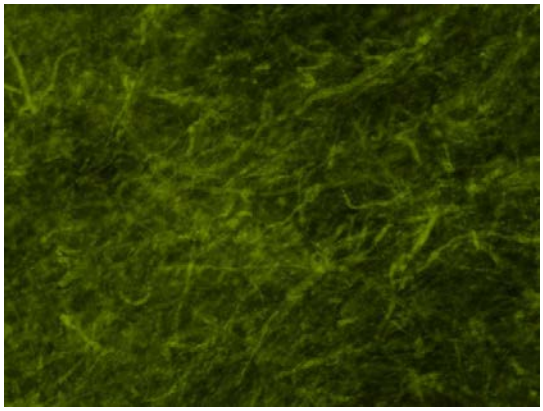

Figure 7-19. Example of negative fluorescence (yellow fluorescence) shown by *Saprolegnia salmonis*

Poor results were obtained from smears prepared from frozen fungal hyphae. Fluorescence was not a bright apple-green as expected, except for some very occasional spots on the smears for *A. invadans*. Smears prepared from *A. invadans* and *Saprolegnia* species had a high background fluorescence. It has been reported that reduction in fluorescence due to degradation of RNA has been found when fungal material has been frozen (Vandersea *et al*., 2006), and this finding was confirmed in our laboratory. At the beginning of the project no viable cultures of *A. invadans* were available and so stored frozen hyphae had been used for the PNA-FISH.

The results of all diagnostic samples, prepared specimens and specificity testing are listed in Table 7-5.

Table 7-5. Results of PNA-FISH using diagnostic and prepared samples for *A. invadans* and specificity testing using other oomycete fungi

Identification	Culture ID	Probe	Sample type	Result
A. invadans, NSW	#24	Ainv-FLU3	Frozen hyphae	Occasional spot of bright fluorescence but background fluorescence was high
A. invadans, NSW 2002	#24	Ainv-FLU3	PE fish tissue seeded with fungal hyphae	Positive Bright apple green fluorescence
Saprolegnia sp	#9	Ainv-FLU3	Frozen hyphae	High background fluorescence
A. invadans, USA	#54	Ainv-FLU3	Frozen hyphae	Occasional spot of bright fluorescence but background fluorescence was high
Universal Positive control (*A. invadans*, WIC, USA)	#54	EuUni1	Frozen hyphae	Occasional spot of bright fluorescence but background fluorescence was high
A. invadans, WA	#36c	No probe	PE culture	Negative
No probe control (*A. invadans*, WIC, USA)	#54	No probe	Frozen hyphae	Negative
No probe control, EUS, diagnostic submission	AS-05-0817-3	No probe	PE section	Negative
A. invadans positive control	#24	EuUni1	PE fish tissue seeded with fungal hyphae	Positive Bright apple green fluorescence
EUS, diagnostic submission, WA	AS-05-0817-3	EuUni1	PE section	Positive Bright apple green fluorescence
A. invadans, WA	#36c	Ainv-FLU3	PE culture	Weak positive
EUS, diagnostic submission, WA	AS-05-0817-1	Ainv-FLU3	PE section	Positive Bright apple green fluorescence
EUS, diagnostic submission, WA	AS-04-2149-1	Ainv-FLU3	PE section	Positive Bright apple green fluorescence
Non-EUS case, negative control	AS-03-1316	Ainv-FLU3	PE section	Negative
A. astaci from AAHL	AS-07-821 PC2	*A. astaci*	Cultured hyphae embedded into paraffin and used to test specificity	Negative
Crayfish plague-infected section from Sweden	AS-07-750 #2	*A. astaci*	Paraffin-embedded section used to test specificity	Negative
Saprolegnia diclina ATCC 36144	#50	Ainv-FLU3	Fresh hyphae	Negative
S. salmonis ATCC 16111	#51	Ainv-FLU3	Fresh hyphae	Negative
S. salmonis AS-03-3827-4	#57	Ainv-FLU3	Fresh cultured hyphae	
S. parasitica AS-03-3827-5	#58	Ainv-FLU3	Fresh hyphae	Negative
S. parasitica/ salmonis AS-04-1735	#59	Ainv-FLU3	Fresh hyphae	Negative
Achlya diffusa	#51	Ainv-FLU3	Fresh hyphae	Negative
Unknown lab contaminant. AS-03-3416	#60	Ainv-FLU3	Fresh hyphae	Negative

Table 7-5, continued

Identification	Culture ID	Probe	Sample type	Result
AS-03-1316	Ainv-FLU3 probe	Negative control	Fish tissue	Negative
EUS, diagnostic submission, NSW	AS-07-0101	Ainv-FLU3	Fresh cultured hyphae	Positive Bright apple green fluorescence
EUS, diagnostic submission, NSW	AS-07-0101	Ainv-FLU3	Fresh cultured hyphae paraffin-embedded	Positive Bright apple green fluorescence in reduced number of hyphae
EUS, diagnostic submission, NSW	AS-07-0189	Ainv-FLU3	Fresh cultured hyphae	Positive Bright apple green fluorescence
EUS, diagnostic submission, NSW	AS-07-0101	Ainv-FLU3	Fresh cultured hyphae paraffin-embedded	Positive. Bright apple green fluorescence in reduced number of hyphae
EUS, diagnostic submission, SA	AS-07-1423	Ainv-FLU3	PE section	Equivocal result. Some staining of hyphae but as bright as background
EUS, diagnostic submission, USA	AS-07-0438	Ainv-FLU3	PE section	Positive. Bright apple green fluorescence in reduced number of hyphae

7.3.2 Fluorescent in situ hybridisation using peptide nucleic acid probes for detection of A. astaci

The PNA-FISH was tested on formalin-fixed, paraffin-embedded sections obtained from Sweden and from non-viable cultures formalin-fixed and sent in ethanol to AHL. At AHL the *A. astaci* material was tested as hyphae or tested after embedding into paraffin blocks. Results from the cultures received from AAHL were variable, but generally a low fluorescent signal was seen. Some cultures did not produce a fluorescent signal. Paraffin-embedded sections from Sweden were difficult to interpret but no bright apple-green fluorescence was seen. One sample showed a small spot of fluorescence. The PCR on these sections was negative, but had been diagnosed as positive for crayfish plague by the submitting laboratory. Overall the paraffin-embedded material was difficult to interpret with only some sections showing a few small areas of bright fluorescence. Most of the hyphae on the slides showed a yellowed fluorescence.

The Asastaci PNAFLU probe was specific as no fluorescence was seen from fresh cultured hyphae from other oomycete fungi such as *Saprolegnia* species and the true fungi such as *Aspergillus* species (Table 7-6) or *A. invadans*.

Table 7-6. Results of PNA-FISH using diagnostic and prepared samples for *A. astaci* and specificity testing using other oomycete fungi and true fungi

Identification	Culture ID	Probe	Sample type	PNA probe result
PE section from Sweden	07-750 #2 *A. astaci*	Aastaci-PNAFLU	Paraffin-embedded tissue	Negative (? Small piece)
A. astaci culture material from AAHL	07-821 Di	Aastaci-PNAFLU	Culture in ethanol	Negative
A. astaci culture material from AAHL	07-821 Pc	Aastaci-PNAFLU	Culture in ethanol	Positive fluorescent signal ++
A. astaci culture material from AAHL	07-821 Yx	Aastaci-PNAFLU	Culture in ethanol	Positive fluorescent signal +
A. astaci culture material from AAHL	07-821 Dc	Aastaci-PNAFLU	Culture in ethanol	Negative
A. astaci culture material from AAHL	07-821 Ti	Aastaci-PNAFLU	Culture in ethanol	Positive fluorescent signal +
A. astaci culture material from AAHL	07-821 Ra	Aastaci-PNAFLU	Culture in ethanol	Positive fluorescent signal +
A. astaci culture material from AAHL	07-821 Pc2	Aastaci-PNAFLU	Culture in ethanol	Negative, ?
07-821 Ra	No probe control	No probe	Culture in ethanol	Negative
07-0101 *A. invadans*, NSW culture	Negative control for *A. astaci*	Aastaci-PNAFLU	Fresh cultured hyphae	Negative
S. salmonis #51 ATCC 16111	Negative control for *A. astaci*	Aastaci-PNAFLU	Fresh hyphae	Negative, dull yellow fluorescence
07-0101 *A. invadans*, NSW culture	Universal positive control	EuUni 1	Fresh cultured hyphae	Positive
07-0101 *A. invadans*, NSW culture	Specificity testing	Aastaci-PNAFLU	Fresh cultured hyphae	Negative
Saprolegnia sp. AS-01-3195	#11	Aastaci-PNAFLU	Fresh hyphae	Negative
Saprolegnia diclina ATCC 36144	#50	Aastaci-PNAFLU	Fresh hyphae	Negative
S. salmonis ATCC 16111	#51	Aastaci-PNAFLU	Fresh hyphae	Negative
S. salmonis AS-03-3827-4	#57	Aastaci-PNAFLU	Fresh cultured hyphae	
S. parasitica AS-03-3827-5	#58	Aastaci-PNAFLU	Fresh hyphae	Negative
S. parasitica/ salmonis AS-04-1735	#59	Aastaci-PNAFLU	Fresh hyphae	Negative
Achlya diffusa	#51	Aastaci-PNAFLU	Fresh hyphae	Negative
Aspergillus species	#48	Aastaci-PNAFLU	Fresh hyphae	Negative

7.4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

7.4.1 Complete trial of A. invadans PCR at participating laboratories

A total of nine laboratories (four international and five national) laboratories were approached and agreed to participate in validation trials of the PCR for *A. invadans*. The kits were sent to all laboratories. Results received from seven laboratories (two international laboratories and five Australian laboratories) are presented in summary in Table 7-7. The raw results and comments received from each laboratory are attached in Appendix G.2.1. The kit was also tested at AHL and the results are presented in Table 7-7 along with the other participating laboratories.

Table 7-7. Results returned from laboratories involved in validating the *A. invadans* primers

Isolate ID	Code used in kit	Expected result	AHL WA	Gribbles, Vic via PIRSA	AAHL Vic-1	AAHL Vic-2	NFRL USA	DPI NSW	MAFF NZ	DPI&F QLD	DPIW Tas
Penicillium sp	13	N	N	N	N	N	N			N	N
Pythium sulcatum	22	N	N	Not sent	N	N	N			N	Not sent
A. invadans (reference strain)	24	++++	++++	++	++++	++++	++++			++++	++++
A. frigidophilus	26	N	N	N	N	N	N			N	N
Saprolegnia sp	33	N	N	N	N	N	N			N	N
A. invadans, (NJM9701 obtained from Thailand	45	++		N	Faint non-specific band	N	N			N	N
Aspergillus niger	48	N	N	N	N	N	N			N	N
Saprolegnia diclina	50	N	N	N	N	N	N			N	N
Achlya diffusa	51	N	N	N	N	N	N			N	N
S. salmonis/parasitica	59										
Positive control		++		++++	++++	++++	++++			++++	++++
Negative control		N	N	N	N	N	N			N	N

Code used in kit refers to the culture identification number; AHL, WA = this laboratory, Animal Health Labs, Dept of Agriculture and Food Western Australia; AAHL = Australian Animal Health Laboratory, CSIRO Vic; DPI & F QLD = Dept of Primary Industries and Fisheries Queensland; DPIW, Tas = Dept of Primary Industries Water and Environment Tasmania; PIRSA (Dept of Primary Industries and Resources South Australia) sent kit to Gribbles Victoria; NFRL USA = National Fish Research Laboratory; ID = identification; + indicates positive reaction; ++ indicates moderate intensity of stained amplified product on gel; ++++ indicates strongly staining amplified product on gel; N = negative reaction.

Correct results were obtained by all laboratories that returned results. All laboratories obtained a strongly staining amplicon for the coded *A. invadans* (#24) and for the *A. invadans* positive control. Cross-reactions were not reported from other fungi, such as other oomycetes, *Saprolegnia* and *Pythium* species, or the other *Aphanomyces* isolates in the test kit. The PCR was robust when evaluated by inter-laboratory testing.

The laboratories returned information on the assay conditions. Both TAE and TBE buffers were used, the amount of loading buffer and product loaded into the gel varied between laboratories. Laboratories also used run time and conditions different to those in the kit. However, all these variables did not affect the outcome of the results. Although the information on the type of thermocycler was not requested, some laboratories provided this information. The use of different machines did not affect the results. This is good information as there have been reported instances of different thermocyclers impacting on the outcome of a PCR (Wilhelm *et al.*, 2000). The reproducibility of the results on different machines

indicates that the primers for *A. invadans* are sufficiently robust to cater for slight differences in ramping time and block temperature reliability that may be encountered on some machines. The PCR was optimised using a Corbet FTS 320, an older model thermal cycler machine, therefore the newer thermal cyclers used by the validating laboratories should be more accurate than the older Corbet.

The NFHRL, USA tested three extra isolates that they had in their laboratory and one isolate (Wicomoco isolate of *A. invadans* from menhaden) was positive, whereas the others were negative. No details were given for the latter isolates.

7.4.2 Complete trial of PNA-FISH for A. invadans at participating laboratories

The PNA-FISH method and reagents for *A. invadans* was sent to DPI NSW at the Elizabeth Macarthur Agricultural Institute (EMAI). The instruction and results sheet that were sent with the PNA-FISH kit are attached in Appendix H.2.

The types of samples, case number and fungal identification with probe type which were sent in the kit are detailed in Table 7-8.

Table 7-8. Samples sent to validating laboratory

Tube	Treatment	Case Number	Identification	Probe	Expected Results
1	PE	AS-07-0821 Yx	*A. astaci*	Ainv-FLU3	-ve
2	PE	AS-07-0189	*A. invadans*	Ainv-FLU3	+ve
3	PE	AS-07-0101	*A. invadans*	Ainv-FLU3	+ve
4	PE +ve crl	AS-04-1316	Negative control	Eu-Uni-1	+ve
5	PE –ve crl	AS-04-1316	Negative control	No probe	-ve
6	Culture	AS-07-0101	*A. invadans*	Ainv-FLU3	+ve
7	Culture	AS-07-0189	*A. invadans*	Ainv-FLU3	+ve
8	Culture	AS-06-1928	*S. diclina* (#50)	Ainv-FLU3	-ve
9	Culture	AS-04-1735	*S. salm/para* (#59)	Ainv-FLU3	-ve
10	Culture	AS-06-1928	*A. diffusa* (#51)	Ainv-FLU3	-ve
11	Culture +ve crl	AS-07-0101	*A. invadans*	Eu-Uni-1	+ve
12	Culture –ve crl	AS-07-0101	*A. invadans*	No probe	-ve

Crl = control; PE = paraffin-embedded; *S. salm/para* = *Saprolegnia salmonis/parasitica*; +ve = positive; -ve = negative.

The validating laboratory returned the results with comments as detailed in Table 7-9.

Table 7-9. Results as received from validating laboratory

Tube	Treatment	Results	Magnification	Notes
1	PE	Negative	40	Nothing observed on slide
2	PE	Positive	40	Not very much evidence of fungi but some short strands of hyphae with evidence of bright green fluorescence in patches
3	PE	Negative	40	Nothing observed on slide
4	PE +ve control	?	40	Bright green glowing mass but did not look like any sort of fungal growth looked more like agar
5	PE -ve control	?	40	Similar to positive control but less intense. Pale green/yellow glowing mass but did not look like any sort of fungal growth looked more like agar
6	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems
7	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems
8	Culture	Positive	40	Hazy cloud like mass with evidence of bright green fluorescence patches particularly at the outer extremes
9	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems

Table 7-10. Summary of expected results and results returned from the validating laboratory

Tube	Treatment	Case Number	Identification	Probe	Expected results	Reported results
1	PE	AS-07-0821 Yx	*A. astaci*	Ainv-FLU3	-ve	-ve
2	PE	AS-07-0189	*A. invadans*	Ainv-FLU3	+ve	+ve
3	PE	AS-07-0101	*A. invadans*	Ainv-FLU3	+ve	-ve
4	PE +ve crl	AS-04-1316	Negative control	Eu-Uni-1	+ve	?
5	PE –ve crl	AS-04-1316	Negative control	No probe	-ve	?
6	Culture	AS-07-0101	*A. invadans*	Ainv-FLU3	+ve	-ve
7	Culture	AS-07-0189	*A. invadans*	Ainv-FLU3	+ve	-ve
8	Culture	AS-06-1928	*S. diclina* (#50)	Ainv-FLU3	-ve	+ve
9	Culture	AS-04-1735	*S. salm/para* (#59)	Ainv-FLU3	-ve	-ve
10	Culture	AS-06-1928	*A. diffusa* (#51)	Ainv-FLU3	-ve	-ve
11	Culture +ve crl	AS-07-0101	*A. invadans*	Eu-Uni-1	+ve	Occ +ve
12	Culture –ve crl	AS-07-0101	*A. invadans*	No probe	-ve	-ve

The returned results were as expected for two of six positives, and for four of six expected negatives. Interpretations were unable to be made for two samples. These were the universal positive control (tube 4) and the no probe control (tube 5) both for paraffin-embedded sections.

7.5 Analyse feedback from participating laboratories on PCR tests and PNA-FISH

7.5.1 Analysis of feedback from A. invadans PCR

All laboratories returned correct results and there were no problems encountered with the technique. DPI&F QLD and AAHL, VIC made comments that they didn't like the DNAzol extraction reagent. DPI&F found the reagent didn't give a template that was free from contaminating proteins and organics, and so were unable to assess the viability of the template. They commented on shearing of the amplicon as being due to the DNAzol extraction reagent, but as the gel was run at high voltage (220v) for only 10 minutes this may have been the cause of shearing rather than the extraction reagent.

Gribbles pathology found that the DNAzol reagent left a particulate suspension in some extractions, which made the spectrophotometer readings unreliable, but commented that the actual procedure was effective.

In project FRDC 2001/621 nine of the most common DNA extraction kits including manual DNA extraction methods were tested for feasibility of extracting DNA from oomycete fungi. The commercial kits that were tested were the Qiagen DNeasy mini plant tissue kit, the DNAzol reagent (Invitrogen), Puregene DNA extraction kit (Gentra), Instagene mix (Bio-Rad), PrepMan (Applied Biosystems), and the manual methods of boiling and grinding in liquid nitrogen. These methods also included the E.Z.N.A plant DNA miniprep kit which was recommended by the manufacturer as being suitable for oomycete fungi. No DNA was obtained using this kit. Some kits did not produce any DNA. Fungi can be difficult to extract DNA from because they are similar to plants in that they have chitin, glucans and other polymers in their cell wall that produces extensive cross-linking which may be difficult to disrupt. Often mechanical disruption of the cell wall is recommended. Only the DNAzol reagent (Invitrogen) and the Qiagen DNeasy mini plant tissue kit produced reliable, unsheared DNA.

DPIW, Tasmania, commented that the protocol was simple and easy to follow and requested a temperature be given when hydrating the DNA in the DNA extraction protocol.

Many laboratories were concerned that they had not achieved the correct results as only a small amount (25 mg) of starting material was given with the kit, and there were a number of tubes to test but only one positive result in addition to the positive control. Our main concern was to test the reliability of the specificity of the primers, therefore most of the samples in the kit were from other oomycete fungi and true fungi that may have produced cross-reaction. However, the PCR primers were robust and no laboratories reported non-specific products either at the molecular weight expected for *A. invadans* or at any other molecular weight. The primers, therefore, were specific when tested under a variety of conditions existing at other laboratories.

7.5.2 Analysis of feedback from A. invadans PNA-FISH

The results of the validation of the PNA-FISH method at another laboratory indicate that further validation and optimisation is needed for the PNA-FISH method. As found in our laboratory for some samples the results were difficult to interpret. This is in part due to the nature of the fungus and partly due to the type of sample. This is discussed in 8.5.2.

7.6 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedure and FRDC Report

An ANZSDP has been written and is attached at the end of this report (Appendix K).

8. DISCUSSION

8.1 Complete validation of *A. invadans* PCR using fresh tissue and wax-embedded tissue

Primers AIF14 + AIR10 are suitable for direct detection of *A. invadans* in fresh fish tissue or lesion material.

Other oomycete fungi, true fungi and bacteria may also be present in a lesion and it is important that the PCR primers are tested for specificity against these organisms. Primers (AIF14 + AIR10) designed in project FRDC 2001/621 were further tested for specificity against bacteria that may be present in a lesion as secondary invaders or on fish skin. Results showed that the primers were specific when used against these bacteria demonstrating that a false positive result would not be produced when any of these bacterial species are present. These primers were specific for only *A. invadans* when tested against other oomycete fungi such as *Achlya* species and *Saprolegnia* species, and when tested against true fungi such as *Penicillin* and *Aspergillus* species that are often present as plate contaminants. The primers did not show cross-reaction when tested against *Trichoderma* species and bacteria such as *Vibrio* species and *Aeromonas* species that may be present on fish tissue or secondary invaders in lesions. It can be difficult to isolate *A. invadans* from lesion material because of the overgrowth by invading bacteria and other fungi. *Aphanomyces* species do not compete well against other fungi and bacteria, therefore using the PCR direct on fish tissue is the best test for achieving a specific diagnosis of EUS. The presence of appropriately sized hyphae that are morphological suggestive of *A. invadans* in histological sections is a good method for diagnosis of EUS but may not be specific in inexperienced hands.

One case of decomposed tissue sent from NSW did not test positive by PCR, although a fresher sample from the same outbreak did test positive. Therefore, it is important to use the PCR on tissue before it decomposes to the stage that the fungus is destroyed during the decomposition process.

Primers AIF14 + AIR10 were compared to primers published during the course of this project. The results confirmed that primers AIF14 + AIR10 were more specific and sensitive in comparative assays. The primers could be used at two annealing temperatures and still produce amplicon at high concentration, where as other primers operated in a very a narrow annealing temperature and outside this temperature non-specific amplification could occur or produce an amplicon of reduced concentration. Other primers had a reduced performance at their reported annealing temperature. These factors are important because heating blocks on thermocyclers may experience temperature drift, or have a slow ramping rate, which could result in reduced performance of the test. Therefore, primers that are robust under various conditions are required for an optimal diagnostic test.

The results completed in this project confirm that the primers are specific and when used for direct detection on fish tissue or on cultured hyphae, are the most specific method for the diagnosis of EUS.

Primers (AIF14 + AIR10) designed in project FRDC 2001/621 were further tested for suitability using formalin-fixed paraffin-embedded material. The primers successfully amplified DNA from *A. invadans* from cultured hyphae or direct on fresh fish tissue. The primers were not successful in amplifying DNA from formalin-fixed tissue in paraffin-embedded sections. The amplified product produced from these primers is 554 base pairs and is borderline for a molecular weight that may not amplify due to cross-linking of DNA by formalin (Hamatani *et al*., 2006). A fourth DNA extraction method using high heat and an alkaline retrieval buffer was tested against cultured hyphae prepared as formalin-fixed,

paraffin-embedded material. High heat and alkaline buffer is said to remove the cross-linkages of the DNA thereby allowing amplification (Shi *et al*., 2004), but this was not apparent with the material tested in this project.

Other primer pairings (AIF14 + AIR15 and AIF14 + AIR16) designed in the course of project FRDC 2001/621 were not as specific as AIR14 + AIR10 but produced a small amplicon of approximately 100 base pairs. These primers were tested against formalin-fixed paraffin-embedded tissue but no product was amplified despite the tissues being positive for *A. invadans* by histological examination. Under these testing regimes it would appear that the product size of 554 base pairs produced by the specific primers AIF14 + AIR10 was not a factor in the non-amplification of *A. invadans* DNA from formalin-fixed paraffin-embedded tissue. Three DNA extraction methods were trialled, but none produced DNA that could be amplified from this material. Formalin cross-links DNA thus preventing the primers from successfully amplifying DNA from formalin-fixed material. It is generally thought that cross-linked DNA can still be amplified if primers produce a small product or amplicon. However, this was not the case for *A. invadans* material fixed in formalin. Recent work suggests using particular buffers in the DNA extraction process to improve the success rate of amplification from formalin material by reversing the cross-linking (Hamatani *et al*., 2006). A quick DNA extraction protocol using an alkaline buffer was investigated at the end of this project, but there was insufficient time in the project to optimise this method but it is hoped that this may be done in the future.

8.2 Complete research to develop a specific PCR for *A. astaci* (crayfish plague)

Four (of the 13) primer pairs developed in this project were suitable for the detection of *A. astaci*, but one primer pair (AAF 21 + AAR25) gave the best result. This primer pair produced a small amplicon but was unable to detect DNA from paraffin-embedded tissue. During the course of this project primers (525f + 640r) were published for the detection of *A. astaci* (Oidtmann *et al*., 2004). These primers had the advantage that they were able to detect *A. astaci* from formalin-fixed paraffin-embedded tissue as tested in this project. In addition they were at least 10-fold more sensitive than AAF 21 + AAR 25, however they had the disadvantage of amplifying a weak product from *A. invadans* and *A. frigidophilus*. To date *A. invadans* has not been reported from freshwater crayfish. *A. frigidophilus* has been implicated as a possible opportunist or contaminant in crayfish with suspect crayfish plague and has only been reported recently in Europe where crayfish plague occurs (Ballesteros *et al*., 2006). Previously, the only report of *A. frigidophilus* was from Japanese char eggs (Kitancharoen and Hatai, 1997).

Recently a newly described species, *Aphanomyces repetans*, was reported from freshwater crayfish (Royo *et al*., 2004). This species will also need to be included in specificity testing.

8.3 Complete research to develop FISH tests for *A. invadans* and *A. astaci*

The FISH for *A. invadans* has been completed for use on fresh cultured hyphae and can also be used on paraffin-embedded material, although fluorescence is reduced. The procedure was adapted from a recently published paper (Vandersea *et al*., 2006) in which a peptide nucleic acid (PNA) probe (Ainv-FLU3) was designed for *A. invadans*. In the published results culture material and fish tissue were tested, but not paraffin-embedded material. In our laboratory the PNA probes produced a bright strong fluorescence using the PNA-FISH technique for *A.*

invadans from fresh cultured hyphae. The fluorescence was reduced when tested on paraffin-embedded material from diagnostic cases, and further optimisation needs to be done to ensure complete confidence with this method when used on paraffin-embedded material.

The FISH using the PNA probe for *A. invadans* (Ainv-FLU3) produced sensitive and specific results from material using freshly cultured fungal hyphae. Results were variable when frozen stored hyphae were used, or from paraffin-embedded material. Fluorescence in fewer hyphae was seen from these samples. Some hyphae showed fluorescence as clumps along the hyphal filament.

Because it is an exotic disease no fresh culture material could be obtained for testing the *A. astaci* (Aastaci-FLU) PNA probe developed during this project. The probe was tested against non-viable culture material and paraffin-embedded tissue. The results were variable with similar results on quality of fluorescence as seen for the *A. invadans* probe when tested on stored hyphae or paraffin-embedded material.

Peptide nucleic acid probes are neutrally charged synthetic probes with a pseudopeptide backbone that mimics the structure of DNA (and RNA). That is, the sugar phosphate backbone of natural nucleic acid is replaced by a synthetic peptide [N-(2-amino-ethyl)-glycine units]. They have a higher melting temperature per base pair than do conventional probes, are resistant to enzymatic cleavage, and form strongly binding complexes with DNA or RNA that have increased specificity. PNA probes are said to be more specific than using an oligonucleotide with a fluorescein molecule attached at the 5 prime end. The increase in specificity was confirmed in this project, as previously (FRDC 2001/621) a fluorescein-conjugated oligonucleotide probe was not specific when tested against other oomycete fungi.

It has been claimed that a single freeze thawing of the fungal hyphae can result in loss of RNA and therefore the fluorescent signal would be reduced (Vandersea *et al*., 2006). This loss of fluorescence was confirmed in this project with a reduction in fluorescent signal observed when frozen material was used compared with freshly cultured hyphae. Likewise reduced signal was seen in hyphae in paraffin-embedded sections. The formalin fixation process cross-links amino groups or cytosine residues so that tissue is preserved, but this process then causes reduced binding of the probe to the RNA target. Further work needs to be done in order to optimise the pH of buffers to reverse cross-linking, to improve probe penetration, and allow further investigations of hybridisation temperature in order to improve the signal from paraffin-embedded material.

8.4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

8.4.1 Complete trial of PCR for A. invadans at participating laboratories

The main objectives for sending the kit to other laboratories was to (1) determine that the PCR was reproducible in other laboratories, (2) determine that the primers remained specific under different laboratory conditions (e.g. equipment and personnel) and (3) obtain feedback on the technique and in particular the DNA extraction method.

The results returned from the participating laboratories showed that the PCR was reproducible and specific in other hands using their equipment and personnel, but using reagents supplied by this laboratory. The returned results also showed that the primers were specific in other hands. The oomycete fungi are very similar in several regions along their DNA such as the 16S rRNA, 18S rRNA and the internally transcribed spacer regions (ITS). The primers were designed from the ITS region as this area shows the greatest variation in DNA sequence. The kit included a variety of oomycete fungi, laboratory contaminants and fungi that may

contaminate a lesion on a diseased fish. The results showed that the primers were specific when used in other laboratories and using different thermocyclers. Some cultures of *A. invadans* that had been sent to us from AARI, Thailand, did not amplify with the *A. invadans* primers, and some had growth morphologies that were not consistent with *A. invadans*. These anomalies were reported in FRDC 2001/621. Culture *A. invadans* NJM9701 was one such culture and so was included in the kit to determine if amplification occurred in other laboratories. No laboratory reported amplification of NJM9701, and we conclude as previously in FRDC 2001-621 that some of the cultures purported to be *A. invadans* may no longer be a true culture, or rather the culture we received is no longer *A. invadans*. Recent results from sequencing of the 16S rRNA gene showed that the culture of NJM9701 that we received from Thailand was not *Aphanomyces* or any similar fungus.

The PNA-FISH was trialled at one Australian laboratory. Results were variable and reduced fluorescence was observed. Some of the samples in the kit were stored frozen hyphae or paraffin-embedded material, and it is thought that this type of sample contributed to the variable results. It was noted that one freeze thaw cycle of the fungal hyphae could cause a reduction in the fluorescence of the PNA probe (Vandersea *et al*. 2006). This needs further investigation to ensure all samples are suitable for PNA-FISH.

8.5 Analyse feedback from participating laboratories on PCR tests and PNA-FISH

8.5.1 Analysis of feedback from A. invadans PCR

Overall the PCR was performed easily and successfully in other laboratories, and should easily be incorporated into these laboratories and others as a routine test. The PCR was specific and robust and this was proved by validation in other laboratories.

Some laboratories were dissatisfied with the DNAzol DNA extraction method. Another alternative DNA extraction reagent that successfully extracts DNA from oomycete fungi is the DNeasy mini plant tissue kit from Qiagen. This was not sent to the participating laboratories, but should provide an extraction method that provides a more pure form of DNA than the DNAzol extraction reagent. There were no other major concerns from the participating laboratories.

The PCR for *A. invadans* for use on fish tissue and culture material is suitable for incorporation into laboratories as a routine diagnostic test specific for EUS.

8.5.2 Analysis of feedback from A. invadans PNA-FISH

Variable results were obtained for the PNA-FISH. This means that further optimisation of the method needs to be done to develop a robust and reliable as a diagnostic method. One of the main problems is the oomycete fungus itself. As the fungus ages the DNA in the hyphae becomes scattered along the hyphae. The most active part of the hyphae is at the tips and is the area recommended for selecting for subculture. Young active hyphae are packed with dense coarse granular cytoplasm. Older hyphae have a vacuolated cytoplasm which may be restricted to the periphery, and very old hyphae can be totally devoid of contents (Alderman and Polglase, 1986). This means that as the hyphae age less DNA will be present in the hyphae. This was shown in this project where the PNA-FISH produced very bright fluorescence, which appeared solid throughout the hyphae from a young actively growing culture. In paraffin-embedded sections fluorescence was reduced and was seen as spots or clumps along the hyphae. This reduction in fluorescence may also be due to the problems associated with formalin fixation, which causes cross-linking of amino acid groups especially cytosine (Srinivasan *et al*., 2002).

The PNA probe binds with high efficiency and strong bonds, more so than an oligonucleotide primer. However, fixation buffers and hybridisation buffers need to be optimised to ensure penetration of the probe through the cell wall to the DNA. Further work needs to be done to optimise the PNA-FISH for paraffin-embedded tissue.

8.6 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedure and FRDC Report

A draft has been submitted with this report (see Appendix K).

9. BENEFITS

PCR for EUS (A. invadans)

The major benefits of the project are the development and validation of the PCR for the detection of *A. invadans* in fresh cultured hyphae and direct from fish tissue to assist in the diagnosis of epizootic ulcerative syndrome (EUS).

EUS caused by the fungus *Aphanomyces invadans* affects freshwater and brackish water 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 a DNA sequence that is specific to *A. invadans*, rather than the subjective diagnosis by observation of clinical signs and histopathology that is reliant on the expertise of the pathologist. Rapid and accurate diagnosis of EUS will be of benefit to 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 mycotic granulomatosis (MG) in Japan, a rapid test will assist in preventing strains of unknown virulence from entering Australia.

The method has been sent to participating laboratories in “kit” form as part of the validation trial conducted in this project. All laboratories returned correct results and, therefore, should have no problems implementing the test into their laboratories. The method has already been sent to EMAI and is available to all diagnostic laboratories. The method will be available to Australian and New Zealand laboratories once the Australian and New Zealand Standard Diagnostic Test Procedure has been reviewed.

Implementation of the PCR for the detection of EUS will greatly improve diagnostic capacity for laboratories. A number of laboratories do not have the required skill level for culture of the fungus. One of the problems is the spasmodic nature of the disease, so that in some areas the disease is rarely seen and staff may not develop sufficient experience. Nowadays most laboratories have molecular biology facilities and the PCR will enable all laboratories with these facilities to improve their diagnosis of the disease.

The research showed that the fungus is difficult to culture because it is easily overgrown by contaminating bacteria and fungi and requires moribund or recently dead fish for successful culture. The PCR overcomes the problem of contaminating bacteria and fungi, and will produce a successful result provided the tissue is not autolysed.

The PCR test is more specific than histopathology, which detects fungal hyphae in the tissues but unless the pathologist has the experience may incorrectly report *Aphanomyces*. Other oomycete fungi such as *Saprolegnia parasitica* can cause disease or opportunistic infection in some fish species and an incorrect diagnosis of EUS may be made. The PCR will complement histopathological diagnosis.

The work carried out in this report examined a number of DNA extraction procedures and found that many were unsuitable for oomycete fungi. The two successful DNA extraction

procedures were both suitable for culture and tissue material and are easy and quick to perform. Other procedures in the test such as optimal annealing temperature, concentration of reagents etc were validated. Almost all veterinary diagnostic laboratories are accredited and have quality assurance programs. The work undertaken in this report reduces the validation that individual laboratories will need to do to implement the test. All reagents are available commercially, including the PCR master mix, which is one of the cheapest available.

A peptide nucleic acid probe for use in the fluorescent *in situ* hybridisation method was tested in this report. The probe is specific for *A. invadans* and is best performed on culture material. A PNA probe will provide a rapid test to confirm culture growth as *A. invadans*. Identification of the fungus requires sporulation tests to be done and this can take several days and requires expertise with mycology, in particular, the oomyctes. The PNA probe for use on fresh cultures will enable a definitive identification to be made within the first few days of appearance of hyphae.

The PNA FISH method was not done on fish tissue, and variable fluorescence was detected on paraffin-embedded sections. Further work needs to be done before the test can be used for PE sections and fish tissue.

10. FUTHER DEVELOPMENT

Some of the objectives for this research were achieved, that is, the development of a specific PCR for the detection of *A. invadans* from culture and from fresh fish tissue. However, a PCR for the detection of the fungus in formalin-fixed paraffin-embedded tissue was not successful and further work needs to be done to achieve this. The primers produce an amplicon (554 bp) that is probably too large for amplification from DNA that has been cross-linked as part of the tissue preservation process. Research needs to be undertaken to investigate DNA extraction procedures using buffers and conditions that can uncross-link the DNA. Other methods of fixation (for example ethanol fixation) for preservation of tissues that retain the DNA in a form suitable for amplification need to be investigated.

A peptide nucleic acid probe for use in fluorescent *in situ* hybridisation for the detection of *A. invadans* was successful on freshly cultured hyphae, but further research needs to be done to optimise the technique for formalin-fixed paraffin-embedded samples and fresh tissue. This is also the case for the PNA-FISH technique for *A. astaci* (crayfish plague).

The PCR for *A. astaci* developed as part of this project need to be further validated against primers published in 2004 and 2006. The project primers are specific but not as sensitive as the 2004 primers. The 2004 primers are not as specific but can amplify DNA from paraffin-embedded tissue. The 2006 primers are specific but work needs to be done to determine if they can amplify DNA from paraffin-embedded tissue and sensitivity needs to be done. It is hoped this work can be completed, although it will be after the project has been completed.

A group in Finland have designed PCR primers for the detection of *A. astaci* using a real time protocol. Field trials beginning in August 2007 will be used to validate the PCR (pers comm, Dr Satu Satu Viljamaa-Dirks, Finnish Food Safety Authority Evira, Kuopio Research Unit). We will continue contact with this group and further research will be necessary to determine which PCR will ultimately be the most sensitive and specific for the detection of crayfish plague from a number of different sample types.

A PCR and a PNA-FISH for each fungus that works on paraffin-embedded material will complement the tests already developed. It will allow retrospective studies to be done on archive material. For current and future samples a PCR and PNA-FISH will confirm the identity of the fungus that is seen in these histology sections.

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 and a specific PNA-FISH method for use on fungal material has contributed to the planned outcomes. The FISH using an oligonucleotide probe was greatly improved by using a peptide nucleic acid probe, which proved to be more specific than the oligonucleotide probe. These rapid diagnostic tests 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 Complete validation of *A. invadans* PCR using fresh tissue and wax-embedded tissue

- 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.
- 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 PCR primers were unable to detect *A. invadans* in paraffin-embedded samples.

12.2 Complete research to develop specific PCR for *A. astaci* (crayfish plague)

- Of the thirteen primer pairs designed in project FRDC 2001/621 to the virulence genes of *Aphanomyces astaci*; putative chitinase gene chi1, subtilisin-like serine proteinase precursor (SP1) gene, trypsin proteinase precursor (SP2) gene, six pairs produced strongly staining amplicon.
- The primers designed to the virulence genes were not as sensitive as primers published by Oidtmann *et al*., (2004, 2006).
- No amplification of DNA from formalin-fixed tissue was achieved using the primers designed to the virulence genes.
- Primers designed by Oidtmann *et al*., (2004) with a product size of 115 bp successfully amplified DNA from paraffin-embedded material, but were not specific because they also produced product from *A. invadans* and *A. frigidophila*.
- Primers designed by Oidtmann *et al*., (2006) were specific (although produced non-specific bands at other molecular weights) but have not been tested on paraffin-embedded tissue.
- DNA extraction could be achieved from formalin-fixed tissue using the Qiagen DNeasy mini plant kit. Other extraction methods were not suitable for oomycete fungi.

12.3 Complete research to develop FISH tests for *A. invadans* and *A. astaci*

- A peptide nucleic acid probe was specific for *A. invadans* (Vandersea *et al*., 2006) when used in a fluorescent *in situ* hybridisation, particularly on fresh cultured hyphae.
- A peptide nucleic acid probe was designed for detection of *A. astaci* in paraffin-embedded tissue using a fluorescent *in situ* hybridisation procedure.
- Fluorescence was diminished if fungal hyphae were frozen and thawed more than once.

- Fluorescence was diminished when tested on non-viable or aged fungal hyphae.

12.4 Complete trial of PCR and FISH tests at Australian and overseas laboratories

- The PCR for the detection of *A. invadans* from cultured hyphae was trialled at five Australian and two international laboratories
- The PNA-FISH test for the detection of *A. invadans* from cultured hyphae and paraffin-embedded tissue was trialled at the Elizabeth Macarthur Agricultural Institute, DPI NSW

12.5 Complete writing of method for the Australian and New Zealand Standard Diagnostic Procedures

- A draft ANZSDP for EUS has been completed and is attached in Appendix K.
- A draft ANZSDP for crayfish plague was commissioned from N. Buller by NAAH-TWG. A draft has been through an editing and review process and the finalised document was submitted in October 2007 to NAAH-TWG.

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Appendix A Intellectual Property

No intellectual property exists for this project and the information and methods are freely available to all as this was the intention for the funding of the development of improved diagnostic tests.

Appendix B Staff List

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

Appendix C Formulae of Buffers, Reagents, and Media

Appendix C.1 Buffers

Appendix C.1.1 PNA-FISH method

Fixative buffer one

95% ethanol	44 ml
deionized water	10 ml
25 x SET buffer	6 ml

Fixative buffer two (for use on tissue)

95% ethanol	44 ml
deionized water	10 ml
25 x SET buffer	6 ml
Tween 20	1.8 ml (3%)

25 x SET Buffer

NaCl (3.75M)	219.15 g
EDTA (25mM)	7.31 g
0.5M Tris HCl (HCl)	78.8 g
DW	1000 ml
pH 7.8	

Hybridization buffer one

5 x SET buffer	100 ml
Igepal-CA630 (0.1% vol/vol)	100 µl
polyA potassium salt (Sigma)	2.5 mg

Hybridization buffer two

Hybridisation buffer one	0.5 ml
20 nM PNA probe	0.5 µl

Hybridization buffer three

Hybridisation buffer one	97 ml
3% Tween 20	3 ml

T-röd fixative from Kemetyl used for fixation of crayfish plague infected material from Sweden

Reagent	**Concentration**
Ethanol	60-100 %
ethyl methyl ketone (2-butanone)	5-10%
acetone	1-5 %
ethyl acetate	1-5 %
denatonium benzoate (Bitrex)	0-1 %

Appendix C.2 Media

Appendix C.2.1 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

Appendix C.2.2 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 Methods for isolation of *Aphanomyces invadans*

Appendix D.1 Culture method for *Aphanomyces invadans*

Materials

GP or IM agar media (in 9 mm Petri dishes).
Sterile washer
Incubator set 20-25ºC.

Method

Select small (2-5 mm) pieces of tissue from the active edge of a lesion and embed into the surface of the agar plate. Do not dry the surface of the plates too much (just enough to get rid of surface droplets), as Oomycete fungi prefer a moist environment and can be destroyed by drying, or won't grow on a dry surface.
Invert plates and incubate at 20-25ºC for 7-10 days. Growth should start to appear after 2-3 days.
Examine plates daily for signs of hyphae. Use a stereomicroscope to look for first signs of growth and subculture as soon as possible to prevent the overgrowth of contaminating bacteria and fungi.
It may be helpful to place the original piece of tissue inside a sterile washer (Figure C-1). *A. invadans* grows within the first 2-3 mm of the agar surface and can grow under the washer and through the agar and away from the contaminants.
A procedure (Willoughby and Roberts, 1994) for isolation of *A. invadans* is given in the Appendix.

Figure D-1. Growth of *Aphanomyces* (*A. invadans*) demonstrating the use of a sterile washer to ensure growth of contaminating *Aspergillus* species is contained within the centre of the washer.

Appendix D.2 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 continuum 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.

Appendix E List of suppliers for reagents

Reagent	Supplier	Catalogue number
DNA ladder 100 bp	Geneworks	DMW-100L
HyperLadder IV 100 bp ladder. Quantitative DNA ladder	Astral Scientific	BioLine BIO-33029
DNAzol DNA extraction reagent	Invitrogen Life Technologies	1050-027
Qiagen Dneasy mini plant kit	Qiagen	69104
Promega PCR Master Mix	Promega	M7502 ($95)
PNA probes	Panagene	

Geneworks
PO Box 299
Hindmarsh SA 5007
1800882555

Panagene
100 Sinsung-dong
Yuseong-gu
Daejeon
305-345 Korea
ph: +82-42-861-9295
Fax: +82-42-861-9297

Promega
PO Box 168
Armidale NSW 2038
1800225123
Fax: 02 95504454

Qiagen Australia
PO Box 641
Doncaster VIC 3108
1800243800

Invitrogen Life Technologies
2A/14 Lionel Rd
Mount Waverley VIC 3149
03-8542 7400

Appendix F Results for further validation of *A. invadans* primers AIF14 + AIR10

Appendix F.1 Specificity testing of primers AIF14 + AIR10 and ITS 11 + ITS 23 using bacteria that may be present in a lesion

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

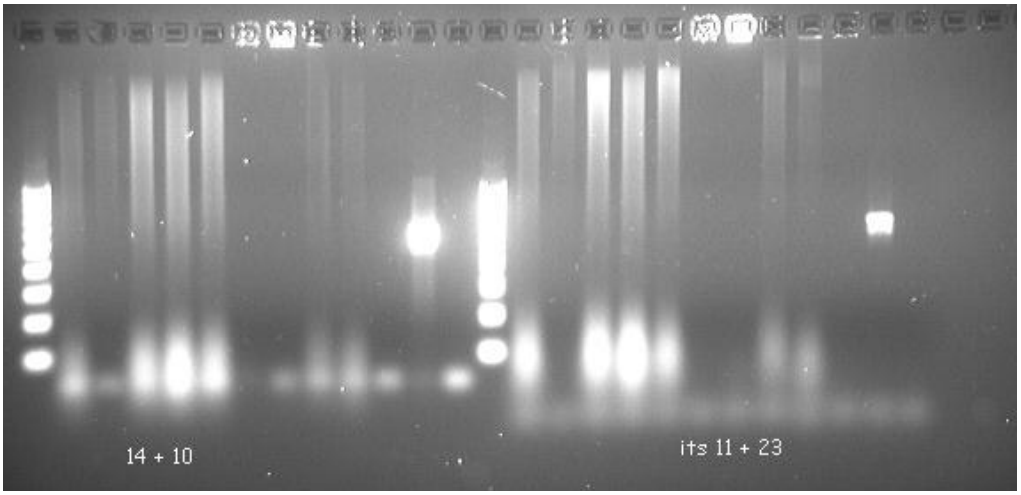

Figure F-1. Results of specificity test of *A. invadans* primers AIF14 + AIR10 (Lanes 2-13) compared to published primers ITS 11 + ITS 23 (Lanes 15-26). Lane 1 = molecular weight marker 100 bp ladder; Lane 2 = *Vibrio alginolyticus*; Lane 3 = *V. cholerae*; Lane 4 = *V. mimicus*; Lane 5 = *Photobacterium damselae* subsp. *damselae*; Lane 6 = *Listonella anguillarum*; Lane 7 = *Trichoderma* sp.: Lane 8 = *Streptococcus iniae*; Lane 9 = *Aeromonas salmonicida* (atypical strain); Lane 10 = *A. hydrophila*; Lane 11 = *S. dysgalactiae*; Lane 12 = positive DNA control; Lane 13 = negative, no DNA control; Lane 14 = molecular weight marker 100 bp ladder; Lane 15 = *Vibrio alginolyticus*; Lane 16 = *V. cholerae*; Lane 17 = *V. mimicus*; Lane 18 = *Photobacterium damselae* subsp. *damselae*; Lane 19 = *Listonella anguillarum*; Lane 20 = *Trichoderma* sp.: Lane 21 = *Streptococcus iniae*; Lane 22 = *Aeromonas salmonicida* (atypical strain); Lane 23 = *A. hydrophila*; Lane 24 = *S. dysgalactiae*; Lane 25 = positive DNA control; Lane 26 = negative, no DNA control

Appendix G Validation trial of *A. invadans* PCR using fungal material

Appendix G.1 Contact and address details for participating laboratories

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E-mail: Jeremy.Carson@dpiw.tas.gov.au
Road Address: Mt Pleasant labs, DPIW, 165 Westbury road Prospect Tas, 7250

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Queensland Department of Primary Industries
180-202 River Boulevard, Oonoonba QLD 4811
PO Box 1085, Townsville QLD 4810
annette.thomas@dpi.qld.gov.au

Nicholas Gudkovs
Experimental Scientist
CSIRO - Livestock Industries
Australian Animal Health Laboratory
5 Portarlington Road
Private Mail Bag 24
Geelong, Victoria, 3220 Australia
email: nicholas.gudkovs@csiro.au

Dr Supranee Chinabut
Aquatic Animal Health Research Institute
Department of Fisheries
Thailand

Dr Colin Johnstone (PIRSA) & Dr Rick McCoy (Gribbles)

Gribbles VetLab, 33 Flemington
Street, Glenside, Adelaide, SA 5065

Suzanne Keeling
MAF Biosecurity New Zealand
Investigation and Diagnostic Centre
Wallaceville, Upper Hutt
New Zealand

Dr David Alderman
CEFAS Weymouth Laboratory,
Barrack Road, The Nothe,
Weymouth, Dorset, DT4 8UB, UK
Tel (44) 1305 206641
Fax (44) 1305 206638

Dr Vicki Blazer and Dr Jeff Shields
National Fish Health Research Laboratory
11649 Leetown Road
Kearneysville, WV 25430 USA

Ian Marsh & Vanessa Saunders
Elizabeth Macarthur Agricultural Institute, NSW DPI
Contact: Ian Marsh Phone02-46-406-502, Fax: 02-46-406-384, E-mail: ian.marsh@agric.nsw.gov.au

Appendix G.2 Instruction sheet sent to participating laboratories

POLYMERASE CHAIN REACTION (*APHANOMYCES INVADANS*)

The PCR involves 3 distinct phases: DNA extraction, DNA amplification and detection and analysis of the reaction product.

KIT CONTENTS:
DNA Extraction:
DNAzol – 10mL labelled McCartney

Table 1 Cultures for extraction (0.2mL tubes):

Label	Colour
13	**BLUE**
22	***Blue***
24	*Green*
26	***Green***
33	*Yellow*
45	**Red**
48	**Purple**
50	**Purple**
51	**Red**
59	Clear

You will need: micropestles (Scientific Specialties Inc), 1.5mL tubes, ice cold 100% and 75% ethanol

DNA amplification
Promega PCR Master Mix – Purple screw lid tube (mmx)
Nuclease-Free Water – Green screw lid tube (H2O)
Forward Primer – Yellow screw lid tubes (F)
Reverse Primer – Orange screw lid tube ®
Positive Control – Red screw lid tube (+ve)
Negative Control – Blue screw lid tube (-ve)
DNA extracted from cultures provided (see Table 1)

You will need: General PCR equipment: thermocycler, 0.2/0.6mL tubes

DNA detection and analysis:
You will need: 2% agarose gel, Ethidium bromide/staining solution, Molecular weight standard for electrophoresis (100 bp increments), Loading buffer for electrophoresis, 0.5M xTBE or 1xTAE buffer, Photography equipment

Results:
Results sheet provided **(see at end of methods)**

METHODS:

DNA Extraction:

There are enough reagents in the kit to repeat the assay or to test your own cultures.

Care should be taken to avoid cross contamination of samples.

DNAzol is used for the isolation of PCR-ready genomic DNA from pure culture. Cultures were grown in GP Broth. The DNA is extracted as detailed below.

1. Remove the ~25mg of fungal hyphae from the 0.2mL tube using flamed forceps and place into a separate sterile 1.5mL labelled tube.
2. 600µl of DNAzol is added to the tube and the tissue is homogenised using a sterile hand-held micro pestle. (Homogenate can be stored for 18 hours at 15-30°C or for 3 days at 2-8°C).
3. Centrifuge for 10 minutes at 13,000rpm. Transfer the supernatant to a sterile labelled 1.5mL tube.
4. Precipitate DNA by adding 400 µl of 100% ice-cold ethanol. Mix by inversion.
5. Centrifuge at 16,060 x *g* (13,000 rpm) for 5 minutes to pellet the DNA. Decant supernatant.
6. Wash pellet with 600 µl of ice cold 75% ethanol. Suspend pellet by gently inverting the tube a few times.
7. Centrifuge for 5 minutes at 16,060 x *g* (13,000 rpm). Decant supernatant.
8. Repeat wash step, decant supernatant.
9. Air dry pellet for a short period of time (too long and the pellet becomes insoluble).
10. Dissolve pellet in either sterile water or 8mM NaOH. (If pellet >2mm diameter add 150 µl of 5mM NaOH, if <2mm add 50 –100µl 8mM NaOH – heating helps dissolve pellet).
11. Quantify DNA.

DNA amplification:

1. Prepare reaction in a pre-PCR clean laboratory. A master mix is prepared according to the total number of tests and controls (Table 2) – 10 cultures, negative and positive control, total of 12 tubes.
2. 23 µl of master mix is placed into a labelled 200 µl thin wall PCR tube.

Table 2. Master mix reaction

Reagent	**X1** (µl)	**X13** (µl)
Promega master mix	12.5	162.5
Forward primer	1.5	19.5
Reverse primer	1.5	19.5
Nuclease-free water	7.5	97.5
Total (µl)	23	299

Add 30 µl of sterile paraffin oil if using a thermocycler that doesn't have a hot lid.

3. Tubes are sealed and labelled.
4. Reaction tubes kept cold and moved to the laboratory area.
5. 2 µl of each culture's eluted DNA is added to each tube. 2µl of positive and negative control DNA samples are added to the appropriate tubes.
6. Tubes are placed into the Thermocycler using the following protocol: denaturation is 95°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 30 seconds, 72°C for 30 seconds a final extension of 72°C for 10 minutes. Hold at 4°C.
7. After the completion of the program, PCR products are separated on the gel to detect DNA fragment of 554 bp .

DNA detection and analysis:

1. Dissolve 2% agarose with either 1x TAE or 0.5M TBE. After melting, ethidium bromide (0.9µl per 45mL gel) can be added if required. Pour melted agarose onto gel tray with comb in place. Allow agarose to set. Remove comb. Wells formed by comb are to be filled with PCR product.
2. Fill electrophoresis tank with 0.5M x TBE or 1 x TAE buffer, ensuring that the entire gel is covered by at least 3 mm of buffer.

3. Mix 5 □L of 2x loading buffer with 5 □L of PCR product and transfer the entire volume to the appropriate well on the gel. Molecular weight markers should be included on all gels for reference.
4. Replace lid to the tank, attach electrodes to the power pack and run at about 90 V for 60 min (until bromophenol blue is at least 40 mm from wells).
5. Remove gel and stain in ethidium bromide at 1mg/mL for 30 minutes (if not previously added to the gel). Examine under UV illumination and photograph.

Results:

DNA fragments are examined and approximate size determined by comparison with the molecular weight markers.

Positive samples: 554 bp amplicon seen on gel.

Negative samples: No DNA fragment seen at appropriate size.

Results sheet sent to participating laboratories

RESULTS:
POLYMERASE CHAIN REACTION (*APHANOMYCES INVADANS*)
DNA Extraction:

Table 1. Cultures for extraction: Results of spectrophotometer reading (if possible)

Label	Colour	Spectro results
13	BLUE	
22	Blue	
24	Green	
26	Green	
33	Yellow	
45	Red	
48	Purple	
50	Purple	
51	Red	
59	Clear	

DNA detection:

Gel Type:
Buffer used:
Loading Buffer used and amounts:
Ethidium in gel/tank:
Molecular weight used and amounts:
Amount of product used:
Volts:
Run time of gel:
Notes:

Results:
Table 2 (please attach copy of gel electrophoresis photo – if possible)

Well	Tube ID	Tube Colour	Result 554 bp
Molecular weight			
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

For Result section (Table 2) use: Positive strong band, positive band, positive weak band, non-specific band, no band, primer-dimer

Any comments on the PCR or kit would be appreciated:

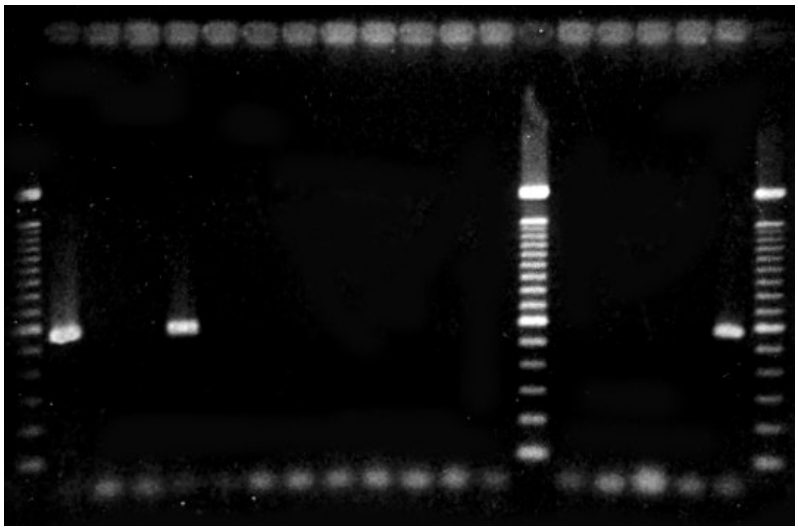
Appendix G.3 Results returned from participating laboratories

Appendix G.3.1 Results returned from Vicki Blazer's lab, National Fish Health Research Laboratory, West Virginia, USA

***Aphanomyces invadans* PCR**

Extracts were prepared from 1) samples provided; 2) four presumptive negative samples (lanes 15-18); and 3) our Wicomoco isolate using the DNAzol procedure as directed. PCR was conducted following the provided protocol using a Hybaid OmniGene thermocycler. A gel photo and sample loading table follow:

Lane	Sample	Lane	Sample
1	100 bp ladder	11	Kit sample # 51
2	Positive control	12	Kit sample # 50
3	Negative control	13	Kit sample # 59
4	Kit sample # 13	14	100 bp ladder
5	Kit sample # 24	15	02-88/2-3
6	Kit sample # #33	16	02-88/2-3
7	Kit sample # 45	17	02-88/2-1
8	Kit sample # 48	18	02-88/2-1
9	Kit sample # 22	19	Wicomoco isolate
10	Kit sample #26	20	100 bp ladder



Appendix G.3.2 Results returned from Suzanne V, from MAF Biosecurity New Zealand, Investigation and Diagnostic Centre- Wallaceville, Upper Hutt, New Zealand

Results from MAF, New Zealand for *A. invadans* PCR testing kit

Well	Tube ID	Tube Colour	Result 554 bp	Expected Result
Molecular weight				
1	Positive Control	Red (screw lid)	Positive band	Positive
2	13	Blue	no band	Negative
3	24	Green	no band	Positive *
4	26	Green	Positive band	Negative *
5	33	Yellow	no band	Negative
6	45	Red	no band	Negative
7	48	Purple	no band	Negative
8	50	Purple	no band	Negative
9	51	Red	no band	Negative
10	59	Clear	no band	Negative
11	NC	Blue (screw lid)	no band	Negative
12	B	NA	no band	Negative

*The results were reversed from those expected, and in discussion with the laboratory it was discovered that the two tubes had been reversed on the gel. It is anticipated that the laboratory would successfully amplify *A. invadans* using their staff and their equipment (thermocycler).

Appendix G.3.3 Results returned from Ian Marsh and Vanessa Saunders, Elizabeth Macarthur Agriculture Institute, NSW

PCR results for *Aphanomyces invadans*

Please note: There was no sample 22 provided in kit.

Test performed at: Elizabeth Macarthur Agricultural Institute, NSW DPI
Contact: Ian Marsh Phone02-46-406-502
Fax: 02-46-406-384
E-mail: ian.marsh@agric.nsw.gov.au

History: Kit arrived at EMAI on Monday 5th March and was stored at 4°C until Monday 19th March. There was concern about the required storage conditions for the Promega PCR Master Mix but we went with 4°C to avoid any chance of freezing/re-thawing. There was no storage conditions provided. However, based on the results for the positive control this appears to have no adverse affect.

The test was run as per the instructions provided with the kit. One minor change was included. After the final wash step with 75% EtOH, the samples were pulsed down and a 200 µL pipette was used to carefully remove remaining EtOH before the samples were air dried. Lane information for each sample is given in Table 1. Lanes 7 and 14 are the molecular weight makers.

DNA quantification: NanoDrop spectrophotometer
Gel type: 2% agarose
Buffer used: 1.0 M TBE
Loading buffer used: 40% sucrose with bromophenol blue
Ethidium in gel or in tank: in gel
Molecular weight used and amount: Roche molecular weight marker VIII, 1.0 µL per lane (lanes 7 and 14)
Amount of amplified product used: 5µL per sample (Figure 1A) approx. 20 µL per sample (Figure 1B)
Volts: 90
Running time of gel: approximately 60 min
Results

Table 1: All results

Sample	ID	Tube Colour	DNA quantification			Electrophoresis	
			A_{260}	Ratio	ng/µL	Lane	Result 554 bp
1	13	Blue	0.076	1.52	3.8	1	No band
2	24	Green	0.045	1.23	2.3	2	Positive band
3	26	Green	0.121	1.33	6.1	3	No band
4	33	Yellow	0.085	1.38	4.3	4	No band
5	45	Red	0.056	1.06	2.8	5	No band
6	48	Purple	0.289	1.71	14.5	6	No band
7	50	Purple	0.993	1.51	49.7	8	No band
8	51	Red	1.734	1.98	86.7	9	No band
9	59	Clear	0.119	1.32	5.9	10	No band
10	PC	n/a	n/a	n/a	n/a	11	No band
11	+ve	n/a	n/a	n/a	n/a	12	Positive strong band
12	-ve	n/a	n/a	n/a	n/a	13	No band

Ratio – A_{260}/A_{280}
PC – process control. DNA extraction procedure without any sample.
+ve – positive control sample red capped tube supplied with kit.
-ve – negative control sample, blue capped tube supplied with kit.
n/a – not applicable.

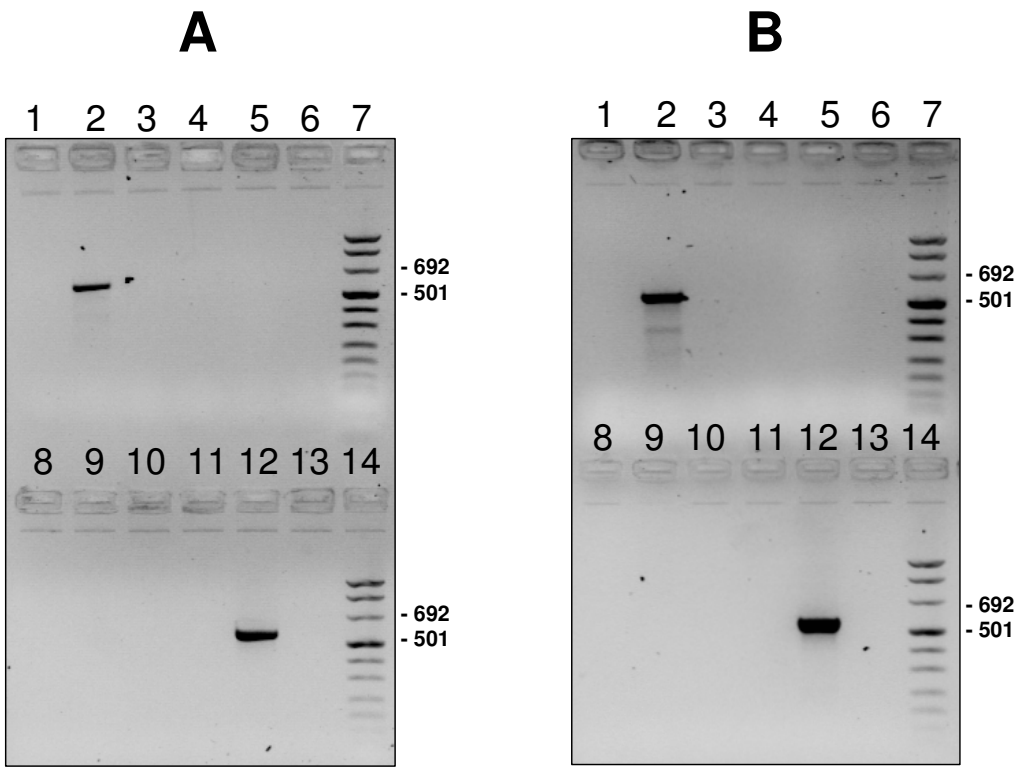


Figure 1: *Aphanomyces invadans* PCR loading 5 µL per sample (A) and 20 µL per sample (B).

Conclusions

We successfully detected only one sample, sample 24 green. The positive and negative controls performed accordingly. We also included a DNA extraction process control (no sample). This was negative as well. The PCR produces a distinct single band when used as described in the kit. A second lower non-specific band can be seen when more amplified product is run on the gel but this can be easily distinguished from the specific product and should not be a problem. Local optimisation should be able to eliminate this further.

Concerns

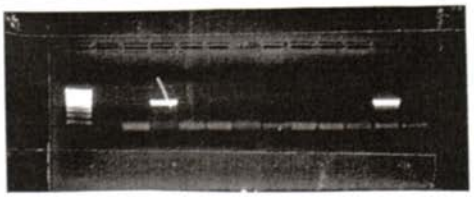
Our major concern with the kit is the DNA extraction procedure. The procedure is a little old school and relatively messy compared to current commercial extraction procedures. Have these been compared with the procedure described in the kit. Matrix-based column extraction may be a little neater. We fear we may have lost some DNA during the washing steps.

Appendix G.3.4 Results returned from Annette Thomas and Jane Oakey, Department Primary Industries and Fisheries, QLD

Results:

Table 2 (please attach copy of gel electrophoresis photo – if possible)

Well	Tube ID	Tube Colour	Result 554 bp
Molecular weight			
1	13	Blue	neg
2	22	Blue	neg
3	24	Green	Pos strong
4	26	Green	neg
5	33	(Yellow)	neg
6	45	Red	neg
7	48	Purple	neg
8	50	Purple	neg
9	57	Red	neg
10	59	Clear	neg
11	+ve		Pos strong
12	-ve.		neg.
13			
14			
15			



For Result section (Table 2) use: Positive strong band, positive band, positive weak band, non-specific band, no band, primer-dimer

Any comments on the PCR or kit would be appreciated:

V. difficult to assess viability of DNA extract from DNAzol because of contaminating proteins & organics. Would have preferred to use a method or kit where concentration & purity could be better determined. At present, can't tell if template material is good enough for PCR, and hence can't determine liklihood of false negatives.

RESULTS:
POLYMERASE CHAIN REACTION (*APHANOMYCES INVADANS*)
DNA Extraction:

Table 1. Cultures for extraction: Results of spectrophotometry (if possible)

Label	Colour	~~Spectro results~~
13	Blue	WEAK, SHEERED
22	Blue	WEAK, SHEERED
24	Green	NEGLIGABLE
26	Green	NEGLIGABLE
33		WEAK, SHEERED
45	Red	PARTIAL SHEERING
48	Purple	PARTIAL SHEERING
50	Purple	PARTIAL SHEERING
51	Red	PARTIAL SHEERING
59	Clear	PARTIAL SHEERING

PREPS ARE NOT SUFFICIENTLY PURE FOR SPECTRO READINGS – HIGH A_{230} and A_{260} VALUES.
1% GEL ELECTROPHORESIS CHECK ON DNA PREPS – SEE COMMENTS.
GEL LOADED WITH 12µl ALIQUOTS SHEERING MAKES ESTIMATION OF CONCENTRATION IMPOSSIBLE.

DNA detection:

Gel Type: DNA GRADE AGAROSE
Buffer used: TBE.
Loading Buffer used and amounts: INHOUSE RECIPE, 6x. USED 2µl.
Ethidium in gel/tank: GEL.
Molecular weight used and amounts: 100bp ladder.
Amount of product used: 10µl.
Volts: 220v
Run time of gel: 10min.
Notes:

Appendix G.3.5 Results returned from Rick McCoy, Gribbles Laboratory on behalf of Colin Johnson, PIRSA, SA

RESULTS:
POLYMERASE CHAIN REACTION (*APHANOMYCES INVADANS*)
DNA Extraction:

Table 1. Cultures for extraction: Results of spectrophotometry (if possible)

Label	Colour	Spectro results µg/mL
13	Blue	3
~~22~~	~~Blue~~	———
24	Green	2.3
26	Green	4.7
33		3.1
45	Red	14.7
48	Purple	6.5
50	Purple	48.3 ?
51	Red	4.5
59	Clear	3.9

DNA detection:

Gel Type: 2% Agarose / TAE
Buffer used: 1x TAE
Loading Buffer used and amounts: Promega 6x, 2µL
Ethidium in gel/tank: Gel
Molecular weight used and amounts: Promega 100 bp ladder, 5µL
Amount of product used: 10µL
Volts: ~170V (150 mAmp)
Run time of gel: ~20 minutes
Notes:

- Very little starting material was provided, in most cases less than 2.5 mg.
- Sample 50 extraction produced large white pellet which when suspended left a particulate suspension (thus the unreliable spectro result)
- the $260/280_{nm}$ ratio for all samples except 24 and 50 were between 1.5 and 1.7.
  - Sample 24 $260/280_{nm}$ ratio = 2.8
  - " 50 " " " = 1.23
- All pellets (some not visible) were resuspended in 50µL of TE except Sample 50 (150µL)

036110

Results:

Table 2 (please attach copy of gel electrophoresis photo – if possible)

Well	Tube ID	Tube Colour	Result 554 bp
Molecular weight	M		
1	13	Blue	~~No Band~~
2	24	Green	Positive band
3	26	Green	No band
4	33	Yellow	No band
5	45	Red	No band
6	48	Purple	No band
7	50	Purple	No band
8	51	Red	No band
9	59	Clear.	No band
10	Positive Control	—	Positive Band strong
11	Negative Control	—	No band
12	H_2O	—	No band
13			
14			
15			

For Result section (Table 2) use: Positive strong band, positive band, positive weak band, non-specific band, no band, primer-dimer

Any comments on the PCR or kit would be appreciated:

No comments re the actual procedure - very effective! I have concerns that my results may not necessarily match what I should have due to a lack of starting material. Let me know how you go and if further participation in trials is required I would be pleased to help

All the best for 2005,

Kirk McG 6/Jan/05

Appendix G.3.6 Results returned from Marianne Douglas and Jeremy Carson, Department of Primary Industries and Water, Tasmania

Sample #	DNA conc(µg/ml)	result
13	14	N
24	14	N
26	14	P
33	34	N
45	26	N
48	61	N
50	14	N
51	18	N
65	26	N

Gel type: 2% Agarose in 0.5M TAE Buffer
Buffer used: 0.5M TAE Buffer

Loading buffer used and amounts:

Molecular Biology Grade Sucrose (Sigma cat#S0389)	0.4 g
0.5M EDTA	20 µl
10% SDS	100 µl
1% Bromophenol Blue	80 µl
Distilled Water	800 µl

Used 4µL per PCR product and loaded 8µL onto the gel
Ethidum in gel: 0.005% (15 µL in 300 mL TAE buffer)
Molecular weight used and amount: Abgene Superladder-Mid 100 bp, cat# SLM 100, used 6 µL
Amount of product used: 8 µL per well
Volts: 72V
Run time of gel: 0.7 hours
Notes:
I thought the protocol was easy to follow. The only comment I could make is to mention the temperature in step 10 (hydration of the DNA). I used 37 °C.
Hope this all helped you!
Cheers,
Marianne Douglas

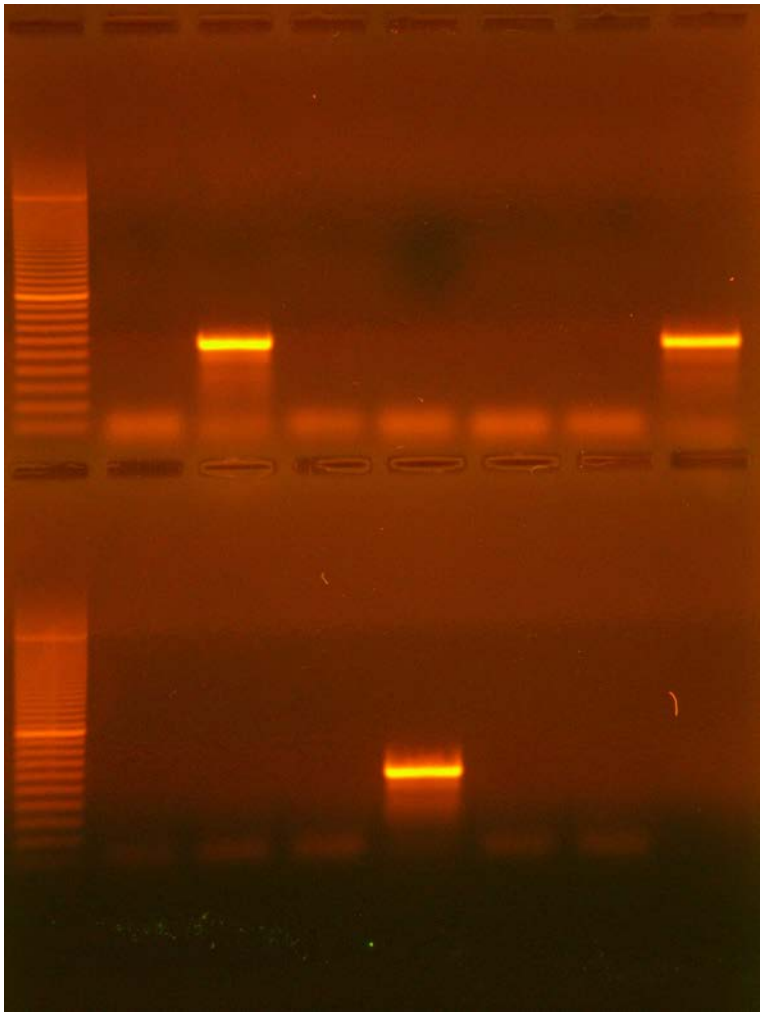


Figure 13-2. Gel results from DPIW, Tasmania

Appendix G.3.7 Results returned from Nick Gudkovs, Australian Animal Health Laboratories, CSIRO, Victoria

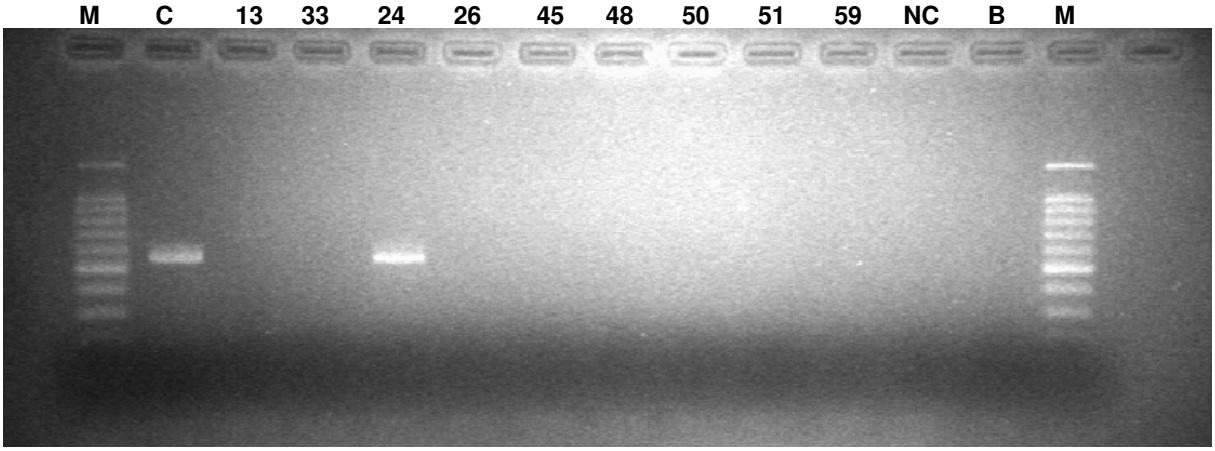


Figure 1. M, 100 bp marker; C, positive control; 13, 24, 26, 33, 45, 48,50,51,59, all sample isolates; NC, negative control; B, blank, M, 100 bp marker

Any comments on the PCR or kit would be appreciated:

- Volume of DNAzol made it difficult to homogenise the fungal material – suggest start with a smaller volume and then increase to required 600µL
- Also send MSDS of DNAzol with kit
- Specify length of time for air drying pellet

Appendix H Validation trial of PNA-FISH for *A. invadans*

Appendix H.1 Contact and address details for participating laboratories

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Appendix H.2 PNA-FISH kit and instructions sent to DPI NSW (EMAI)

PEPTIDE NUCLEIC ACID- FLUORESCENT *IN SITU* HYBRIDISATION (*APHANOMYCES INVADANS*)

The PNA-FISH involves 4 distinct phases: Removing the paraffin (PE sections only), Fixation, Hybridisation and Detection and analysis of the reaction product.

KIT CONTENTS:

A Deparaffinising:
You will need: xylene, 100% ethanol

B Fixation:
Fixative 1 (Culture tubes 6-12) – Labelled McCartney ((44mL of 95% ethanol, 10mL of deionised H2O, 6mL of 25X SET buffer [3.75M NaCl, 25mM EDTA, 0.5M Tris HCl {pH 7.8}])

Fixative 2 (PE section tubes 1-5) – Labelled McCartney (44mL of 95% ethanol, 10mL of deionised H2O, 6mL of 25X SET buffer [3.75M NaCl, 25mM EDTA, 0.5M Tris HCl {pH 7.8}], 3% polyoxyethylene sorbitan monolaurate [Tween 20])
You will need: Sterile MQ water

C Hybridisation:
Hybridisation buffer 1 (Culture tubes 6-12) – Labelled McCartney [5XSET, 0.1% Igepal-CA630 (Sigma, St. Louis, Mo), and 25µg·ml-1 poly (A) potassium salt (Sigma)]
Hybridisation buffer 2 (PE section tubes 1-5) – Labelled McCartney (5XSET, 0.1% Igepal-CA630 (Sigma, St. Louis, Mo), and 25µg·ml-1 poly (A) potassium salt (Sigma), 3% polyoxyethylene sorbitan monolaurate [Tween 20])

D Probes:
A. invadans probe – Green 0.2mL tube
Positive control probe – Red 0.2mL tube
Negative control probe – Blue 0.2mL tube

E Detection and Analysis:
You will need: fluorescent microscope
Silane coated slides
Cover slips

F Results:

Results sheet provided

Table 1. Samples sent (1.5mL tubes):

Tube	Treatment
1	PE
2	PE
3	PE
4	PE + control
5	PE –ve control
6	Culture
7	Culture
8	Culture
9	Culture
10	Culture
11	Culture +ve control
12	Culture –ve control

METHODS:

Deparaffinising Samples 1-5:

Care should be taken to avoid cross contamination of samples. The paraffin is extracted as detailed below.

1 Three 12µm tissue sections were cut from paraffin embedded blocks and placed into a 1.5 mL microfuge tube.
2 Add one millilitre of xylene to the tube and rock for 5 minutes at room temperature before centrifuging at 16,060 x *g* (13,000rpm) for 3 minutes to pellet the tissue.
3 Remove the supernatant and repeat the process twice.
4 After the last xylene wash, add 1mL of 100% ethanol and mix by inversion, again the tissue was pelleted (as above) and the supernatant removed.
5 Repeat the process twice and dry the final pellet at 60ºC for about 10 minutes.

Fixation Samples 1-5:

1 Rinse the dried deparaffinised tissue twice with 1mL of sterile ultrapure or deionized water and agitate at room temperature for 10 minutes.
2 After the second rinse, add 1mL of **fixative 2** and agitate at room temperature for 30 minutes.
3 Pellet the tissue by centrifugation 16,060 x *g* (13,000rpm for 3 minutes) and remove the fixative.

Fixation Samples 6-12:

1 Remove ~20mg of cultured hyphae from the tubes, taking care to leave any agar behind. Place into new microtubes.
2 Rinse twice with 1mL of sterile ultrapure or deionized water and agitate at room temperature for 10 minutes.
3 After the second rinse, add 1mL of **fixative 1** and agitate at room temperature for 30 minutes.
4 Pellet the tissue by centrifugation 16,060 x *g* (13,000rpm for 3 minutes) and remove the fixative.

Hybridisation Samples 1-5:

1 Add 0.5mL **hybridisation buffer 2** to the tubes and agitate at room temperature for 15 minutes, pellet the tissue 16,060 x *g* (13,000rpm) for 3 minutes and remove the buffer
2 Repeat the procedure.
3 *****After this point light should be kept as low as possible *****
4 0.5 mL of **hybridisation buffer 2** containing 0.5µl of the *A. invadans* probe is added to each tube from 1-3 and left in the dark at 60°C for 1 hour.
5 0.5 mL of **hybridisation buffer 2** containing 0.5µl of the positive control probe is added to tube 4 and left in the dark at 60°C for 1 hour.
6 0.5 mL of **hybridisation buffer 2** containing 0.5µl of the negative control probe is added to tube 5 and left in the dark at 60°C for 1 hour.
7 After 1 hour the material was pelleted [16,060 x *g* (13,000rpm) for 3 minutes] and the probe solutions removed.
8 Rinse twice in 1mL of 60°C 5XSET buffer and pellet [16,060 x *g* (13,000rpm) for 3 minutes] before adding the pellet to the silane slide and overlaying with a cover slip.

Hybridisation Samples 6-12:

1 Add 0.5mL **hybridisation buffer 1** to the tubes and agitate at room temperature for 15 minutes, pellet the tissue [16,060 x *g* (13,000rpm) for 3 minutes] and remove the buffer
2 Repeat the procedure.
3 *****After this point light should be kept as low as possible ******
4 0.5 mL of **hybridisation buffer 1** containing 0.5µl of the *A. invadans* probe is added to each tube from 6-10 and left in the dark at 60°C for 1 hour.
5 0.5 mL of **hybridisation buffer 2** containing 0.5µl of the positive control probe is added to tube 11 and left in the dark at 60°C for 1 hour.
6 0.5 mL of **hybridisation buffer 2** containing 0.5µl of the negative control probe is added to tube 12 and left in the dark at 60°C for 1 hour.
7 After 1 hour the material was pelleted [16,060 x *g* (13,000rpm) for 3 minutes] and the probe solutions removed.
8 Rinse twice in 1mL of 60°C 5XSET buffer and pellet [16,060 x *g* (13,000rpm) for 3 minutes] before adding the pellet to the silane slide and overlaying with a cover slip.

Detection and analysis:

Ensure that microscope and or camera settings for the epifluorescence analysis are held constant so that direct comparisons can be made between intensity of the slides.

Results:

Positive samples: Each positive should be compared to its own samples only. Bright apple green fluorescence should be seen when compared to the negative control.

Negative samples: Each negative should be compared to its own samples only. The negatives may have some background fluorescence usually seen as a yellowish fluorescence. This may be brighter for cultured samples than for the PE samples.

References:

Deparaffinising method adapted from:
Wright, D.K. and Manos, M.M. 1990. Sample preparation from paraffin-embedded tissues. p. 153-158. In Innis, M., Gelfand, D., Sninsky, J.and White, T (ed.), PCR protocols: A guide to methods and applications, 1990. Academic Press Inc, San Diego, California

FISH method adapted from:
Vandersea, M.W., Litaker, R.W., Yonnish, B., Sosa, E., Landsberg, J.H., Pullinger, C., Moon-Butzin, P., Green, J., Morris, J.A., Kator, H., Noga, E.J., Tester,P. Molecular Assays for Detecting *Aphanomyces invadans* in Ulcerative Mycotic Fish Lesions. Applied and Environmental Microbiology. 2006:72:1551-1557.

RESULTS:
PEPTIDE NUCLEIC ACID-FLUORESCENT IN SITU HYBRIDISATION (*APHANOMYCES INVADANS*)

Table 2 – Results of PNA-FISH

Tube	Treatment	Results	Magnification	Notes
1	PE			
2	PE			
3	PE			
4	PE +ve crl			
5	PE –ve crl			
6	Culture			
7	Culture			
8	Culture			
9	Culture			
10	Culture			
11	Culture +ve crl			
12	Culture –ve crl			

Microscope used:
Filter used:
Notes:
Any comments on the PNA-FISH method or kit would be appreciated:

Appendix H.3 Results of PNA-FISH from DPI NSW (EMAI)

Table 2 – Results of PNA-FISH

Tube	Treatment	Results	Magnification	Notes
1	PE	Negative	40	Nothing observed on slide.
2	PE	Positive	40	Not very much evidence of fungi but some short strands of hyphae with evidence of bright green fluorescence in patches.
3	PE	Negative	40	Nothing observed on slide.
4	PE +ve control	?	40	Bright green glowing mass but did not look like any sort of fungal growth looked more like agar.
5	PE -ve control	?	40	Similar to positive control but less intense. Pale green/yellow glowing mass but did not look like any sort of fungal growth looked more like agar.
6	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems.
7	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems.
8	Culture	Positive	40	Hazy cloud like mass with evidence of bright green fluorescence patches particularly at the outer extremes.
9	Culture	Negative	40	Small amounts of hyphae with yellowish/pale green specks within hyphae stems.
10	Culture	Negative	40	Hazy cloud like mass with no sign of fluorescence.
11	Culture +ve control	Positive (?)	40	Not very much evidence of fungi on slide but small traces of hyphae with evidence of green fluorescence.
12	Culture –ve control	-	40	Nothing observed on slide.

Microscope: Leitz Diaplan
Filter used: Blue 450-490
Notes:

- We were not sure if we had any sample in tubes 6, 7, 9 and 11. These tubes had liquid media and agar plugs but no apparent growth. Although we did see evidence of growth on the slides, there was not a lot.
- Any comments on the PNA-FISH method or kit would be appreciated:
- We found it difficult to determine if we had retained the sample during the procedure in some tubes.

Any comments on the PNA-FISH method or kit would be appreciated:
There were some steps in the methods that we thought were a little confusing. I have included a revised version of the method with changes to clarify. Please do not take this is a criticism just some suggestions.

Appendix I Glossary

Abbreviation	Name
AAHRI	Aquatic Animal Health Research Institute
AHL	Animal Health Laboratories
ATCC	American Type Culture Collection
AQIS	Australian Quarantine Service
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
DAFWA	Department of Agriculture and Food 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
EMAI	Elizabeth Macarthur Agricultural Institute
EUS	Epizootic Ulcerative Syndrome
FISH	Fluorescent *in situ* hybridisation
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
NAAH-TWG	National Aquatic Animal Health Technical Working Group
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PNA-FISH	Peptide nucleic acid fluorescent *in situ* hybridisation
QLD	Queensland
rRNA	Ribosomal ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RSD	Red Spot Disease
SCAHLS	Sub-Committee on Animal Health Laboratory Standards
SDS	Sodium dodecyl sulfate
subsp.	Subspecies
TBE	Tris/boric acid/EDTA buffer
TE	Tris/EDTA buffer
Tm	Melting temperature
U	Units of enzyme activity
UV	Ultraviolet
V	Volts
WA	Western Australia

Appendix J Contact with other laboratories in the course of sourcing material for *A. invadans* and *A. astaci*

Twelve laboratories including four from Australia and eight international laboratories were contacted in an attempt to obtain wax-embedded material infected with *A. astaci* and fresh cultures of *A. invadans* (Table 1).

Table J-1. Scientists and laboratories contacted to obtain Aphanomyces material

Scientist	Laboratory	Isolate	Notes
Susan Kueh	Aquatic Animal Health Branch, Agri-Food and Veterinary Authority, Singapore	*A. invadans*	Not available
Dr Supranee Chinabut & Dr Ackaban	Aquatic Animal Health Institute, Thailand	*A. invadans*	Thailand cultures contaminated. Trying to contact Dr Ackaban but no answer to date
Professor Hatai	Department of Biology, Tohoku University, Japan	*A. invadans*	No reply
Matt Landos	Private company, NSW	*A. invadans*	Fish with ulcers sent to DAFWA but negative for EUS
Teresa Wilson	Diagnostic Services, Dept of primary Industries Water and Environment, Tasmania	*A. invadans*	No cultures
Vicki Blazer	Fisheries Biologist, Leetown Science Center, US Geological Surveyl USA	*A. invadans*	Testing stocks but none viable to date
Annette Thomas	DPI&F QLD	*A. invadans*	Testing stocks but none viable to date
American Type culture collection	ATCC, USA	*A. invadans*	None available
CABI culture collection, UK		*A. invadans*	None viable
Dick Callinan	DPI NSW	*A. invadans*	None viable
CBS (Centraalbureau voor Schimmelcultures)	Netherlands Culture collection	*A. invadans*	None in stock
Suzanne Keeling	MAF, New Zealand	*A. invadans*	Not isolated
Professor Ludwig Buckup	Universidade Federal do Rio Grande do Sul, Department of Zoology, Brazil	*A. astaci* blocks	No material
Japo Jussila	Crayfish Innovation Centre, Finland	*A. astaci* blocks	Last reply several weeks ago – speaking to supervisor

Table J-1 continued

Scientist	**Laboratory**	**Isolate**	**Notes**
Jeff Shields	Virginia Institute of Marine Science, USA	*A. astaci* blocks	Paraffin-embedded material initially diagnosed as crayfish plague was *Saprolegnia* when identified by DNA sequencing
Birgit Oitmann and David Aldermann	CEFAS, UK	*A. astaci blocks, A. invadans*	Searching for blocks from CP outbreak 20 years ago, and obtaining *A. invadans* from Germany. D. Alderman retiring at end of November 2006.
Nick Gudkovs	AAHL, Geelong, Vic	*A. astaci*	Stocks held but appear to be non-viable. Possibility of using material to embedded into paraffin blocks for FISH. Unable to process until late December
Dr Kenneth Söderhäll,	Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, Sweden	*A. astaci* blocks	None available
Dr Lage Cerenius	Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, Sweden	*A. astaci* blocks	None available
DVM Satu Viljamaa-Dirks, Finland satu.viljamaa-dirks@evira.fi	Finnish Food Safety Authority Evira, Kuopio Research Unit, PO Box 92, 70701 Kuopio, tel. 358-20-7724 962 fax 358-20-7724 970	*A. astaci* blocks	To be sent in August 2007 so that further validation of the PCR and PNA-FISH for crayfish plague can be completed.

EUS

Appendix K Draft ANZSDP for Epizootic Ulcerative Syndrome

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SUMMARY

Epizootic ulcerative syndrome (EUS) is an ulcerative skin disease affecting wild and farmed freshwater and estuarine finfish throughout Australia, Papua New Guinea, Asia (Japan, Malaysia, Indonesia, Thailand, India, Pakistan) and USA. EUS has not been reported from Europe where it is an exotic disease.

A working group proposed re-naming EUS to epizootic granulomatous aphanomycosis (EG), but at present this is a synonym for EUS[1].

The primary causative agent is the oomycete fungus Aphanomyces invadans. *Little is known about the ecology of the fungus. The disease has been known by different names and the fungus has had several names. In Japan the disease was called mycotic granulomatoses (MG) and the organism A. piscicida*[2]*, whereas in Australia it was first called red spot disease (RSD) because of the distinctive red lesions*[3]*. In North America a condition called ulcerative mycosis from menhaden was reported*[4] *but is now considered to be synonymous with EUS. The organism was named* Aphanomyces invaderis[5] *but this was scientifically incorrect and has been corrected to* A. invadans[6]*. Currently epizootic ulcerative syndrome is the accepted term for the disease*[6].

In countries where EUS occurs, over 100 species of fish are reported to be susceptible as confirmed by histological diagnosis, but some important culture species, including tilapia, milk fish and Chinese carp, are resistant[7]*. EUS tends to be a seasonal disease occurring during periods of low temperatures and after periods of heavy rainfall*[8]*, conditions which favour sporulation of* A. invadans[9]*. The acidity from acid sulphate run-off after heavy rain damages the skin and makes it vulnerable to infection*[10]*. Other factors may be involved in damage to the dermis, and Rhabidoviruses,* Aeromonas *species,* Vibrio *species and other Gram-negative organisms have been implicated in complicating the aetiology.*

Clinical signs in infected animals are large red oval or circular ulcerated lesions or dermal ulcers up to 4 cm in diameter that extend into the underlying muscle to varying degrees[11].

Control of EUS in natural waters is considered impossible. In outbreaks occurring in small, closed water-bodies, liming water and improving water quality, together with removal of infected fish, is often effective in reducing mortalities.

EUS

Status of Australia and New Zealand: EUS is endemic to Australia and exotic to New Zealand. OIE status = other significant.

EUS

Part 1. Diagnostic Overview

Aetiology

Epizootic ulcerative syndrome (EUS) was defined in 1994 at the Department for International Development (DFID) regional seminar on EUS in Bangkok as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response"[12]. The causative agent of the disease is the aquatic fungus, *Aphanomyces invadans*.

The *Aphanomyces* genus comprises water moulds belonging to the family Saprolegniaceae[13] many of which are pathogens or saprophytes on fish, plants and animals. The Oomycetes are members of the Kingdom Chromalveolata and are not directly related to the true fungi, the Eumycota, but are more related to the brown algae.

The disease causes high mortality and up to 80% of the fish may be affected in a disease outbreak. The large red ulcerated lesions make fish unsaleable.

A. invadans is a non-septate, sparsely branching fungus with wide hyphae of 11.7-16.7 micron when *in situ*, but narrower (8.3 micron) hyphae are seen in young growing cultures[5]. The hyphae become wavy or undulating as they die, either in diseased fish or culture.

Using random amplification of polymorphic DNA (RAPD)[14], epizootic ulcerative syndrome, mycotic granulomatosis and red spot disease were all shown to be the one disease caused by the one agent, *A. invadans*. The results indicated that a single clonal type was responsible for the rapid spread of the disease throughout south-east and south Asia, and Australia. Restriction fragment length polymorphism (RFLP) showed that *A. invadans* is more closely related to *A. astaci*, the aetiological agent of crayfish plague, than any other *Aphanomyces* species[14]. Each fungal species is host-specific and unable to cause infection in the opposite host[15]. The organism isolated from the initial report of ulcerative mycosis in North America[4] was distinct from *A. invadans* (later thought to be *A. laevis*), but the more recently isolated, slow-growing *Aphanomyces* species cultured from muscle tissue of menhaden (*Brevoortia tyrannus*) in Chesapeake Bay, USA are indistinguishable morphologically and genetically from *A. invadans* associated with EUS[16].

Clinical Signs

During a disease outbreak mass mortalities of a number of different fish species may be seen at the same time and within a short time-frame (two weeks) after a significant rain event. Fish have a necrotising dermatitis that progresses to characteristic red skin ulcers that erode the underlying tissues. In some cases the lesion erodes the cranium leaving the brain exposed. The majority of fish will have one lesion but some can have multiple small lesions of different size. Dermal ulcers occur more frequently on the posterior and dorsal areas of the body surface compared to the anterior and ventral areas[11]. Lesions rarely occur on the fins.

In infection trials the infected fish were reported to be lethargic, stayed on the bottom of the tank, had loss of appetite and were anorexic[17]. Other signs include dark appearance of fish, hyperactivity or jerky movements, or floating below the surface of the water[1].

EUS

Epidemiology

EUS is an episodic, seasonal condition that affects over 100 species of freshwater and estuarine finfish throughout Asia, extending from Japan, Philippines, Malaysia, Indonesia, Thailand, Papua New Guinea, India, Sri Lanka, Bangladesh, Pakistan and Australia. It was first reported from Japan in 1971 from farmed ayu (*Plecoglossus altivelis*)[2] and a year later reported from eastern Australia in grey mullet (*Mugil cephalus*) and other estuarine fish[3]. The disease continued to spread to Papua New Guinea and into south-east and south Asia, then to west Asia, and to Pakistan[18, 19]. Outbreaks of ulcerative disease occurred in menhaden (*Brevoortia tyrannus*) from Chesapeake Bay in the United States of America (USA) and the causative fungi are genetically similar to those isolated in Asia[16]. Earlier isolations of *Aphanomyces* from menhaden[4] were genetically different to the later isolates (and there is some indication that this isolate was *A. laevis*).

In Australia EUS has been reported from Northern Territory, Queensland, New South Wales, Western Australia, and Victoria. In South Australia, fungal elements suspected of being EUS have been seen in histopathology sections, but the fungus has not been isolated and the disease cannot be confirmed. In 1985, ulcer disease was reported from cod (*Pseudophycis bachus*) in the Tamar river in Tasmania, and was thought to be due to river pollution[19, 20] otherwise EUS has not been reported from Tasmania.

In Australia, species that are affected include bream (*Acanthopagrus butcheri*), silver perch (*Bidyanus bidyanus*), eastern states cod (*Maccullochella ikei*), sea mullet (*Mugil cephalus*), sand whiting (*Sillago ciliata*), and rainbow trout (*Oncorhynchus mykiss*) In Asia, important fish species such as snakehead (*Channa striata*), catfish (*Pangasius* species) and ayu (*Plecoglossus altivelis*) are extremely susceptible, with the bottom-dwelling fish such as the snakehead (*Channidae*), being particularly susceptible. Aquarium fish such as three-spot gouramis (*Trichogaster trichopterus*), rosy barb (*Puntius conchonius*) and goldfish (*Carassius auratus*) are susceptible and the disease can enter Australia via the aquarium trade. Species of fish have different susceptibilities to *A. invadans*. Carp (*Cyprinus carpio*) and stickleback fish (*Gasterosteus aculeatus*) are resistant to infection, whereas tilapia and roach (*Rutilus rutilus*) are generally resistant but can succumb to infection if immunosupressed[21].

A number of causal factors combine to result in an EUS outbreak. Infection can only occur once the dermis is exposed[7], and outbreaks occur mostly during periods of low temperatures (18-22°C) and high rainfall[8]; conditions that favour the sporulation of *A. invadans*[9]. The low temperatures inhibit the inflammatory response to fungal infection[22, 23]. Outbreaks of EUS usually occur after periods of prolonged heavy rainfall with associated acid sulphate run-off. Fish exposed to acidified water can develop lesions, and it is thought that the propagules of *A. invadans* can then invade the dermis through these lesions[24, 25].

Genetic analysis through random amplification of polymorphic DNA (RAPD) indicated that *A. invadans* isolated throughout Asia, Australia and also the *A. invadans* isolated from menhaden from Chesapeake Bay in the USA were genetically similar and constituted a clonal group. The *A. invadans* isolates were distinctly different from *Saprolegnia* species and *Achlya* species that had been isolated as secondary invaders or contaminants from fish with EUS[14, 26]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

produced similar peptide bands between 14 to 94 kDa for Philippine and Australian isolates[10]. Other Asian strains were not tested at this time.

Incubation period

The appearance of cutaneous lesions generally occurs 2-3 weeks after a significant rain event[11, 24]. *A. invadans* inoculated under the skin of snakefish (*Channa striatus*) showed penetration of fungal hyphae deep into the tissues after five days and typical chronic granulomatous lesions occurred by twenty days post-inoculation[27].

Strategies used by the fungus for survival and maintenance

Typical of most Saprolegniaceae *A. invadans* produces motile zoospores that can swim in the water body to attach to another host. How the spores survive between seasonal outbreaks is not known.

Methods of transmission

Very little is known about the ecology of *A. invadans*. Horizontal transmission occurs by motile zoospores that swim and attach to and infect a new host under conditions optimal for the fungus[28]. It is thought that zoospores can remain viable for a short time (hours) on damp contaminated fishing gear and equipment.

Other oomycete fungi present on freshwater finfish

Oomycete fungi are widespread in fresh water and are saprophytic opportunists on fish that are stressed, infected, immunocompromised or injured. Saprophytic *Aphanomyces* species, *Saprolegnia* species and *Achlya* species may be present as contaminants in lesions and on skin of fish, and also contaminants in lesions caused by EUS. Infections caused by *Saprolegnia* species can be seen as circular or crescent-shaped white cotton-like mycelium around the head, caudal and anal fins. The infection can spread over the body radially and lesions can merge. In Australia, *Saprolegnia parasitica* is a pathogen of silver perch and in other countries has caused disease in brown trout, carp and catfish[29, 30].

Saprophytic *Aphanomyces* species are rapid growers especially at 10-30°C compared to the slow-growing *A. invadans*. The saprophytes can grow at 37°C whereas *A. invadans* dies at this temperature[27].

Occurrence and Distribution

EUS is endemic in most States of Australia, and throughout Asia extending from Japan to Pakistan. The disease also occurs in menhaden from Chesapeake Bay, USA. It is exotic to New Zealand and Europe.

Gross Pathology

The first stage of lesion development is a necrotising dermatitis that develops two to five weeks post-infection. These lesions are 1-4 cm in size, oval, domed, and yellowish-grey to red with an absence of scales. Dermal tissue is macerated and swollen. “Cotton-wool” type fungal growth is not seen, but hyphae can be seen when the tissue is immersed in water. Cotton-wool type fungal growth on diseased tissue is usually associated with *Saprolegnia* species. Sloughing of the central necrotic tissue occurs as the lesion progresses. The final stage of the lesion is a dermal ulcer that has a sharply defined margin, with exposure of skeletal muscle 1 cm below the surface[11] (Figure 1).

EUS

Figure 3 Black bream with dermal ulcer caused by EUS

Discrete, pale, firm circular or linear areas 1 x 4 mm can be seen on the ulcerated surface and extending into the underlying soft tissue. Bone or viscera may be exposed.

Evidence of healing of dermal ulcers can be seen 4-16 weeks after a rain event. Areas of pale, greyish-yellow with irregular reddening and a smooth surface can be seen at the edges of a healing ulcer. Limited scale and tissue regeneration occurs. Lesions classified as erythematous dermatitis usually heal progressively during an outbreak[11]. Atrophy of exocrine pancreas can occur.

Diagnostic Tests (General)

The diagnosis of EUS is confirmed using clinical findings, presence of typical fungal elements on histology sections, typical morphology on culture and sporulation. A PCR is available for direct use on fish tissue and for culture material.

Transport of samples to the laboratory

The fungus is killed by drying and exposure to temperatures below 7°C, therefore consideration needs to be given to appropriate packaging and transportation conditions of fish and samples to the laboratory. Ideally, samples (whole fish) should be collected within 12 hours of death and transported to the laboratory at temperatures between 7-25°C.

Culture and identification

Diseased fish or freshly dead fish are selected for testing. Autolyzed tissues are not suitable for culture as the fungus will not be viable. Tissue and underlying muscle surrounding a lesion is sampled for culture and wet preparations. Hyphae in the centre of a lesion are likely to be dead and devoid of internal components. The best sites for selection of material for culture are the leading edge of a lesion and the tissue beyond this edge. Fungal isolation is most successful from muscle that appears yellow and soft[31]. Tissue surrounding a lesion needs to be sterilized by either by washing with several rinses of sterile water, or seared with a hot spatula or scalpel blade. The seared skin is removed and small (2 x 2 x 2 mm) pieces of tissue or muscle are removed aseptically and inoculated into the agar (2-3 mm below the surface) of a culture plate. Plates are incubated at either room temperature or 15-25°C (using a refrigerated incubator) and examined daily for up to

ten days. Use of a stereomicroscope can assist in detecting and removing emerging hyphae. *A. invadans* does not compete well against contaminants and to prevent overgrowth by bacteria and other fungi, emerging hyphae should be subcultured regularly to new plates, in specific media without antibiotics. Detailed culture protocols are given in the Appendix.

Wet preparation

Small tissue samples from the leading edge of the lesion or from a freshly growing culture are placed into a few drops of distilled water on a glass slide and flattened under a coverslip. The tissue is examined for the presence of non-septate branching fungal hyphae approximately 11.7-16.7 micron in width (Figure 2). Fungal hyphae are strong and difficult to break apart.

Figure 4. Wet preparation taken from lesion on black bream

Histopathology

Only select tissue from euthanased live or moribund animals. Tissue from the leading edge and adjacent underlying muscle is selected for histopathology. Fungal elements may be difficult to visualise using haematoxylin and eosin stain (H&E), but fungal elements are clearly seen using Grocott's modification of Gomori's hexamine silver method[32, 33] (Figure 3). H&E with a silver counterstain can also be used.

EUS

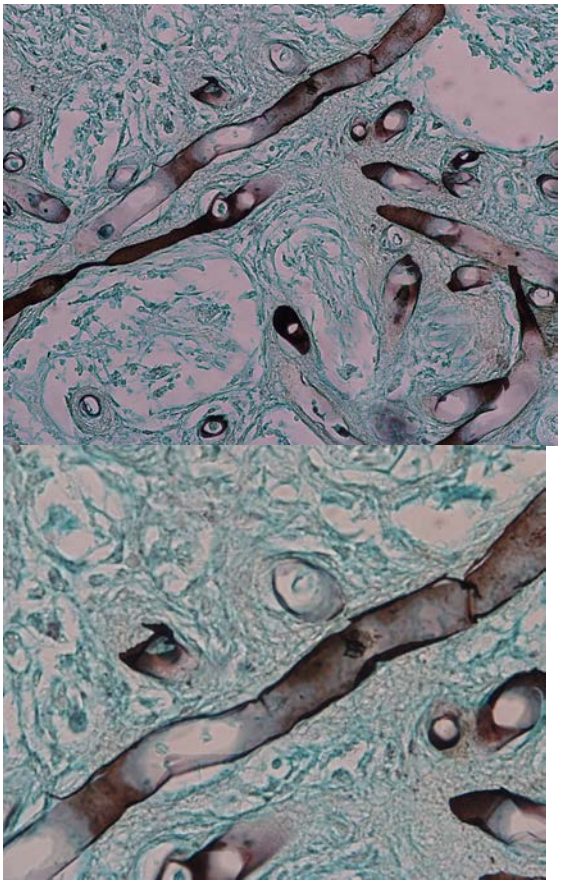

Figure 5. Formalin-fixed paraffin-embedded section stained with Grocott-Gomorri stain with malachite green counter stain showing fungal elements stained black, at x20 and x 40 magnification. Photograph courtesy Dr Brian Jones.

Tissues infected with *A. invadans* show a necrotising dermatitis, a severe locally extensive granulomatous lesion, that invades the dermis where numerous non-septate hyphae 12-18 micron in diameter are seen. The hyphae can extend throughout the stratum spongiosum, stratum compactum, underlying subcutaneous tissue and skeletal muscle. Skeletal muscle fibres show a severe floccular degeneration. Epidermis may be absent from affected areas. Scales at the edge of the lesion may show fractures containing areas of haemorrhage and osteoclasts[11].

In mature lesions the hyphae are often dead and can be surrounded by a thick layer of chronic inflammatory cells, primarily macrophages and epithelioid cells[27].

Lesions classified as erythematous dermatitis (which is a mild to severe, focal, chronic active dermatitis) show no fungal involvement. These lesions need to be differentiated from the dermal ulcers characteristic of EUS. Erythematous dermatitis lesions are usually less than 1 cm in diameter, involve one or two scales and epidermis is present at the edge of the lesion and irregularly present over the lesion. The epidermis may be hyperplastic, oedematous and mononuclear cells are present. These lesions usually heal once the initial acid sulphate assault has abated[11].

Serology

No serological tests are available.

EUS

Nucleic acid detection

The PCR developed through the Fisheries Research and Development Corporation project FRDC 2001/621 and FRDC 2004/091 is in use at the Department of Agriculture and Food Western Australia. The PCR is suitable for direct detection from fresh fish tissue or from culture. The PCR is not suitable for detection using formalin-fixed, paraffin-embedded sections because of the large size (554 bp) of the amplicon.

A fluorescent *in situ* hybridisation (FISH) test using peptide nucleic acid (PNA) probes is available. This is suitable for use on freshly cultured hyphae. The DNA within the hyphae deteriorates quickly as the hyphae age, and in some aged hyphae no internal components can be seen. Thus the PNA FISH can only be used on fresh samples. The PCR and PNA FISH methods are given in the Appendix.

Other reading

Epizootic Ulcerative Syndrome (EUS) Technical Handbook (Lilley et al., 1998) is available free of charge from the Aquatic Animal Health Research Institute, Thailand. See their website for order information, http://www.fisheries.go.th/aahri/Health_new/index.htm

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Part 2. Test Methods

Culture from fresh tissue

Principle of the test

Only tissue from live or moribund fish should be selected. Mature lesions often contain only dead hyphae and can be contaminated by opportunistic and saprophytic fungi and bacteria. *A. invadans* is slow growing compared to saprophytic fungal species such as *Saprolegnia* species, *Achlya* species and other non-pathogenic *Aphanomyces* species. For *A. invadans* optimal growth is 26-30ºC, with no growth at 37ºC. *A. invadans* does not grow on Sabouraud dextrose agar, cornmeal agar or malt extract agar. Growth on Czapek Dox agar can be poor compared to GPY (Glucose Peptone Yeast) agar[15].

Materials

GP-POX, or GPY-PS, IM agar, Czapek Dox agar (with penicillin and streptomycin) can be used for initial isolation. GPY agar is best for ongoing subculture. (See Appendix for procedure and media recipes).
Sterile washer
Incubator set to 20-25ºC.

Method

The tissue surrounding an ulcer is sterilised by washing with several rinses of sterile water, or searing with a hot spatula or scalpel blade. Fungal isolation is most successful from muscle that appears yellow and soft[31]. The seared skin is removed and small (2 x 2 x 2 mm) pieces of tissue or muscle are removed aseptically and embedded into the surface of an agar plate. The agar plate is not dried before inoculation as Oomycete fungi prefer a moist surface and can be destroyed by drying, or won't grow on a dry surface.

The plates are inverted and incubated at 20-25ºC for 7-10 days. Growth should start to appear after 2-3 days.

Plates are examined daily for signs of hyphae. A stereomicroscope can be used to look for first signs of fungal hyphae, which are subcultured as soon as possible to prevent overgrowth by contaminating bacteria and fungi. *A. invadans* does not compete well against contaminants and to prevent overgrowth by bacteria and other fungi, emerging hyphae should be subcultured regularly to new plates, in particular media without antibiotics.

It may be helpful to place the original piece of tissue inside a sterile washer (Figure 4). *A. invadans* grows within the first 2-3 mm of the agar surface and can grow under the washer and through the agar and away from the contaminants.

A procedure[34] for isolation of *A. invadans* is given in the Appendix.

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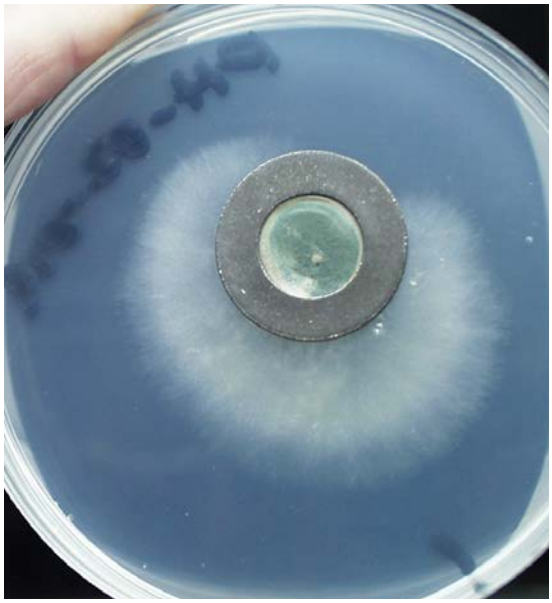

Figure 6. Growth of *Aphanomyces invadans* demonstrating the use of a sterile washer to ensure growth of contaminating *Aspergillus* species is contained within the centre of the washer.

A. invadans is slow growing and can take more than ten days to reach the edge of a 9 mm Petri dish. Growth is contained within the agar surface and aerial hyphae are not seen. Appearance is creamy-white in colour and the culture can appear moist (Figure 5).

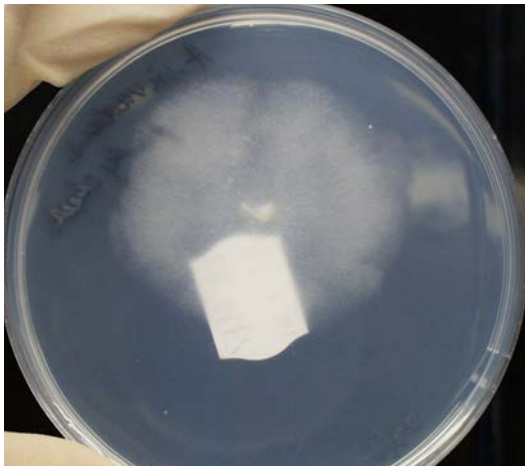

Figure 7. *A. invadans* on IM media after 9 days growth

Examination of fungal hyphae using wet preparation

Hyphae from plate or broth culture are placed into a drop of lactophenol blue and examined microscopically for typical morphology. It can be helpful to "pick up" hyphae from a culture plate using a piece of sticky tape and placing this on top of a drop of lactophenol blue (Figure 6).

Hyphae from culture material are smaller (8 micron) than hyphae seen in tissues. Fresh cultured hyphae are non-septate, branching with fairly straight edges. Dead mycelium tend to be wavy[27].

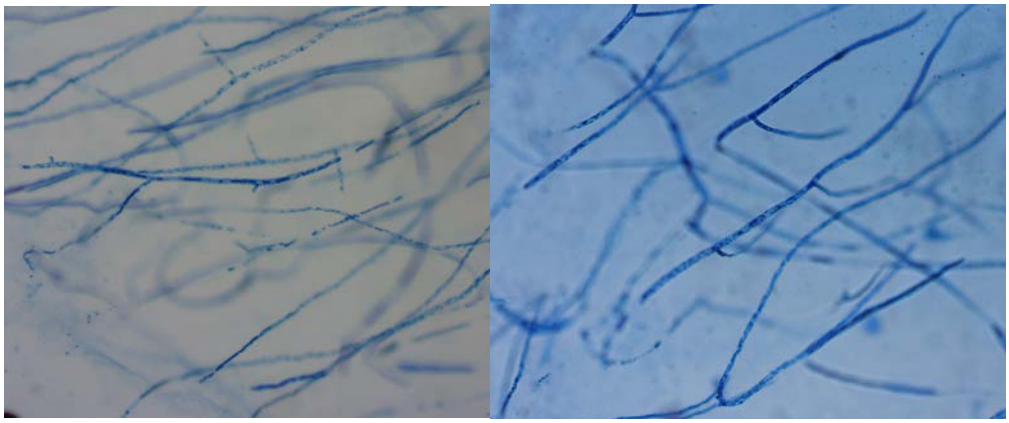

Figure 8. Wet preparation of *A. invadans* hyphae from culture, stained with Lactophenol blue

The hyphae may be densely packed with coarse granular cytoplasm with highly refractive globules (young active hyphae), or vacuolate with cytoplasm restricted to the periphery (older hyphae) or entirely devoid of contents (very old hyphae)[35].

Sporulation for the identification of A. invadans

Principle of the test

The identification of Oomycetes to genus level is based on sporangial morphology, and to species level on the morphology of the sexual reproductive stages (oogonia and antheridia). The latter cannot be done in *Aphanomyces* species as they reproduce asexually. Instead the process of sporulation, where spores are produced to discharge from the hyphal tip and encyst before producing motile spores (zoospores) that swim away, is used to identify *A invadans*. Sporulation is induced by exposure to freshwater or low salinity.

Materials

Culture plate of actively growing fungus
Sterile scalpel blade or cover slip
Sterile Petri dish 9 mm
Autoclaved river water or GPY or GP broth
Incubator set to 20-25°C
Light microscope or stereomicroscope

Test procedure[28]

Using a sterile scalpel blade (or sterile coverslip) cut a thin slice (2 cm x 2 cm x 2 mm) from the edge of an actively growing culture and place into 25 ml GP-POX

broth and incubate at 25°C for 5-7 days. Sporulation is induced by rinsing twice in sterile river water and incubating for 12-24 h at 25°C. Sporulation occurs at salinities of 0-2 $^{0}/_{00}$ and within a temperature range of 15-25°C (25°C being optimal). Sporulation occurs 1-3 days after induction and zoospores (spores with flagella) can remain motile for 12-18 hours. The process begins with the transformation of a long piece of hyphae into a sporangium, which is delineated by a highly refractive septum. Primary spores (cytoplasmic units) are formed from the sporangium's contents and line up as elongated spores linked by thin strands of cytoplasm[31]. Spores are released from the sporangial discharge tube, either terminally or laterally.

Encystment of spores occurs after 12-18 hours, but vigorous shaking causes immediate encystment of spores[28]. In the encystment phase the spores become round and a cell wall develops to enclose a cluster of 30-50 spores[31]. Secondary motile zoospores will form from the cysts.

A stereomicroscope, or light microscope with a stage that can accommodate a Petri dish, are used to view the sporulation process.

Quality control aspects

All three stages must be observed, but be aware that conditions must be optimal for sporulation to occur.

Interpretation of results

Subtle differences in sporangial types and release patterns are observed between the genera and species of the *Saprolegniaceae*, but may only be recognised by an experienced mycologist. Genera include *Saprolegnia*, *Leptolegnia*, *Achlya*, *Thraustotheca*, and *Aphanomyces*.

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Appendix

Isolation and growth media for Aphanomyces invadans

Glucose peptone (GP) Agar[27]

Reagents	**Amount**
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 (DW)	1000 ml

Prepare trace elements as a stock solution (described below). Add 1 ml of trace element mix to 1l ml 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.

Preparation of trace elements stock solution

A stock trace element solution is prepared composed of the following; $CaCl_2.2H_2O$ (0.029 g/L), $FeCl_3.6H_2O$ (2.4 mg/L), $MnCl_2.4H_2O$ (1.8 mg/L), $CuSO_4.5H_2O$ (3.9 mg/L), $ZnSO_4.7H_2O$ (0.4 mg/L).

This is done by preparing a 100 x stock solution (100 ml) of the trace element solution using the amounts in the last column of Table 1 as follows.

Table 1. Calculations for amount of trace element to weigh for 100 X stock solution of trace elements

Compound	**Formulae weight**	**Molecular weight of trace element**	**Final concentration required**	**Amount to weigh for 100 x stock solution**
$FeCl_3.6H_2O$	270.29	55.85	0.5 mg	**242 mg**
$CaCl_2.2H_2O$	110.98	40.08	8 mg	**2.215 g**
$MnCl_2.4H_2O$	197.91	54.94	0.5 mg	**180 mg**
$ZnSO_4.7H_2O$	161.45	65.38	0.1 mg	**43.97 mg**
$CuSO_4.5H_2O$	249.686	63.546	0.1	**39.29 mg**

Example of calculation:

$FeCl_3.6H_2O$. Formula weight = 270.29
Formula weight obtained from:
[MW of $FeCl_3$= 162.20]

MW of $6H_2O$ = 6 x [(2 x 1.008) + (1 x 15.99)] = 108.09.
FW of $FeCl_3.6H_2O$ = 270.29 (= 162.20 + 108.09).

Prepared from the following:

Want Fe at a final concentration of 0.5 mg (in 1L).
Therefore, 270.30/55.85 x 0.5 = 2.42 mg. For 100 x stock solution (100 ml) weigh out **242 mg** into stock solution.

Note: Compounds with water attached are usually easier to dissolve than the anhydrous solutions.

Glucose peptone broth (GP)[27]

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[27]

Reagents	**Amount**
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[27]

Reagents	**Amount**
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[27]

Reagents	**Amount**
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

Media for *Aphanomyces* species

Isolation Medium (IM)[35]

This medium is used for the isolation of *A. astaci* (crayfish plague), but is also suitable for *A. invadans* and *Saprolegnia* species.

Reagents	**Amount**
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.).

Czapek Dox agar containing antibiotics[31]

Czapek Dox agar (Difco) containing 100 units ml^{-1} penicillin G and 100 ug ml^{-1} treptomycin sulphate.

Czapek Dox agar without antibiotics is used as maintenance medium.

EUS

Quality Control

Quality control for fungal growth should be tested using either *A. invadans* or *Saprolegnia* species. (*Saprolegnia* sp. are easier to keep alive using the 'under oil' storage method – see later).

***Method of isolation of A. invadans from fish tissue*[34]**

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 continuum 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

At this stage colonies will be about 4 mm in diameter. Larger colonies are likely to be an *Aphanomyces* saprophyte.

Stage 4

Transfer the 4mm colonies to GPY-PS agar. If done too early or too late, growth may not occur or overgrowth of contaminants may occur.

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.

Storage of cultures

Aphanomyces species cannot be freeze-dried or placed at –80°C for long-term storage as this treatment renders them non-viable. Cultures are maintained on GP or IM medium prepared as sloped agar in 20 ml sterile plastic McCartney containers (or "universal" containers). A small plug of an actively growing culture is placed into the centre of the slope and incubated at 22-25°C until the growth almost reaches the sides of the container. The container is then filled with sterile paraffin or mineral oil and the culture stored in the dark at 7°C or room temperature (25°C). Cultures must be subcultured every six months, or every three to four months if stored at 25°C. (D. Alderman, personal communication, 2003). Mineral oil appears to lower the metabolic activity of the organism[36].

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Nucleic acid detection of *A. invadans* (FRDC 2001/621 & FRDC 2004/091)

Polymerase Chain Reaction (PCR)

Principle of the test

A number of PCR primers have been published for the amplification of *A. invadans*[14, 37, 38]. In the course of projects FRDC 2001/621 and 2004/091 primers (AIF 14 + ARR 10 producing amplicon of 554 bp in size) were developed and were found to be more specific than the aforementioned published primers. Many commercially available kits and manual methods for the extraction of DNA are not suitable for Oomycete fungi especially *A. invadans*. Only two methods were deemed suitable; the DNeasy mini plant tissue kit (Qiagen) and the DNAzol reagent (Invitrogen).

Reagents and materials

Liquid nitrogen
Sterile disposable micropestle
DNeasy tissue kit (Qiagen) or DNAzol extraction reagent (Invitrogen)
Primers AIF 14 + AIR 10 (Table 1)
Master Mix (Promega)
Microfuge tubes
Bench top microfuge
Thermocycler

Test procedure

DNA extraction from fish tissue

DNA from *A. invadans* is extracted directly from fish tissue using the DNAzol reagent (Invitrogen) according to the manufacturer's instructions.

DNA can also be extracted using the DNeasy mini plant tissue kit (Qiagen) according to the manufacturer's instructions.

DNA extraction from cultured hyphae

Both kits used for extraction of DNA from fish tissue can also be used to extract DNA from cultured hyphae.

Hyphae must be grown in a broth culture. Culture material from an agar plate is unsuitable because it is impossible to separate the agar from the fungal hyphae and agar interferes with the PCR reaction, causing false negative results.

The DNAzol method is followed according to the manufacturer's instructions.

For the DNeasy kit (Qiagen) best results are obtained if the hyphae are ground in liquid nitrogen before using the kit. The fungal mat is removed from a broth culture and moisture is removed between layers of filter paper. The mycelia are then placed into a mortar and ground to a fine powder in liquid nitrogen. This stage can be difficult to achieve as any moisture causes the liquid nitrogen to "spit" creating possible cross-contamination of samples. A sterile disposable micropestle and a 1.5 ml microfuge tube can also be used for this stage.

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Primers and preparation of PCR master mix

A stock solution of each primer (AIF 14 and AIR 10, Table 2) is prepared to 100 pmol using sterile ultra pure water. A working solution of 20 pmol is prepared in sterile ultra pure water. Solutions are stored at -20ºC.

Table 2. Primers AIF 14+AIR 10 for amplification of *A. invadans*

Primer name	Sequence 5' – 3'
AIF 14	CTG ACT CAC ACT CGG CTA GC
AIR 10	ATT ACA CTA TCT CAC TCC GC

A 25 µl PCR master mix is prepared according to Table 3.

Table 3. 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
Total volume	25

Thermocycling protocol

DNA is denatured at 94ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min, with a final cycle containing an extension step of 72ºC for 7 min.

Gel electrophoresis

Amplified product (5 µl) plus 5 µl of loading buffer is loaded into wells of a 2 % agarose gel prepared with chromosomal grade agarose (Bio-Rad) and 0.5M TBE buffer. Separate amplified DNA by electrophoresis using 100 volts for 60 minutes. A 100 base pair ladder (Geneworks) is loaded on the first and last well of the gel. Stain gels for 30 min by immersion in a one litre solution of ethidium bromide in distilled water at a concentration of 50 µl/l. Photograph the gel over ultraviolet light. The expected product size for *A. invadans* is 554 bp.

Quality control

A positive control of *A. invadans* DNA must be used for each PCR test. If possible infected tissue should be stored at -80ºC for use as a DNA extraction control. A DNA-free control, consisting of water used to prepare the master mix, must be included with each run.

Interpretation of results

Primers AIF 14 + AIR 10 produce an amplicon of 554 bp. If further confirmation is needed, the amplified product can be sequenced, or the extracted DNA can be

amplified and sequenced using universal oomycete primers to the internally transcribed spacer regions, ITS1 and ITS4[13, 39].

Specificity

The primers are specific when tested against other oomycete fungi, true fungi and bacteria likely to be laboratory contaminants or contaminants on diseased fish tissue. The primers do not amplify product from *A. astaci*, *Saprolegnia diclina*, *S. parasitica*, *S. salmonis*, *Achlya diffusa*, *Pythium sulcatum*, *Penicillium* species, *Aspergillus niger*, *Aspergillus fumigatus*, and *Trichoderma* species. The primers are also specific against the following bacteria; *Aeromonas hydrophila*, *Aeromonas salmonicida* (atypical strain), *Listonella anguillarum*, *Photobacterium damselae* subsp. *damselae*, *Streptococcus dysgalactiae*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio cholerae,* and *Vibrio mimicus.*

Fluorescent *in situ* hybridisation using peptide nucleic acid probes (FRDC 2004/091)

Principle of the test

Peptide nucleic acid probes (PNA) are DNA mimics and form stronger bonds than DNA-DNA hybridisation. PNA probes are also highly specific being capable of detecting a one base pair change.

A PNA-FISH developed for *A. invadans*[40] produced sensitive and specific results from material using freshly cultured fungal hyphae. Results can be variable when frozen stored hyphae are used, or from paraffin-embedded material (FRDC 2004/091). In these types of samples fluorescence may be seen in fewer hyphae. Some hyphae show fluorescence as clumps along the hyphal filament. This is probably due to the nature of the fungus and to the fact that as the hyphae age they become devoid of contents.

According to Vandersea *et al*., (2006) the method was used on infected fish tissue with good results, however the use of tissue was not validated in project FRDC 2004/091.

Reagents and materials

- Microscope with fluorescent attachment and excitation filter of 450 nm and an emission filter of 510 nm
- PNA probes (Table 4)
  ordered from Panagene, Korea (100 Sinsung-dong, Yuseong-gu, Daejeon, 305-345 Korea, ph: +82-42-861-9295, Fax: +82-42-861-9297).

Table 4. PNA probes used for *A. invadans*

Probe name	Target	Sequence 5' to 3'
Ainv-FLU3	*A. invadans*	FLU-GTA CTG ACA TTT CGT
EuUni-1	Universal positive	FLU-ACC AGA CTT GCC CTC C
PpiscFLU	Universal negative	GAA AGT GAT ATG GTA

FLU = fluorescein conjugate

- Fixation and hybridisation buffers

Fixative buffer one

95% ethanol	44 ml
deionized water	10 ml
25 x SET buffer	6 ml

Fixative buffer two (for use on tissue)

95% ethanol	44 ml
deionized water	10 ml
25 x SET buffer	6 ml
Tween 20	1.8 ml (3%)

25 x SET Buffer

NaCl (3.75M)	219.15 g
EDTA (25mM)	7.31 g
0.5M Tris HCl (HCl)	78.8 g
DW	1000 ml
pH 7.8	

Hybridization buffer one

5 x SET buffer	100 ml
Igepal-CA630 (0.1% vol/vol)	100 µl
polyA potassium salt (Sigma)	2.5 mg

Hybridization buffer two

Hybridisation buffer one	0.5 ml
20 nM PNA probe	0.5 µl

Hybridization buffer three

Hybridisation buffer one	97 ml
3% Tween 20	3 ml

Test procedure

PNA probes

For the FISH assay, controls include a positive (EuUni-1), negative (PpiscFLU-1), and a no-probe control (Table 3). The universal positive control probe EuUni-1 hybridizes to the SSU rRNA of all eukaryotes and is used to ensure that the RNA has not been degraded and that the probe has penetrated the cell.

Preparation using cultured hyphae

The PNA-FISH method using culture material according to Vandersea *et al*., (2006) is followed with the exception that the test is carried out in microfuge tubes rather than 24-well microtitre trays. Fungal hyphae are removed from a broth culture and

placed into a 1.5 ml microfuge tube and rinsed with 1 ml of ultrapure or deionized water with agitation for five minutes at room temperature. The fungal material is pelleted by centrifugation at (16,060 x *g*) (13,000 rpm) for three minutes.

To the fungal hyphae add 1 ml of fixative buffer and incubate at room temperature for 30 mins. Pellet the tissue by centrifugation at 16,060 x *g* (13,000 rpm) for 3 min and remove the fixation buffer by aspiration. Add 0.5 ml hybridisation buffer and place the tubes onto a rocking platform for 15 minutes at room temperature. Pellet the hyphae or tissue by centrifugation as before and remove the buffer by aspiration. From this point onwards in the procedure, the light is kept as low as possible to prevent loss of fluorescence of the probe. Add 0.5 ml hybridisation buffer containing 0.5 µl of probe to each tube and incubate in the dark at 60°C for one hour. The appropriate probe is added to either the positive control or negative control. After incubation centrifuge the tubes at 16,060 x *g* (13,000 rpm) for three minutes at room temperature. Remove the supernatant by aspiration rinse the pellet twice in 1 ml of 5X SET buffer (heated to 60°C). After the final centrifugation step remove the pellet of hyphae from the tube and place onto a microscope slide. Place a coverslip over the hyphae and apply gentle pressure to spread the hyphae in a thin layer. The use of a fluoro retardant is recommended to prolong the fluorescence; particularly needed when taking a photograph.

The slides are kept in the dark (to reduce loss of fluorescence) and examined using a microscope (Olympus BX50) with fluorescence capabilities with an excitation filter of 450 nm and an emission filter of 510 nm suitable for the detection of fluorescein isothiocyanate. The results can be recorded photographically using a camera attachment on the microscope. The results for the test samples are compared to the positive control, and contrasted with the results from the negative control. Positive results show a bright apple-green fluorescence (Figure 7).

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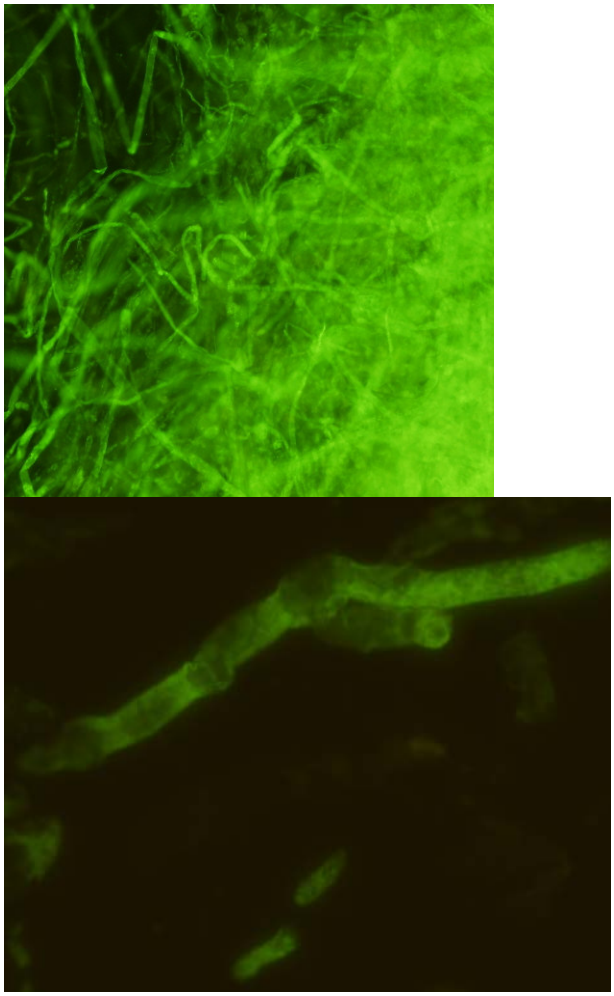

Figure 9. Results of PNA-FISH on fresh cultured hyphae of *A. invadans* showing bright apple-green fluorescence, x 10 and x40 magnification.

Quality control

A positive control of a freshly growing culture of *A. invadans* is used for each test. The positive probe control, universal positive probe and a no probe control are performed with each batch.

Interpretation of results

Young hyphae of *A. invadans* produce a bright apple-green fluorescence. Older hyphae or hyphae that have had a single freeze thaw will show a loss of fluorescence[40]. *Saprolegnia* species will show a yellow fluorescence of greatly reduced intensity.

Specificity

The PNA-FISH probe for *A. invadans* is specific in tests against other oomycete fungi, including *A. astaci*, *A. frigidophilus*, *Saprolegnia ferax*, *S. diclina*, *S. parasitica*, *S. salmonis*, and *Achlya diffusa* (FRDC 2004/091).