A PCR-BASED TECHNIQUE FOR THE IDENTIFICATION OF *Aphanomyces invadans*

Varinee Panyawachira[1], James H. Lilley[2], Douglas Hart[3] and Somkiat Kanchanakhan[1]

[1] Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Kasetsart University Campus, Jatujak, Bangkok 10900, Thailand
[2] Institute of Aquaculture, University of Stirling, FK9 4LA, Scotland
[3] Department of Veterinary Pathology, University of Glasgow, G61 1QH, Scotland

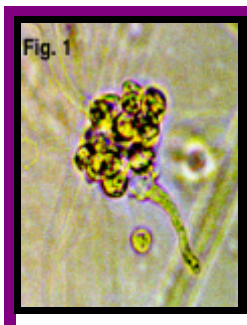

Fig.1 Typical *Aphanomyces* cluster of encysted primary zoospores.

Fig. 2 Lesion developing on striped snakehead (*Channa striata*) after intramuscular injection with *A. invadans* zoospores.

Introduction

Infection by the Oomycete fungus, *Aphanomyces invadans* (=*A. piscicida*), and production of distinctive granulomas, are the diagnostic features of EUS (epizootic ulcerative syndrome) (=MG: mycotic granulomatosis) in Asian fish. Identification *of A. invadans* isolates is based on demonstration of *Aphanomyces* sporulation morphology (Fig.1), slow growth rates in culture (Fig.3) and distinctive pathogenicity in EUS-susceptible fish (Fig.2). This poster describes the use of a polymerase chain reaction (PCR)-based test to identify *A. invadans* cultures.

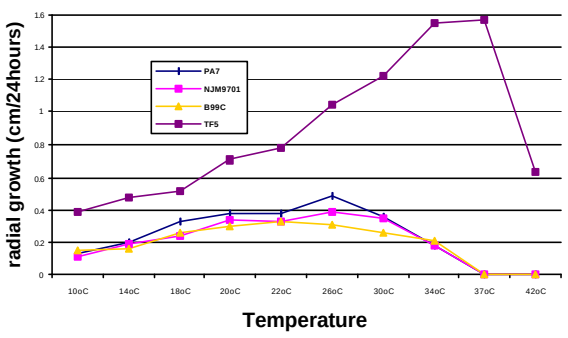

Fig. 3 Temperature growth profiles showing thermolabile characteristic of *A. invadans* isolates (PA7, NJM9701 & B99C) compared to *Aphanomyces* saprophyte (TF5).

Methodology

Primers

Primers were designed based on the DNA sequence of the internally transcribed spacer (ITS) region between the 18S and 5.8S rRNA genes of *A. invadans* (Fig.4). The nucleotide sequences of the primers are:

Aph3 forward 5'-ata agg ctt gtg ctg agc
Aph4 reverse 5'-cat ttc tga tgg cta tag g

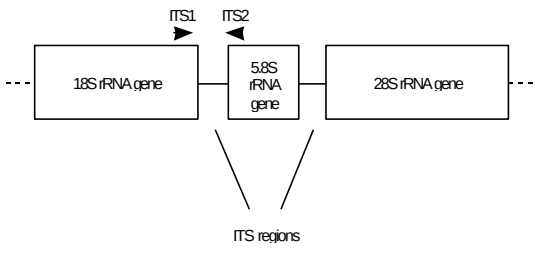

Fig.4 Structure of the rRNA gene cluster. The cluster is split into coding (18S, 5.8S and 28S genes) and non-coding (ITS) regions. The position of the PCR primers and their direction of synthesis are indicated by arrows.

PCR reaction

Each 50 µl reaction tube contained the reagents listed in Fig.5. These were overlayed with 2 drops of mineral oil.

Reagent	Working concentration	Volume	Final concentration
H_2O		29µl	
buffer	10x	5µl	1x
$MgCl_2$	25mM	5µl	2.5mM
dNTP	2.5mM	4µl	200µM
Primer 1	10pmol	2.5µl	25pmol
Primer 2	10pmol	2.5µl	25pmol
Taq	5U/µl	0.25µl	1.25U
DNA	30ng/µl	2µl	60ng
Total		**50.25µl**	

Fig.5 Reagents used for PCR reactions

PCR reactions were performed in a Hybaid Omnigene thermocycler using the temperature cycle illustrated in Fig.6. Amplification products were separated on 1.5% agarose gels stained with ethidium bromide and visualised under UV illumination.

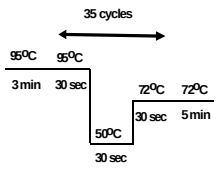

Fig.6 Temperature cycle for PCR reaction

Fungus isolates

Six *A. invadans* isolates (previously shown to have *A. invadans* morphology, growth and pathogenicity characteristics) (Fig.7), and a strain panel of eleven other *Aphanomyces*, *Achlya*, *Saprolegnia* and *Phytophthora* species (Fig.8) were compared using the PCR test. DNA was extracted from fungal mycelium using the Nucleon BACC2 kit (Amersham Life Sciences, England).

Isolate	Host	Date	Location	Worker
RF6	*Channa striata*	Jan 92	Suphanburi, Thailand	L.G. Willoughby
PA7	*Channa striata*	Jan 95	Nonthaburi, Thailand	J.H. Lilley
B99C	*Cirrhina reba*	Mar 99	Mymensingh, Bangladesh	J.H. Lilley & M.H. Khan
33P	*Channa striata*	Dec 91	Cent Luzon, Philippines	J.O. Paclibare
24P	*Mugil cephalus*	May 90	Nth Queensland, Australia	A. Thomas
NJM9701	*Plecoglossus altivelis*	Aug 97	Shiga Prefecture, Japan	K. Hatai

Fig.7 Origin of *Aphanomyces invadans* isolates (all recovered from internal tissues of EUS/MG affected fish)

Species	Isolate	Isolated from	Disease	Date	Location	Worker
Aphanomyces astaci	FDL458	*Austropotamobius pallipes*	crayfish plague	?	Herefordshire, UK	D.J. Alderman
Aphanomyces sp	84-1240	*Brevoortia tyrannus*	UM*	May 84	Nth Carolina, USA	M.J. Dykstra
Aphanomyces laevis	ASEAN3	fish pond water	-	Jan 94	Kasetsart, Bangkok	L.G. Willoughby
Aphanomyces sp	TF5	*Channa striata*	EUS**	Jan 91	Thailand	S. Chinabut
Aphanomyces sp	TF33	*Fluta alba*	EUS**	Dec 91	Udon Thani , Thailand	L.G. Willoughby
Aphanomyces sp	SA11	*Channa striata*	EUS**	Jan 95	Nonthaburi, Thailand	J.H. Lilley
Aphanomyces sp	F3ExtAph	*Channa striata*	external fungal infection	Mar 99	AAHRI wet lab, Bangkok	J.H. Lilley
Aphanomyces sp	EN1	*Eichhornia crassipes*	leaf blight disease	?	Bangkok, Thailand	S. Kiewong
Saprolegnia diclina	TF27	*Channa striata*	EUS**	Dec 91	Udon Thani , Thailand	L.G. Willoughby
Achlya sp.	F3ExtAch	*Channa striata*	external fungal infection	Mar 99	AAHRI wet lab, Bangkok	J.H. Lilley
Phytophthora cinnamomi	IMI335418	Macadamia	plant pathogen	1989	Australia	P.M. Wood

Fig.8 Origin of other fungal isolates
*probably external fungal opportunist on ulcerative mycosis-affected fish
**external fungal opportunist on EUS-affected fish

Results and Discussion

The Aph3-Aph4 primers reacted with DNA extracted from mycelium of a range of *A. invadans* isolates from different countries and host fish species. A 98bp product was produced from each reaction. A few non-specific bands were also produced. The saprophytic *Aphanomyces* species and other Oomycete fungi tested also produced a range of band patterns, but none succeeded in producing the 98bp band. The PCR test is therefore a useful means of differentiating *A. invadans* cultures from other fungal species.

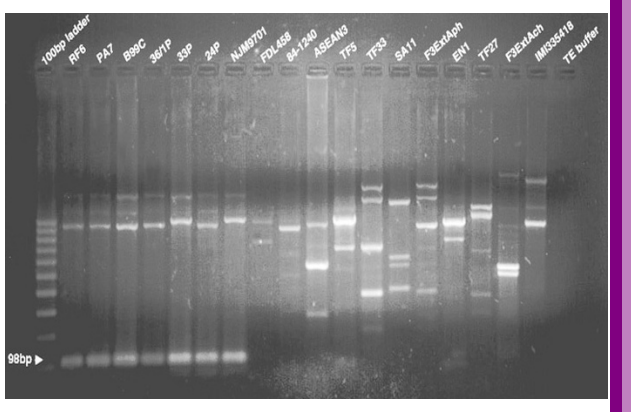

Fig.9 PCR products showing 98bp band amplified from all *A.invadans* isolates

Use of the probe to detect *A. invadans* in the environment

Efforts have been made to recover *A. invadans* from natural water bodies using culture-based techniques, but these have proved problematical due to colonisation of baits and culture medium by faster-growing saprophytic fungi. As a result, important aspects of the natural ecology of *A. invadans* (e.g. persistence of the fungus in ponds and fungal viability on carrier fish and non-fish substrates) have not been adequately studied. Therefore, a technique was developed using the PCR primers for the proxy detection of *A. invadans* DNA in water samples and other substrates. Fungal zoospores were recovered on filter paper and DNA extracted using an ethanol-based procedure. The technique was tested on *A. invadans* zoospore suspensions (i) in sterile water, (ii) in tank water containing EUS-susceptible fish, and (iii) in pond water collected from active EUS outbreaks. The primers succeeded in detecting zoospores of *A. invadans* in experiment (i) but not in (ii) or (iii). This was probably due to the high levels of debris, and low levels of *A. invadans* DNA in the water.

Other applications of the PCR probe

*The PCR-based test is being used to show the relationship to *A. invadans* of new invasive fungus isolates, obtained from ulcerative disease outbreaks on the east coast of USA.
*Attempts to use the DNA probe in an *A. invadans*-specific histological stain using *in situ* hybridisation have not yet been successful.
*A procedure has been developed to test for *A. invadans* DNA in paraffin-embedded tissues using the PCR primers. This has enabled examination of historical tissue samples (Blazer, pers. com.).