Vol. 30: 187-197, 1997 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published September 30

Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis

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ABSTRACT: Fungal mycelium extracts were subjected to gel electrophoresis and Western blot analysis with various lectins and polyclonal antisera. These techniques succeeded in identifying bands specific to pathogenic *Aphanomyces* strains from fish affected by epizootic ulcerative syndrome, redspot disease and mycotic granulomatosis when compared to various other Oomycete fungi. It is concluded that these isolates are conspecific under the name *Aphanomyces invadans* [previously described by Willoughby et al. 1995 (J Fish Dis 18:273-275) as *A. invaderis*] and the various protein and carbohydrate bands identified may provide useful species-specific taxonomic markers. Besides the distinctive band profiles for *A. invadans* on gels subjected to various stains, lectins revealed a specific 45 kDa component, and polyclonal antisera highlighted an immunogenic 10 kDa band. Peroxidase and fluorescein conjugated antisera provided an effective diagnostic tool for identifying hyphae in infected fish tissue.

KEY WORDS: Epizootic Ulcerative Syndrome · Fungus · *Aphanomyces invadans* · SDS-PAGE · Western blot analysis · Lectins · Immunohistochemistry

INTRODUCTION

Aphanomyces invadans is the main aetiological component of a serious disease of Asian freshwater and estuarine fish stocks known as epizootic ulcerative syndrome (EUS). It was described by Willoughby et al. (1995) under the name *Aphanomyces invaderis*, but is now listed in the Index of Fungi (1997) as *A. invadans*. Recent work has shown that this fungus appears to be pathogenically and culturally identical to similar *Aphanomyces* isolates from fish suffering from redspot disease (RSD) in Australia and mycotic granulomatosis (MG) in Japan (Lilley & Roberts 1997). Strains from the latter disease, however, have been generally referred to as *Aphanomyces piscicida* (Hatai 1980). Previous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by Thompson et al. (1997) and Callinan et al. (1995) reported similarities between the SDS-PAGE profiles of *Aphanomyces* sp. isolated from fish infected with EUS, RSD and MG. Western blot analysis with immunized snakehead fish *Channa striata* sera supported this finding (Thompson et al. 1997).

Other studies have employed protein electrophoresis as a means of establishing interspecific and intraspecific relationships between Oomycete fungi (Chen et al. 1991, Latorre et al. 1995). In the present study, a wide range of EUS, RSD and MG isolates were compared by SDS-PAGE, as well as a variety of Oomycete saprophytes and fungi from other diseases of aquatic animals, namely ulcerative mycosis (UM) (Dykstra et al. 1989), crayfish plague (Alderman 1993) and saprolegniasis (Bruno & Stamps 1987). An attempt was made to characterize strain-specific bands using various stains, lectins and polyclonal antibodies.

MATERIALS AND METHODS

Fungal isolates. A list of the fungi used in the present study is given in Table 1 and further details can be found in Lilley & Roberts (1997).

Preparation of fungal extracts. To obtain protein-rich fungal extracts, 45 ml zoospore suspensions were produced in Petri dishes as described by Willoughby & Roberts (1994) and added to an equal volume of double strength glucose-peptone-yeast (GPY) broth (Willoughby & Roberts 1994). Germlings were allowed

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Table 1. Fungal strains used in this study

Isolates	Description
TA1, RF6, RF8, S1PA, G2PA, PA4, PA5, PA7, PA10	*Aphanomyces invadans* from EUS-affected fish in Thailand
BH, BS	*A. invadans* from EUS-affected fish in Bangladesh
36/1P	*A. invadans* from an EUS-affected fish in Indonesia
10D, 33P	*A. invadans* from EUS-affected fish in the Philippines
3P, 4P, 10P, 24P	*Aphanomyces* sp. from RSD-affected fish in Australia
NJM9030	*Aphanomyces piscicida* from a MG-affected fish in Japan
84-1240 (= ATCC 62427)	*Aphanomyces* sp. from an UM-affected fish in the USA
FDL457, FDL458	*Aphanomyces astaci* from plagued crayfish in the UK
TF5, TF33, TF41, F3SA, SSA, SA11	*Aphanomyces* sp. saprophytes from EUS-affected fish in Thailand
T1SA	*Aphanomyces* sp. saprophyte from a diseased turtle in Thailand
WSA	*Aphanomyces* sp. saprophytes from EUS-affected pond water in Thailand
ASEAN1	*Aphanomyces laevis* saprophytes from EUS-affected pond water in Thailand
W2BAC	*Achlya diffusa* saprophyte from EUS-affected pond water in Thailand
S2AC, AC2	*Achlya* sp. saprophytes from EUS-affected fish in Thailand
TF29, TF31	*Saprolegnia* sp. saprophytes from EUS-affected fish in Thailand
S.AUST	*Saprolegnia australis* saprophyte from a fish in the UK
P32	*Saprolegnia ferax* saprophyte from lake water in the UK
TP41 (= ATCC 42062)	*Saprolegnia parasitica* from a saprolegniasis-affected fish in the UK

to develop for 1 to 3 d at 22°C, depending on the growth rate of the isolate, so that thin mycelial mats formed on the bottom of each Petri dish. Growth medium was decanted and the samples washed once in 500 ml sterile distilled water. Samples were harvested on sterile Whatman 541 filter paper and excess water removed using dry sterile filter paper. Fungal mats were ground in liquid nitrogen using a pestle and mortar. The resulting powder was then homogenized in 1 ml Wood's (1988) extraction buffer (85 mM Tris HCl, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.198 g l^{-1} ascorbic acid and 1 g l^{-1} glycerol at pH 7.5) with the addition of 5 µM phenylmethylsulphonylfluoride (PMSF) (Sigma). The homogenate was centrifuged twice at 13000 × *g* for 5 min and the protein concentrations of the extracts were estimated by ultraspectrophotometric readings at OD_{280} (OD: optical density). These were adjusted to 5 mg ml^{-1} with sample buffer and the extracts were frozen at –70°C. Prior to adding sample buffer, a 100 µl aliquot was taken of each extract and stored at –70°C for subsequent protein digestion. This involved incubating the aliquots with 100 µl of a 10 µl ml^{-1} solution of proteinase K (Sigma) for 1 h at 60°C before diluting in sample buffer.

Preparation of extracellular products (ECP). Media (500 ml GPY) in which the fungi had been cultured were collected, placed in dialysis tubing with a molecular weight cutoff of 10 kDa (Fisons) and concentrated using polyethylene glycerol (8 kDa, Sigma). Concentrates were dialyzed using 3 changes of 2 l phosphate buffered saline (PBS: 0.02 M $NaH_2PO_4 \cdot 2H_2O$, 0.02 M $Na_2HPO_4 \cdot 2H_2O$, 0.15 M NaCl, pH 7.2) over 24 h at 4°C.

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

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

Immunization schedule. Three New Zealand White rabbits were immunized with mycelium extracts from either (a) a saprophytic *Aphanomyces* isolate F3SA, (b) *A. invadans* PA7 or (c) the electroeluted band from PA7. The extracts (300 µg protein) were mixed 1:1 with Freund's complete adjuvant (Sigma) and 1.5 ml was delivered subcutaneously (SC) at 2 sites. Each rabbit was given 2 further SC booster injections of fungal extract in Freund's incomplete adjuvant 4 and 8 wk later. A final injection of 1 ml fungal extract in sterile saline was given intravenously in Week 12. The rabbits were bled out by cardiac puncture 10 d later.

Western blot analysis. The polyclonal antisera were used to screen each isolate for antigenic bands by Western blot analysis. Samples were applied to each lane and subjected to electrophoresis as described above. The samples were transferred from the gel to a sheet of nitrocellulose membrane by a wet blotting system (Hoefer) at 50 V for 1 h. Following transfer, the

nitrocellulose membrane was washed with 2 changes of high salt wash buffer (HSW: 0.02 M Tris, 0.5 M NaCl, 0.1% Tween 20, 0.01% methiolate, pH 7.8) and blocked for 2 h with 1.0% (w/v) bovine serum albumin in distilled water. The membrane was then washed twice in HSW and antisera (diluted 1/100 in PBS) were applied to the nitrocellulose membrane and incubated for 1 h at 4°C. After 2 washes in HSW, goat anti-rabbit IgG-Horseradish Peroxidase (HRP) conjugate [SAPU: Scottish Antibody Production Unit, Carluke, UK; (diluted 1/100 in 0.5% (w/v) casein in PBS] was applied to the membrane and left for a further hour. Unbound conjugate was removed from the membrane by washing twice in HSW followed by 1 wash with Tris buffered saline (TBS: 10 mM Tris, 0.15 M NaCl, pH 7.5). The blot was developed with 4-chloro-1-naphthol (Bio-Rad) and the reaction stopped with distilled water.

Lectins were used to examine carbohydrate moieties in each of the fungal extracts. For these studies the nitrocellulose membranes were incubated for 1 h with lectins labelled with biotin (Sigma) (see Table 2) diluted to 20 µg ml^{-1} in low salt wash buffer (LSW: 0.02 M Tris, 0.038 M NaCl, 0.05% Tween 20, 0.01% merthiolate, pH 7.4) in place of the rabbit polyclonal antisera. The blots were washed and incubated for 1 h with streptavadin-peroxidase (SAPU) diluted 1/100 in LSW buffer. Finally, the membranes were washed 5 times with HSW buffer and the reaction developed as described above.

Immunohistochemistry (IHC). The technique used was based on the method of Adams & Marin de Mateo (1994). Fixed blocks of muscle from snakehead fish experimentally infected with *Aphanomyces invadans* isolate TA1 were embedded in paraffin wax and sectioned at 5 µm. The tissue sections were dewaxed, encircled with a wax PAP pen (Merck) and fixed for 10 min with methanol containing 10% v/v hydrogen peroxide to bleach endogenous peroxidases. The slides were then washed 3 times with TBS. Normal goat serum diluted in TBS (10% v/v) was added to the slides which were then incubated for a further 20 min. The serum was poured off, the slides were placed in a moist chamber and each of the 3 rabbit sera (1/100 in TBS) were added to the sections for 1 h at 20°C. Normal rabbit serum was used as a negative control. The slides were washed as above. Goat anti-rabbit-HRP conjugate (1/50 in TBS) was added to the slides for 1 h and the slides washed as previously described. To visualize the reaction, slides were incubated for 10 min with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) in the presence of hydrogen peroxide (H_2O_2) [100 µl of 1% H_2O_2 to 0.5 ml (1.5 mg ml^{-1}) DAB and 5 ml TBS]. The reaction was stopped by immersing the slides in tap water. The slides were counterstained with haematoxylin for 3 to 4 min then dehydrated and mounted. Microscopically, positive tissue appeared brown in colour.

Indirect fluorescent antibody technique (IFAT). IFAT was carried out on TA1 tissue sections according to Neelam et al. (1995) using 1/100 dilutions of the rabbit antisera. Normal rabbit serum was used as the negative control. A 1/100 dilution of fluorescein isothiocyanate (FITC)-donkey anti-rabbit IgG (SAPU) was used as the secondary antibody.

RESULTS

All the isolates listed in Table 1 were tested using SDS-PAGE and a selection of isolates was included in Western blot studies but, given the volume of data generated, only selected results are illustrated here.

SDS-PAGE analysis

Coomassie Brilliant Blue R stained SDS-PAGE gels gave very similar banding patterns for all the EUS, RSD and MG isolates (Fig. 1) and these were distinct

Table 2. Lectins used in this study

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

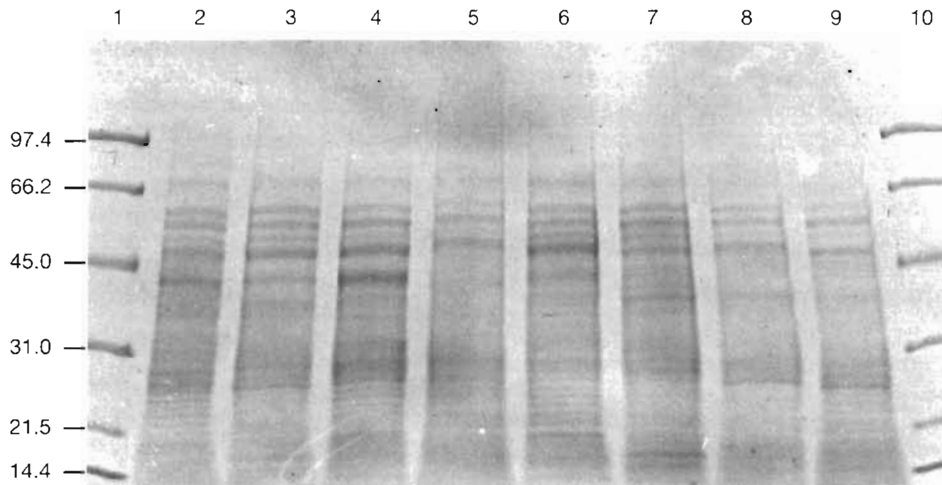

Fig. 1. Coomassie Brilliant Blue R stained SDS-PAGE gel (4–20%) of various isolates of *Aphanomyces invadans* (Lanes 2 to 7) and RSD-*Aphanomyces* (Lanes 8 and 9) showing almost identical polypeptide band patterns. Lanes: (1 and 10) BioRad low molecular weight markers; (2) RF6; (3) G2PA; (4) PA7; (5) BH; (6) 36/1P; (7) 10D; (8) 4P and (9) 24P

from all other fungi tested. Bands shown by these isolates visualized using Coomassie Brilliant Blue R consistently occurred at 48, 56 and 61 kDa. However, banding patterns were generally rather faint, therefore the more sensitive silver stain was used to further highlight bands. Using silver stain, the intensity and number of bands visualized were greater for the EUS, RSD and MG isolates compared with the other fungi. In order to compare isolates from different gels a pictorial representation of the silver stain bands was constructed (Fig. 2a). Bands specific to all the EUS, RSD and MG isolates were located at approximately 10, 84, 195 and 240 kDa. The 10 kDa band was electroeluted, and its presence in the resulting sample was verified using SDS-PAGE (Fig. 2b). This sample was used to prepare the third polyclonal antiserum (αband). While the bands mentioned above remained constant, molecular weights of other bands were inconsistent when gels were stained with the silver stain reagent, even between different gels run using the same fungal extracts. Silver staining also showed similarities between other fungal isolates, with clear groups being identified among some of the saprophytic *Aphanomyces* (TF5, TF41, F3SA, SSA and T1SA), 2 of the *Achlya* isolates (S2AC and AC2) and 2 *Saprolegnia* isolates (TF29 and TF31).

Silver-stained proteinase K-treated samples and Schiff's-stained gels revealed high levels of the low molecular weight carbohydrate around 10 kDa in *Aphanomyces invadans* (as shown for PA7 in Fig. 3a, Lanes 1 and 2 respectively) and at approximately 5 and 14 kDa in most of the saprophytic *Aphanomyces* (as shown for F3SA in Fig. 3b, Lanes 1 and 2). These bands can also be identified on the untreated silver-stained gels (Fig. 2a). Another carbohydrate band of note revealed by Schiff's staining was the 100 kDa band which was apparent on EUS (Fig. 3a, Lane 2), RSD and MG isolates (data not shown) but not on any of the other fungi.

Western blot analysis

Lectins

Fig. 3 also shows the bands of PA7 and F3SA recognised by each lectin. The bands described above could not be positively identified by lectin staining studies, although the 100 kDa band visualized using Schiff's stain may equate with a band of similar size revealed by the lectin LEA on EUS, RSD and MG isolates. The relative affinities of each lectin for the fungal carbohydrates varied, as indicated by the time taken for bands to develop in 4-chloro-1-naphthol. This ranged from 10 s for Con A to 14 h for LEA.

There was again remarkable consistency between EUS, RSD and MG isolates in terms of the bands revealed by lectin binding on the Western blots. Fig. 4a illustrates this consistency with regard to HGA. The lectin UEA-1 gave a very similar banding pattern to HGA. Out of the 9 lectins tested, only ECA revealed any differences between *Aphanomyces invadans* isolates, with 36/1P and 10D showing possible additional bands (Fig. 4b, Lanes 5 and 6). For all the EUS, RSD and MG isolates, a band at approximately 45 kDa was recognised by Con A, ECA, HGA, BS-1, LEA and UEA-1. WGA recognised a region between 33 and 123 kDa, producing an area of continuous staining.

For F3SA and a few apparently similar saprophytes, 2 bands were generally recognised at 55 and 90 kDa

a

M TA1 RF8 S1PA PA4 PA5 PA7 BH BS 33P 3P 4P 10P 24P NJM 9030 84-1240 FDL 457 FDL 458

97.4 – 66 – 45 – 31 – 21.5 – 14.5 –

M TF5 TF33 TF41 F3SA SSA SA11 T1SA WSA ASE-AN1 W2-BAC S2AC AC2 TF29 TF31 S. AUST P32 TP41

97.4 – 66 – 45 – 31 – 21.5 – 14.5 –

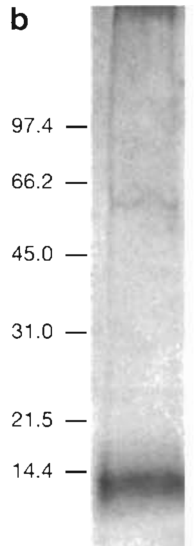

Fig. 2. (a) Pictorial representation of silver-stained SDS-PAGE gels (4–20%) of selected isolates. (b) Silver-stained electroeluted 10 kDa band (on 4–20% SDS-PAGE gel)

for all lectins tested except LEA. Similar banding patterns were obtained with GMA, BS-1, UEA-1 and AHA among the saprophytes.

Polyclonal antisera

Western blot analyses showing the response of the 3 polyclonal antisera against fungal extracts are presented in Fig. 5. The anti-saprophyte (αF3SA) and anti-*Aphanomyces invadans* (αPA7) serum showed a high degree of cross-reactivity with all isolates tested, with both antisera recognising similar bands on a given

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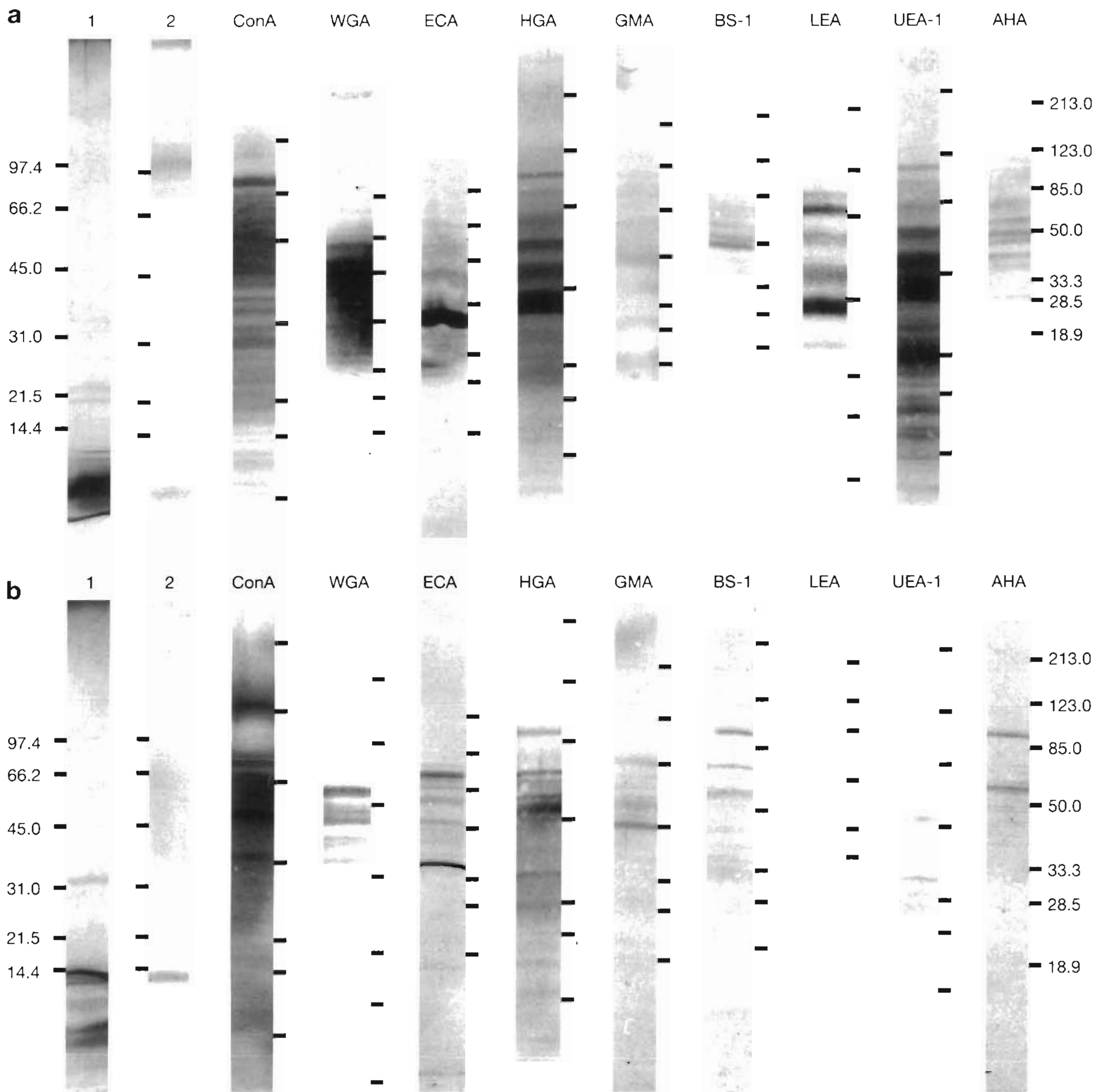
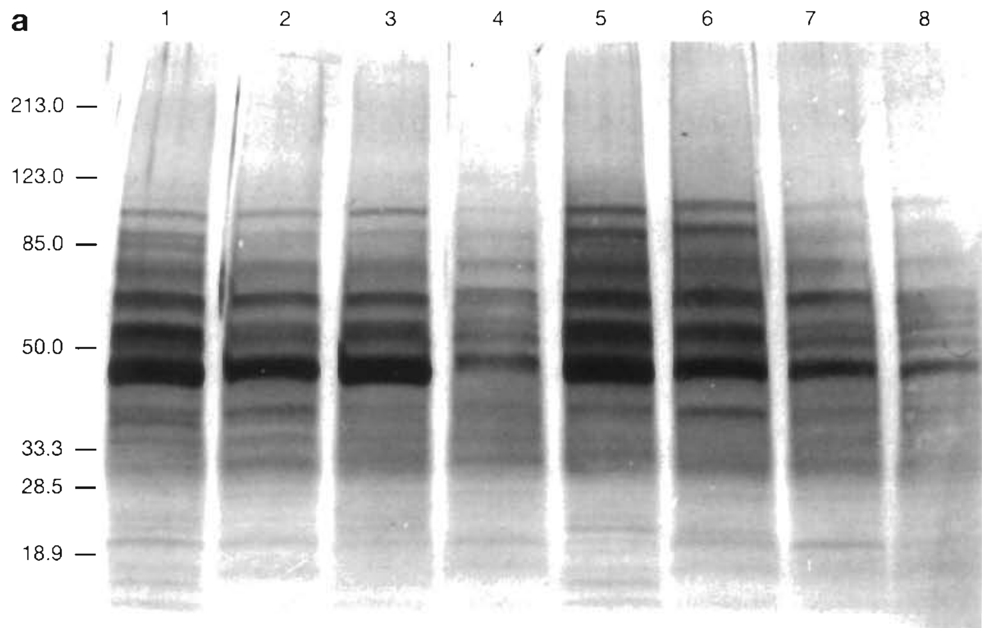


Fig. 3. Polysaccharide banding patterns of SDS-PAGE gels (4–20%) (a) *Aphanomyces invadans* isolate PA7 and (b) saprophytic *Aphanomyces* isolate F3SA. Lanes: (1) proteinase K-treated silver-stained gel; (2) Schiff's-stained gel; (3 to 11) Western blots stained with various lectins. BioRad low-range markers are indicated on the left of Lanes 1 and 2 and BioRad broad-range markers are indicated on the right of lanes 3 to 11

isolate (Fig. 5a, b). However, the αF3SA serum recognised a band at 45 kDa on PA7 and a band at 42 kDa on F3SA which the αPA7 serum only faintly recognised. There was also a substantial amount of staining of low molecular weight material in the F3SA extract by the αF3SA serum which was not recognised by the αPA7 serum. Both the αF3SA and αPA7 sera recognised the 10 kDa band of PA7 and were also able to recognise this band in the pathogenic MG isolate NJM9030 (RSD isolates were not tested with these antisera).



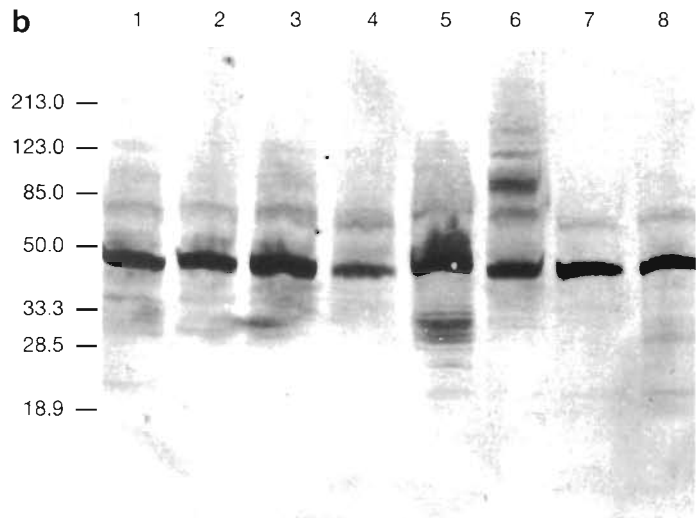


Fig. 4. Western blots of various isolates of *Aphanomyces invadans* (Lanes 1 to 6) and RSD-*Aphanomyces* (Lanes 7 and 8) stained using (a) HGA and (b) ECA. Lanes: (1) RF6; (2) G2PA; (3) PA7; (4) BH; (5) 36/1P; (6) 10D; (7) 4P; and (8) 24P

Antiserum raised against the PA7 electro-eluted band (αband) recognised only 2 bands found solely in extracts of fungi from the EUS, RSD or MG group (Fig. 5c). However neither band appeared at the molecular weight of the original 10 kDa band, but instead at around 50 kDa.

The reaction of the polyclonal antisera with fungal ECP is shown by Western blots in Fig. 6. There was little response of either the αF3SA or the αPA7 serum with the ECP from the saprophyte F3SA (Fig. 6a); however, both antisera reacted strongly with the ECP of the *Aphanomyces invadans* isolate PA7 (Fig. 6b). Six major bands were observed at about 20, 35, 45, 50, 60 and 85 kDa in the PA7 ECP. Anti-PA7 band serum did not react with ECP of either PA7 or F3SA (Fig 6c).

Histochemical analysis

Fungal hyphae in tissues of snakehead fish infected with *Aphanomyces invadans* isolate TA1 were positively labelled with both αF3SA and αPA7 when the secondary antibody was conjugated with either HRP (Fig. 7a, b respectively) or FITC (Fig. 8a, b), with the αF3SA serum eliciting the stronger response of the two. Hyphae were only very faintly labelled with αPA7 band serum (Figs. 7c & 8c), while no reaction occurred with the negative control (Figs. 7d & 8d).

DISCUSSION

Representative EUS, RSD and MG fish-pathogenic *Aphanomyces* isolates were shown to be very similar in terms of their protein and carbohydrate components, as indicated by SDS-PAGE banding patterns visualized using various stains, lectins and polyclonal antibodies. Furthermore, these techniques distinguished this group of pathogens from a variety of other Oomycete fungi that have been previously shown to be non-pathogenic to EUS-susceptible fish (Lilley & Roberts 1997). The results are consistent with other studies using random amplified polymorphic DNA (RAPDs) and pyrolysis mass spectrometry (PyMS) which also

Fig. 5. Western blot analyses showing the response of various polyclonal antisera with different fungal extracts. (a) Rabbit anti-saprophyte (αF3SA), (b) rabbit anti-*Aphanomyces invadans* (αPA7), and (c) rabbit anti-PA7 electroeluted band (αband). Lanes: (1) extract from *A. invadans* strain PA7; (2) extract from saprophytic *Aphanomyces* F3SA; (3) extract from MG strain NJM9030; (4) extract from *Aphanomyces astaci* strain FDL458; and (5) extract from UM strain 84-1240

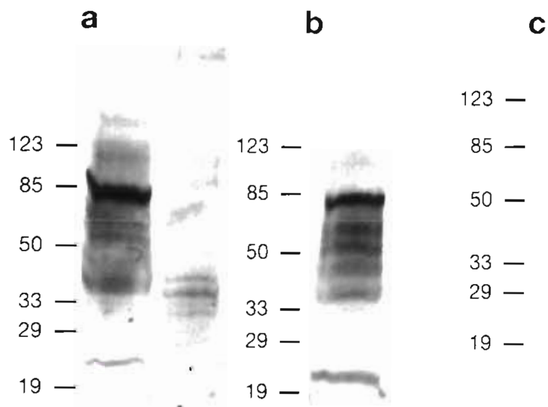


Fig. 6. Reaction of polyclonal antisera with fungal extracellular products (ECP) by Western blot analysis. (a) Rabbit anti-saprophyte (αF3SA); (b) rabbit anti-*Aphanomyces invadans* (αPA7), and (c) rabbit anti-PA7 electroeluted band (αband). Lanes: (1) ECP from *A. invadans* strain PA7; and (2) ECP from saprophytic *Aphanomyces* F3SA

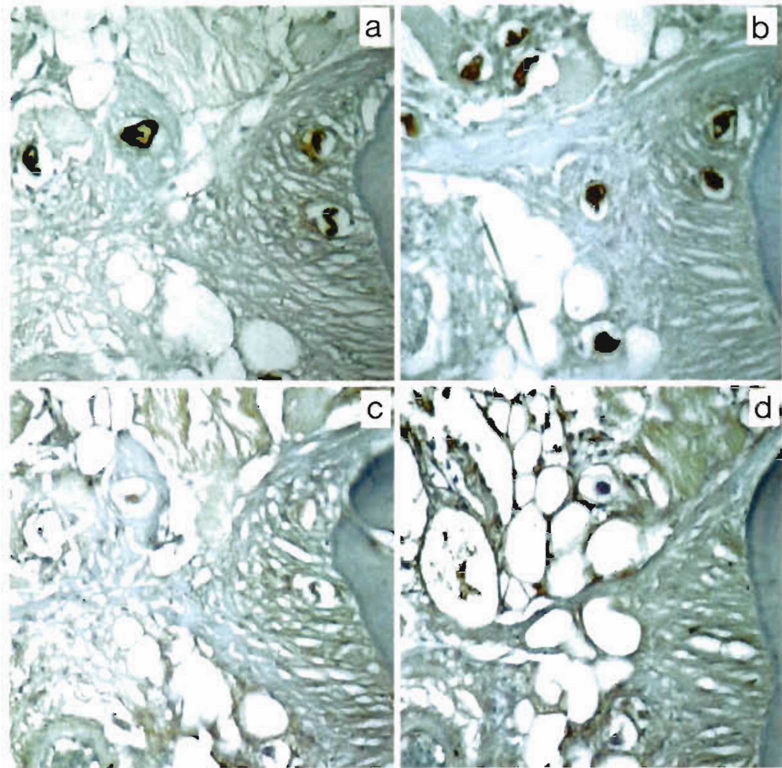


Fig. 7. Reaction of rabbit polyclonal antisera with tissues from snakehead fish infected with *Aphanomyces invadans* (strain TA1) by immunohistochemistry. Plates show the reactions obtained with (a) rabbit anti-saprophyte (αF3SA), (b) rabbit anti-*A. invadans* (αPA7), (c) rabbit anti-PA7 electroeluted band (αband), and (d) negative control (normal rabbit serum). Magnification is 100-fold

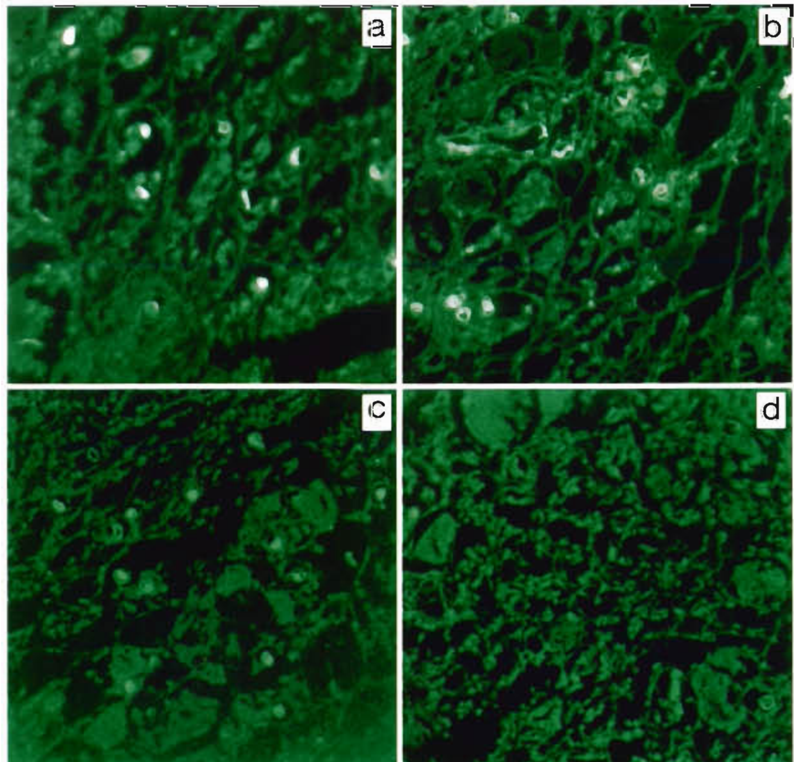


Fig. 8. Reaction of rabbit polyclonal antisera with tissues infected with *Aphanomyces invadans* (strain TA1) by the indirect fluorescent antibody technique. Plates show the reactions obtained with (a) rabbit anti-saprophyte (αF3SA), (b) rabbit anti-*A. invadans* (αPA7), (c) rabbit anti-PA7 electroeluted band (αband), and (d) negative control (normal rabbit serum). Magnification is 100-fold

indicate that the EUS, RSD and MG isolates form a homogenous group and are probably conspecific (J. Lilley et al. unpubl.). As *Aphanomyces piscicida*, the name used to describe MG isolates, is not valid under the International Code for Botanical Nomenclature (Korf 1995), the name *Aphanomyces invadans* will therefore be used here to describe all the EUS, RSD and MG fungal pathogens.

Protein profiles, as revealed by Coomassie Brilliant Blue R and silver staining, were complex and difficult to reproduce consistently. However, it was possible to identify specific bands that could act as taxonomic markers for *Aphanomyces invadans*, although they differed between the 2 stains. It may be the case that the 3 bands identified in the Coomassie Brilliant Blue R stained gels of *A. invadans* equate with the 3 major bands in EUS and RSD samples that are shown between the 43 and 87 kDa reference markers in Callinan et al. (1995). The latter workers, however, grew their fungi for 15 d at 30°C; whereas, in the present study, mycelium was grown from zoospores for a maximum of 3 d at 22°C to ensure a high proportion of protein-rich growing tips. This difference in the age of the samples would affect the biochemical composition of the cells, and thus may be reflected in the different SDS-PAGE profiles. This is also an important consideration when comparing different fungi of greatly varying growth rates. In the present study, growth times were adjusted to between 1 to 3 d so as to produce similar weight yields for each fungus. Despite this adjustment, the slower-growing *A. invadans* isolates still showed more intense coloration than the other fungi with both Coomassie Brilliant Blue R and silver stain.

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

Western blots using lectins gave a more sensitive analysis of the specific carbohydrate moieties in each sample and provided a robust technique for distinguishing *Aphanomyces invadans* samples from other fungi. Only one lectin (ECA) showed any differences between the *A. invadans* isolates and may provide a useful means of strain-typing. Some lectins with different carbohydrate specificities revealed similar banding patterns, which may be explained in that each of the bands visualized were composed of complex polysaccharides with different residues accessible to the various lectins.

Other workers have used lectin-binding properties to characterise the surface components of Oomycete zoospores, cysts and germlings (Burr & Beakes 1994). Con A-binding material (mannose and glucose) was shown to be associated with the glycocalyx of the propagules of a variety of Oomycete species (Burr & Beakes 1994). The high reactivity of Con A with all the fungi tested here suggests that there are significant amounts of these saccharides in the mycelium of saprolegniaceous species as well.

The polyclonal antisera, αF3SA and αPA7, showed a great lack of specificity, with αF3SA reacting more strongly with PA7 than homologous antiserum. These antisera also reacted strongly with bands from *Achlya* and *Saprolegnia* samples (data not shown). This is consistent with the results of Bullis et al. (1990, 1996), which showed that mouse anti-*Saprolegnia parasitica* sera even cross-reacted with some non-Oomycete fungi. However, Western blots exposed to these antisera gave simple banding patterns that were consistent for *Aphanomyces invadans* isolates and distinct from other *Aphanomyces* spp. tested. The 10 kDa band was clearly identified on *A. invadans* samples by both the αF3SA and the αPA7 rabbit antisera. This band was also identified by artificially and naturally infected snakehead fish sera (Thompson et al. 1997). Thus, the 10 kDa band has been shown to be immunogenic in fish and rabbits. However, the antiserum prepared by injecting the electroeluted 10 kDa band into rabbits did not recognise the 10 kDa band by Western blot. Instead, 2 bands at around 50 kDa were recognised on *A. invadans* samples. These bands were also faintly visible on silver-stained gels of the electroeluted 10 kDa band (Fig. 2b), and their appearance may be due to conformational changes to the 10 kDa band as a result of electroelution. Nonetheless, these bands were specific to *A. invadans* as αband serum did not cross-react with other fungus samples.

The serum raised against mycelium extract from a saprophytic *Aphanomyces* (αF3SA) did not recognise ECP secreted by homologous fungus but did recognise the same secreted products from PA7 as αPA7. This suggests that *A. invadans* isolate PA7 secreted greater quantities of ECP than the saprophyte F3SA, which may represent the release of proteolytic enzymes relevant in the pathogenesis of *A. invadans*. The molecular weights of the bands in the ECP recognised by the antisera did

not directly correspond to bands found in the mycelium extract. However, the antisera were raised against mycelium extract and therefore bands recognised in the ECP should correspond to components found in the mycelium extract. If this is the case, the difference in molecular weight of the bands revealed between the 2 samples may be explained by ECP components being altered in some way, such as being cleaved on secretion. As the αF3SA serum recognised the PA7 ECP, this illustrates the non-specific nature of the antisera. However, the lack of any reaction with the αband serum suggests that the electroeluted band is not secreted into the ECP or was lost on dialysis during sample preparation.

The rabbit antisera proved an effective diagnostic tool for identifying fungal hyphae in fish tissue, particularly by IFAT, and compares favourably with conventional Grocott staining in terms of ease of use. It would be interesting to use this technique on sections of UM-affected fish tissue to compare reactivity of the invasive fungus involved in that disease with that of *Aphanomyces invadans*. A UM *Aphanomyces* isolate was shown here to have very different protein and carbohydrate profiles from *A. invadans*, but it is possible that this is not actually the invasive pathogen involved in that disease (Lilley & Roberts 1997). Lectins were used on sections of infected fish tissue in an attempt to obtain a more specific stain for *A. invadans*. This technique has been used for other fish diseases (Marin de Mateo et al. 1993), but the lectins tested here gave no discernible reactivity. Raising monoclonal antibodies against *A. invadans* hyphal material would provide a means of developing a more specific probe for use in the immunohistochemical diagnosis of EUS.

Acknowledgements. J.H.L. and K.D.T were funded by the Overseas Development Administration of the United Kingdom. We thank the following for the provision of fungal isolates: Dr L. G. Willoughby, Dr S. Chinabut, Dr D. Bastiawan, Dr J. O. Paclibare, Dr G. C. Fraser, Dr A. Thomas, Professor K. Hatai, Professor E. J. Noga, Dr D. J. Alderman and Miss W. Valairatana.

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Editorial responsibility: David Bruno, Aberdeen, Scotland, UK

Manuscript received: February 28, 1997
Revised version accepted: May 27, 1997

Erratum

Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis

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Diseases of Aquatic Organisms 30: 187–197, 1997

- Fig. 5 on page 194 was incorrect — the same gel was shown three times. The corrected figure and its caption appear here.

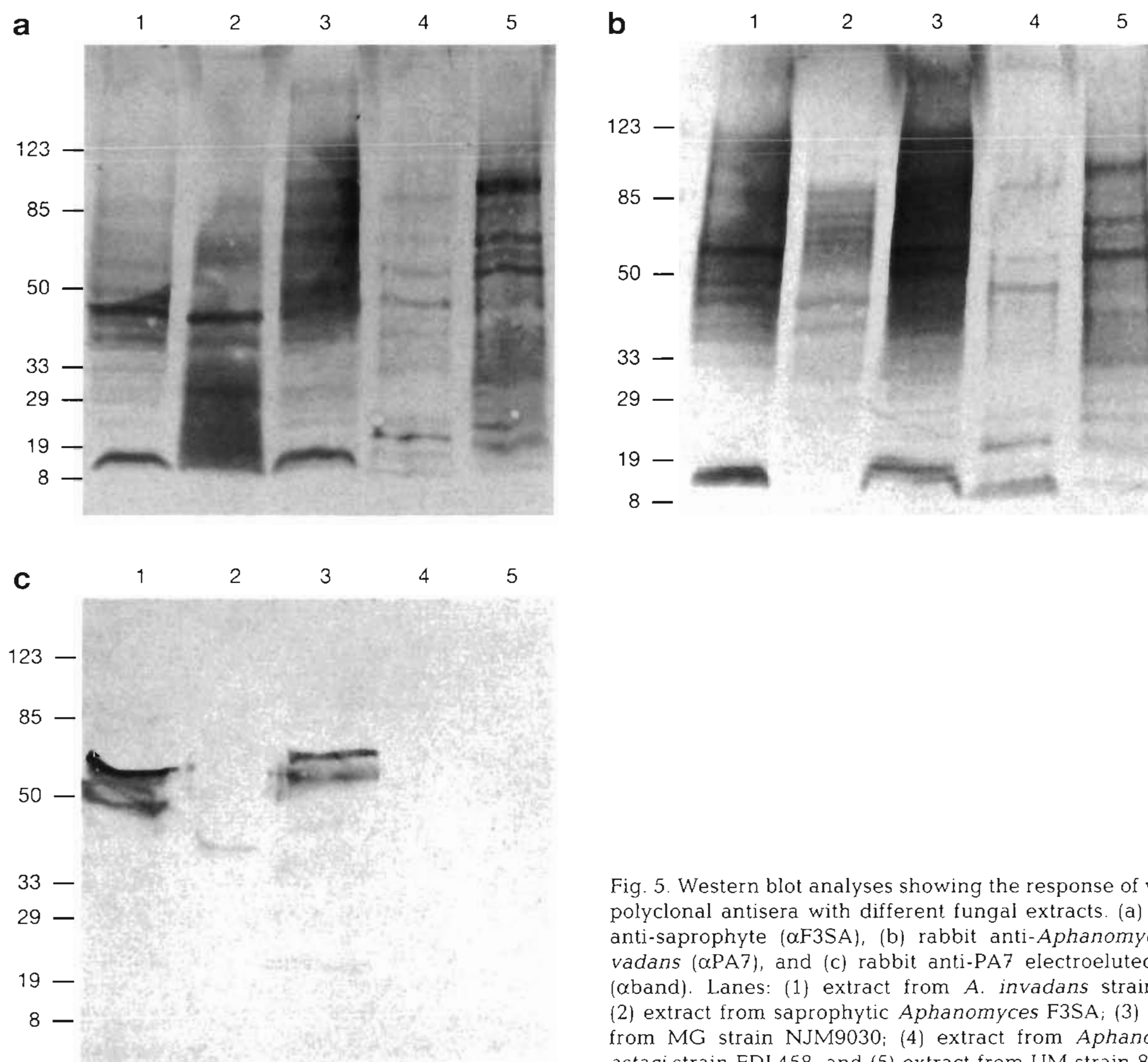

Fig. 5. Western blot analyses showing the response of various polyclonal antisera with different fungal extracts. (a) Rabbit anti-saprophyte (αF3SA), (b) rabbit anti-*Aphanomyces invadans* (αPA7), and (c) rabbit anti-PA7 electroeluted band (αband). Lanes: (1) extract from *A. invadans* strain PA7; (2) extract from saprophytic *Aphanomyces* F3SA; (3) extract from MG strain NJM9030; (4) extract from *Aphanomyces astaci* strain FDL458; and (5) extract from UM strain 84-1240