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Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel

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ABSTRACT: European catfish *Silurus glanis*, European eel *Anguilla anguilla* and rainbow trout *Oncorhynchus mykiss* were challenged by intramuscular injection of zoospores of *Aphanomyces invadans*, the oomycete associated with epizootic ulcerative syndrome (EUS). The tropical three-spot gourami *Trichogaster trichopterus* is known to be highly susceptible and was used as a positive control. European catfish were highly susceptible and rainbow trout had moderate to low susceptibility, whereas eels appeared largely unaffected. Inflammatory host response in European catfish deviated from the effects seen in most other susceptible fish species and was characterised by a more loosely arranged accumulation of macrophages, small numbers of lymphocytes and multinucleated giant cells without occurrence of EUS-characteristic mycotic granulomas. Semi-nested and single round PCR assays were developed for this study to detect *A. invadans* DNA in clinical samples of experimentally infected fish. The detection limit of the assays equals 1 genomic unit. Specificity was examined by testing the DNA of various oomycetes, other relevant pathogens and commensals as well as host DNA. The single round assay used was fully specific, whereas cross-reaction with the closely related *Aphanomyces frigidophilus* was observed using the semi-nested assay. Analysis of samples by PCR allowed detection prior to detectable histopathological lesions. Two other published PCR protocols were compared to the PCR protocols presented here.

KEY WORDS: *Aphanomyces invadans* · Epizootic ulcerative syndrome · EUS · PCR · *Silurus glanis* · *Anguilla anguilla* · *Oncorhynchus mykiss* · *Trichogaster trichopterus*

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is a disease affecting a wide range of wild and farmed freshwater and estuarine fish. It has spread rapidly especially across Asia and Australia and is now also found in some parts of the eastern USA and more recently in Africa (OIE 2007). Due to its ability to infect a wide range of fish species and its epizootic trait, EUS poses a potential threat to European freshwater and estuarine fish (Lilley et al. 1998).

The agent associated with EUS is the oomycete *Aphanomyces invadans* (Lilley & Roberts 1997) (also known as *A. piscicida*). Other diseases, e.g. red spot disease (RSD) in Australia (Callinan et al. 1989), mycotic granulomatosis (MG) in Japan (Egusa & Masuda 1971) and ulcerative mycosis (UM) in the USA (Dykstra et al. 1986), are now recognised as the same disease (Lilley & Roberts 1997, Blazer et al. 2002). Typical clinical signs of the early stages of the disease are petechial hemorrhagic lesions of the skin that continue to develop into deep necrotic ulcers (Chinabut 1998). Microscopically, the lesions are characterised by severe myonecrosis and extensive granulomatous myositis associated with penetrating hyphae of *A. invadans* (Noga et al. 1988). Presumptive diagnosis of EUS can be based on clinical signs or demonstration of the EUS-characteristic mycotic granulomas. Identification of the primarily responsible pathogen, *A. invadans*, is essential for definitive diagnosis (Chinabut & Roberts 1999, OIE 2006).

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Since morphologic characteristics are not sufficient for accurate species determination of *Aphanomyces invadans*, identification may be achieved by demonstration of *Aphanomyces* spp. sporulation morphology (Scott 1961), combined with the temperature–growth profile in culture and distinctive pathogenicity in EUS susceptible fish species (Lilley & Roberts 1997). However, isolation of the primary pathogen from infected fish tissues poses a problem. Ulcerative skin lesions are frequently colonised by opportunistic water moulds (Blazer et al. 1999) including other *Aphanomyces* spp. (Roberts et al. 1993). They are likely to overgrow *A. invadans*, due to its slow growth rate compared to other species. Furthermore, bacteria can inhibit growth of *A. invadans.* PCR based approaches are extremely useful as diagnostic methods, where conventional methods of pathogen identification fail (Oidtmann et al. 2004, 2006).

Little is known about the susceptibility of European fish species to EUS (Khan et al. 1998). Among European fish species, Crucian carp *Carassius carassius* is noted to be naturally susceptible (Miyazaki 1994, cited in Lilley et al. 2001) and rudd by experimental challenge (Hatai 1980 cited in Baldock et al. 2005). Several introduced fish species are known to be susceptible, including goldfish (Phadee et al. 2004), bluegill, channel catfish (Hawke et al. 2003) and rosy bitterling (Hatai 1980 cited in Baldock et al. 2005).

The potential impact of an introduction of the pathogen into Europe on wild and farmed fish populations is unclear. European eels and European catfish have not previously been experimentally challenged. The objectives of this study were (1) to test the susceptibility to EUS of selected freshwater fish species, which are relevant in the European aquaculture industry, and (2) to establish a PCR method suitable for the detection of *Aphanomyces invadans* directly from clinical specimens. After completion of the majority of the work for the current study, 2 PCR assays for the detection of *A. invadans* were published (Phadee et al. 2004, Vandersea et al. 2006). We compared those methods to the new methods presented here.

MATERIALS AND METHODS

Susceptibility challenges of fish. ***Oomycete culture and sporulation:*** Zoospores for fish challenge experiments were obtained from *Aphanomyces invadans* isolate NJM9701 (isolated Ayu, from Japan, 1997, Phadee et al. 2004). All *A. invadans* isolates were cultured at 20°C on glucose-peptone agar (GPA) (OIE 2003). For spore production, an improved method (Marshall 1998) was adopted and slightly modified: *A. invadans* mycelium was allowed to grow on sterile hemp seeds placed on agar. The covered hemp seeds were placed in V8 broth (5% Campbells V8 Juice, 0.2% $CaCO_3$) at 20°C for 3 d and washed 3 times in autoclaved pond water (APW). The seeds were then placed in 2 ml Eppendorf tubes containing APW with 0.05% $CaCO_3$ and left for 24 h. The number of zoospores in the suspension was calculated by using a Neubauer counting chamber and was adjusted to the required concentration.

Fish: Fish used for challenge experiments were European catfish *Silurus glanis* (12.1 ± 6.8 g), European eels *Anguilla anguilla* (5.1 ± 3.6 g), rainbow trout *Oncorhynchus mykiss* (10.4 ± 7.4 g), and the Southeast Asian species three spot gourami *Trichogaster trichopterus* (4.7 ± 2.1 g). Rainbow trout were from our own specific pathogen free stocks, whereas European catfish and European eels were purchased from a local fish farm. Three-spot gouramis were obtained from a local ornamental fish wholesale trader.

Challenge experiments: European catfish, European eels and rainbow trout were tested for their susceptibility to *Aphanomyces invadans*. Three-spot gouramis, which are known to be susceptible to EUS (Catap & Munday 2002), were used as a positive control to confirm the pathogenicity of *A. invadans* isolate NJM9701, which had been in culture for several years. Isolate NJM9701 was chosen for the challenge experiments since it readily produces spores, which has not been seen in the same way in the other isolates. Three replicate challenge experiments and one control were run for each fish species, using 12 fish per tank (50 l tanks equipped with air stones and aquarium heaters). The fish were allowed to acclimate to the test tanks for a minimum of 7 d. Rainbow trout were kept in a flow-through system at 18°C, while the other fish were kept at 23°C with partial daily water changes. Anaesthetised fish (tricane methanesulphonate, MS-222, Thomson & Joseph) received 0.1 ml (eels and gouramis) or 0.2 ml (catfish and rainbow trout) of a 10 000 spores ml^{-1} suspension, which was administered intramuscularly into the left dorsal trunk muscle just below the anterior end of the dorsal fin using a 26 gauge needle. Control fish were inoculated with equal quantities of APW. The fish were monitored daily throughout the duration of the trial. One fish from each tank was sampled at 1, 2, 4, 6, 8, 10, 12, 14, 17, 21, 28 and 35 d post-injection (p.i.). Dead and heavily affected fish were collected additionally when necessary. The sampled fish were killed with an overdose of MS-222, and eels were additionally decapitated. All of the fish were examined for external lesions, and 4 mm cubes of skin and attached muscle tissue were excised using sterile scalpel blades from the injection site for DNA isolation and histopathological examination.

Histopathological processing: The excised tissues were fixed in 10% buffered formalin solution for at

least 24 h, dehydrated with ethanol and embedded in paraffin. The paraffin blocks were sectioned to 3 µm thick slices with a rotary microtome. Slides were stained with hematoxylin and eosin (H&E) and with Grocott's methenamine-silver nitrate (GMS).

PCR assays. *Oomycetes and other organisms tested:* Seven *Aphanomyces invadans* isolates were included in the study (see Table 1). To test primer sets for possible cross-reaction, we examined several closely related oomycetes, bacterial species known to be associated with skin lesions and inflammation in fish, and some ectodermal fish parasites. Specificity was also tested against DNA from all fish species employed in this study (see Table 1).

DNA preparation: Methods used to extract DNA from pure oomycete and fungal cultures have been described previously (Oidtmann et al. 2004). DNA extraction from infected and non-infected fish tissues, bacterial cultures and from zoospores of *Aphanomyces invadans* NJM9701 was performed using a DNeasy tissue kit (Qiagen) according to the manufacturer's protocol. Fish tissue samples were processed immediately after sampling. DNA from fish tissues was routinely extracted from 25 mg of tissue and suspended in 100 µl of the buffer provided with the kit.

Sequencing: In order to generate sequence data for *Aphanomyces invadans* NJM9701 in the target region, the ITS1, 5.8S ribosomal DNA and ITS2 region was amplified using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen) following the manufacturer's instructions. Sequencing was carried out by GENterprise.

Design of Aphanomyces invadans-specific primers: The ITS1, 5.8 ribosomal DNA and ITS2 sequences of *A. invadans* isolates NJM9701 (accession no. EU422990), RF6 (AY283642), and NJM9801 (AY455773) were aligned with sequences of some closely related species: *A. astaci* M96/1 (AY310499), *A. laevis* (AY283648), *A. laevis* CBS107.52 (AY310497), *A. repetans* Se (AY683897), *A. repetans* FA (AY683892), *A. euteiches* (AY647190), *A. helicoides* CBS210.82 (AY310496), *A. stellatus* CBS578.67 (AY310498), *A. stellatus* (AY455774), *Achlya bisexualis* NJM9905 (AY647189), *Saprolegnia diclina* ATCC90215 (AY455775), *S. parasitica* CBS540.67 (AY310504), and *Aphanomyces* sp. 84–1240 (AF396683) using Megalign (DNAstar, Lasergene) (Fig. 1). Regions unique to *A. invadans* were identified by eye within heterologous sections of the ITS1 and ITS2 regions. Each primer was subjected to NCBI Blast searches to determine the individuality of the primer sequence. The primers chosen for evaluation in the PCR assay were BO73 (5′-CTT GTG CTG AGC TCA CAC TC-3′; forward primer) and BO639 (5′-ACA CCA GAT TAC ACT ATC TC-3′; reverse primer) for the first round PCR. For the second round PCR primers were BO487 (5′-TGT GTT GAT ATT ACA CGA CT-3′; forward primer) and BO639 (reverse primer). The primers were designed prior to the identification of *A. frigidophilus* (Ballesteros et al. 2006, B. Oidtmann & S. Geiger unpubl. data), and hence its similarity to *A. invadans* was not taken into account at the time.

Semi-nested PCR assay: For first round PCR, reactions were performed in a 50 µl reaction volume containing ReddyMix PCR Mastermix (ABgene AB-0575), 0.6 µM of each primer and 2.5 µl of DNA template. Amplifications were carried out in a thermal cycler with the following cycling parameters: initial denaturation at 96°C for 5 min; 20 cycles of 1 min at 96°C, 1 min at 58°C and 1 min at 72°C; followed by a final extension at 72°C for 5 min. A negative control containing all reaction components except DNA template was included. Second round PCR amplifications were performed using the same reaction mixture as described for the first PCR round, except for the amount of template DNA, which was 1 µl of PCR product from the first round PCR, and the second round primers. Cycling parameters were as follows: initial denaturation at 96°C for 5 min; 30 cycles of 1 min at 96°C, 1 min at 56°C, and 1 min at 72°C; followed by a final extension at 72°C for 5 min. Aliquots of 7.5 µl from each reaction were electrophoresed on a 1.3% agarose gel containing 0.5 mg ml^{-1} of ethidium bromide.

Fish tissues from the challenge experiments were initially analysed using the semi-nested PCR assay and later retested using the single round PCR.

Single round PCR assay: Conditions were as described above for the first round PCR, except that 50 cycles were run.

Other published PCR assays: Primers used were ITS11/ITS23 (Phadee et al. 2004) and Ainvad-2F/Ainvad-ITSR1 (Vandersea et al. 2006). PCR reactions were performed in a 50 µl reaction volume containing ReddyMix PCR Mastermix (ABgene AB-0575). Amplifications using the primer set described by Phadee et al. (2004) were carried out with a primer concentration of 0.6 µM per primer and using the following program: 5 min at 94°C; 40 cycles each consisting of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C; and final extension of 5 min at 72°C. Primer concentration for the method described by Vandersea et al. (2006) was 0.5 µM per primer and amplifications were carried out using the following thermocycling program: 2 min at 95°C; 35 cycles each consisting of 20 s at 95°C, 30 s at 54°C, and 45 s at 72°C; final extension of 5 min at 72°C.

Sensitivity: In order to determine the lowest limit of detection of the semi-nested PCR assay, serial dilutions of pure DNA extracted from mycelium and zoospores of *Aphanomyces invadans* NJM9701 were analysed.

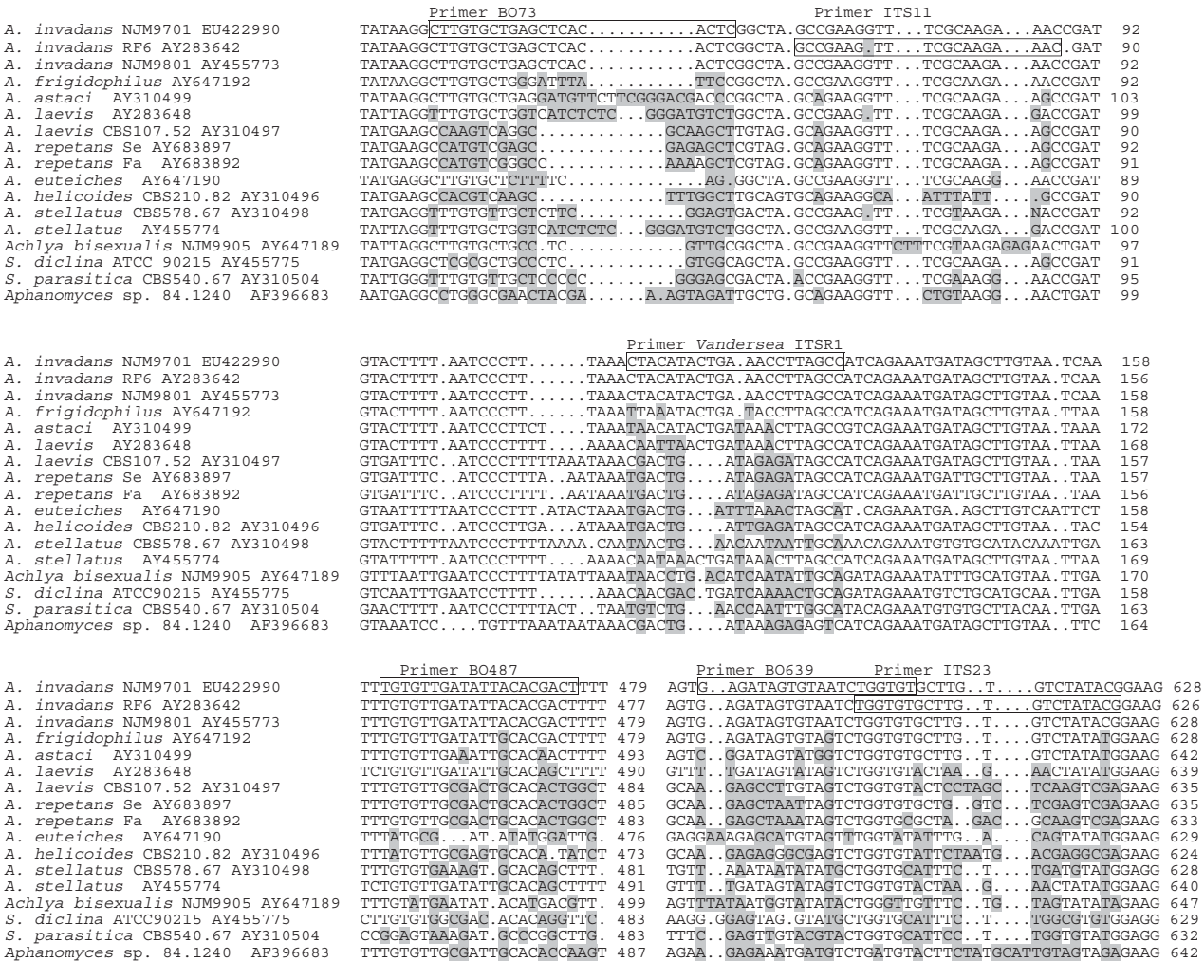

Fig. 1. *Aphanomyces* spp. and other oomycetes. Alignment of partial internal transcribed spacers 1 and 2, showing positions of primers BO73, BO639, BO487, ITS11, ITS23 (the latter two from Phadee et al. 2004), and Ainvad-ITSR1 (Vandersea et al. 2006). GenBank accession nos. of sequences provided. Nucleotides deviating from primer sequences are shaded in grey. Dots stand for absence of nucleotide in the respective position. For full species names see Table 1

DNA was extracted from mycelium of cultured *A. invadans* and DNA concentration of the eluate was measured with a spectrophotometer. A 10 ng μl^{-1} stock solution was prepared and 10-fold serially diluted. Of each dilution, 2.5 µl was submitted to PCR. For zoospores, a 200 µl spore suspension containing 10 000 *A. invadans* zoospores was subjected to DNA extraction and suspended in 100 µl of the buffer provided with the kit. The 100 µl eluate was 10-fold diluted and 2.5 µl of each dilution was submitted to PCR. Additionally, sensitivity was tested using DNA extracted from fish tissue spiked with *A. invadans* spores. A 10000 spores stock solution was prepared and 10-fold serially diluted. Samples of 25 mg uninfected fish tissue were spiked with 10 000, 1000, 100, 10 and 1 spore respectively prior to DNA extraction.

To determine the detection limits of the single round assay presented here and the PCR assays described by Phadee et al. (2004) and Vandersea et al. (2006), 10-fold serial dilutions of pure DNA extracted from mycelium were submitted to PCR.

Specificity: To determine specificity, the primer sets presented here were tested against various strains of *Aphanomyces invadans*, a panel of related oomycetes, a panel of organisms known to parasitise fish skin or known to be associated with skin lesions in fish, and host DNA (see Table 1). Once a culture of *A. frigidophilus* was available (isolated in our own laboratory from signal crayfish in 2006, B. Oidtmann & S. Geiger unpubl. data), we also tested the specificity of the PCR assays on DNA of *A. frigidophilus.* The primer sets described by Phadee et al. (2004) and

Vandersea et al. (2006) were submitted to a reduced set of specificity tests (see Table 1). To ensure sufficient amounts of DNA of the organisms tested for specificity of the primers BO73, BO487 and BO639, control PCRs were run using primers known to produce an amplicon with these organisms. Primers used to amplify fish DNA in the positive controls were ChordVf and ChordVr (Jarman et al. 2004), primers for bacterial DNA were EUB f933 and EUB r1387 (Ji et al. 2004), primers for DNA of ectoparasitic protozoans were NS1 and NS2 (Dams et al. 1988), primers for DNA of *A. astaci* were BO525 and BO640 (Oidtmann et al. 2004) and primers for DNA of oomycetes and fungi were ITS1 and ITS4 (White et al. 1990).

RESULTS

Susceptibility and infection of challenged fish

Three-spot gourami. Both zoospore-injected fish and control fish showed reddening and scale loss on Days 1 to 3, most likely attributed to traumatic injury caused by the insertion of the needle, and dark patches from Day 4 at the site of inoculation. Changes in behaviour in the groups that received spore injections first occurred on Day 6 p.i. and consisted of forward and backward teetering movements. Behaviour of control fish appeared normal during the course of the trial. Deeply penetrating focal ulcers that exposed the underlying musculature started to appear from Day 8 p.i. (Fig. 2A). EUS-characteristic mycotic granulomas were found in 18 of the 36 injected fish from Day 6 p.i. (Fig. 3A, Table 2); hyphae of *Aphanomyces invadans* (diameter 5 to 10 µm) were encapsulated by layers of epitheloid cells, which again were surrounded by a fine layer of fibtroblasts. The muscle tissue was infiltrated with inflammatory cells and muscle fibres were degenerating. The intensity of epitheloid cell reaction increased over time. The presence of grossly visible lesions was usually combined with the presence of mycotic granulomas except for 1 individual sampled on Day 8 p.i. This fish was found dead, showing a skin ulcer 0.4 cm in diameter at the site of injection; granulomas were not present in the lesion area, but numerous hyphae could be demonstrated in GMS-stained histological sections. In all trial groups PCR indicated the presence of *A. invadans* DNA. Of 36 fish infected with *A. invadans* spores, 29 tested clearly positive by PCR (Table 2). All gouramis with mycotic granulomas and GMS-stained hyphae tested positive by PCR. Fish sampled later than Day 14 showed neither mycotic granulomas, nor was *A. invadans* DNA detected by PCR (Fig. 4A). Control fish tested negative by PCR and neither hyphae nor mycotic granulomas were found in histological sections.

European catfish. On Day 2 p.i. both fish that had received a zoospore injection and control fish showed minor swelling at the site of injection which increased in size over time in the fish challenged with *Aphanomyces invadans* spores. The lesions developed into deeply penetrating focal ulcers exposing the

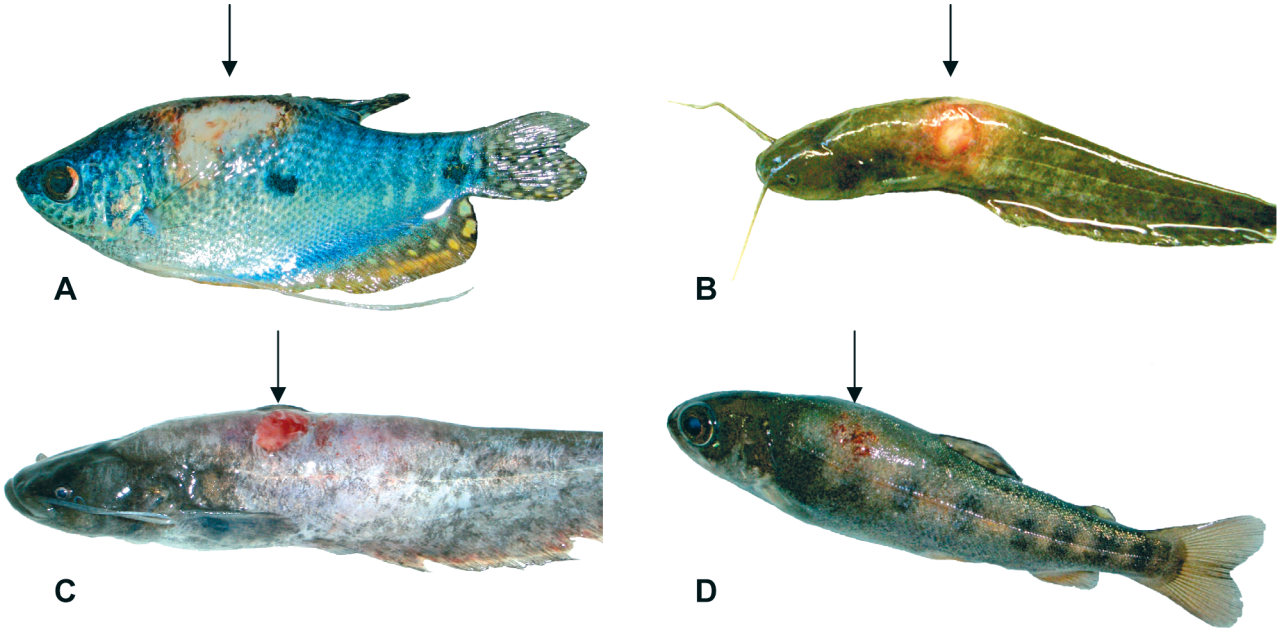

Fig. 2. *Aphanomyces invadans*. Skin lesions (arrows) of fish injected with *A. invadans* spores: (A) three-spot gourami, (B) and (C) European catfish, and (D) rainbow trout

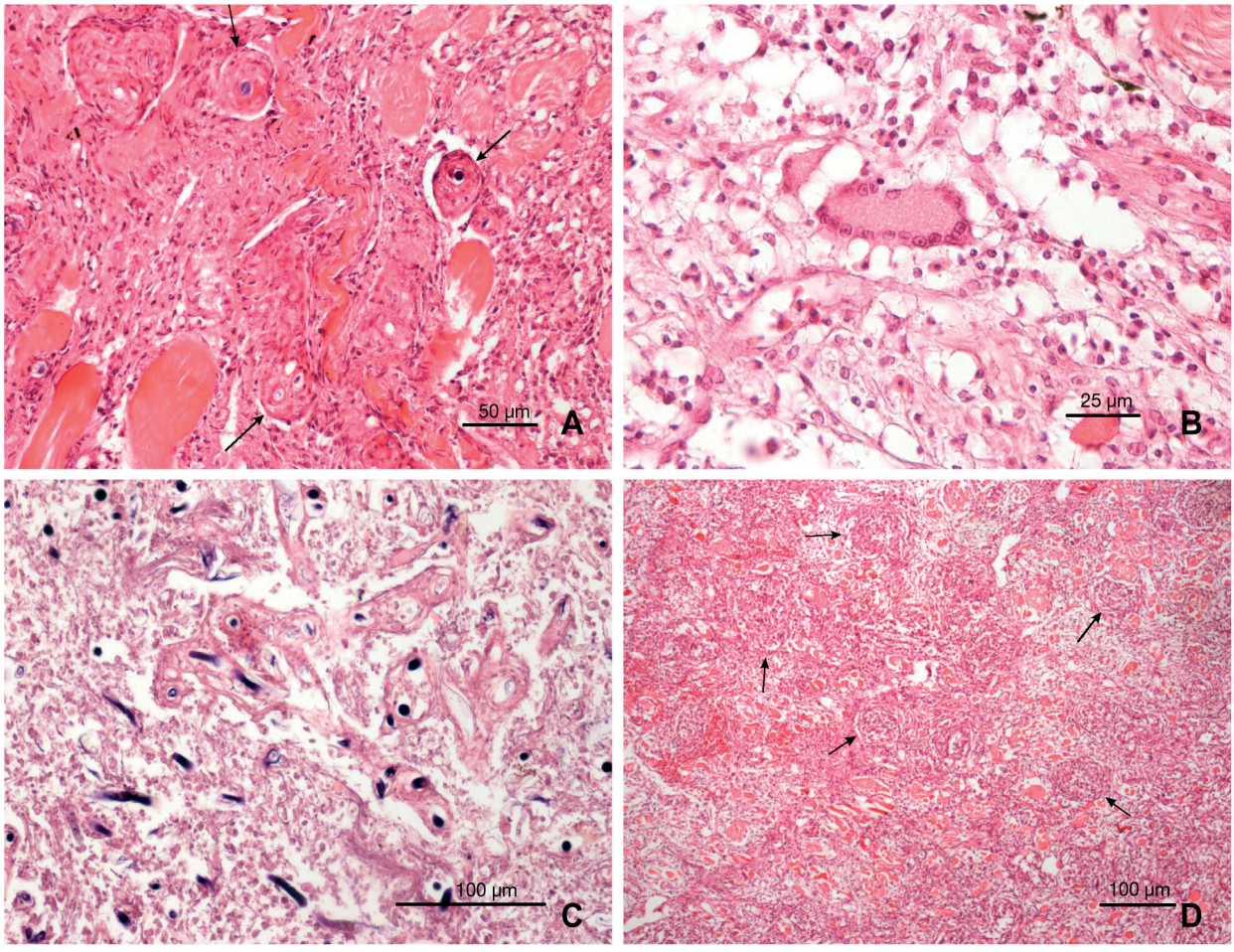

Fig. 3. *Aphanomyces invadans*. Skin and muscle histopathology of infected fish: (A) mycotic granuloma (arrows) in a three-spot gourami, H&E, (B) multinucleated giant cell, European catfish, H&E, (C) stained hyphae, rainbow trout, Grocott and (D) mycotic granuloma (arrows), rainbow trout, H&E

underlying musculature (Fig. 2B,C). In histopathological sections, no typical mycotic granulomas were found. However, multifocally disseminated accumulations of unencapsulated hyphae (diameter 5 to 10 µm) were present. Degenerated and necrotic muscle fibres were infiltrated by a few lymphocytes and macrophages; formation of giant cells (Langhans type, Fig. 3B) were detected from Day 10 p.i. All fish showing hyphae in GMS-stained histological sections were also positive by PCR (Fig. 4B, Table 2). In the fish of the control group no amplification products could be visualised and no histopathological changes were found.

Rainbow trout. No grossly apparent lesions could be seen in the first 3 d of the trial. In the following days, clinical signs observed in the area of infection were reddening, swelling and limited scale loss (Fig. 2D). At no time did lesions develop into open ulcers such as those observed in three-spot gouramis and European catfish. EUS-characteristic mycotic granulomas in the musculature, with hyphae (diameter 5 to 10 µm) surrounded by epithelioid cells, could be demonstrated in 4 of 36 rainbow trout infected with *Aphanomyces invadans* spores (sampled on Days 12, 14, 21 and 28, Fig. 3C,D, Table 2); the degrading muscle tissue was heavily infiltrated by inflammatory cells. In 7 further fish, fungal hyphae were detected between muscle fibres without granulomatous inflammatory response (Days 6 to 28). All 11 fish, which stained GMS-positive for hyphae located in the musculature, were positive for *A. invadans* DNA by PCR (Fig. 4C). Fish of the control group did not show any grossly visible lesions or histopathological changes and tested negative for presence of *A. invadans* by PCR.

European eels. In contrast to the other 3 species, no inflammatory response such as reddening, swelling, erosion, ulcers or granuloma formation were found in European eel except in 1 individual. This eel, sampled on Day 2 p.i., showed erythema at the site of injection

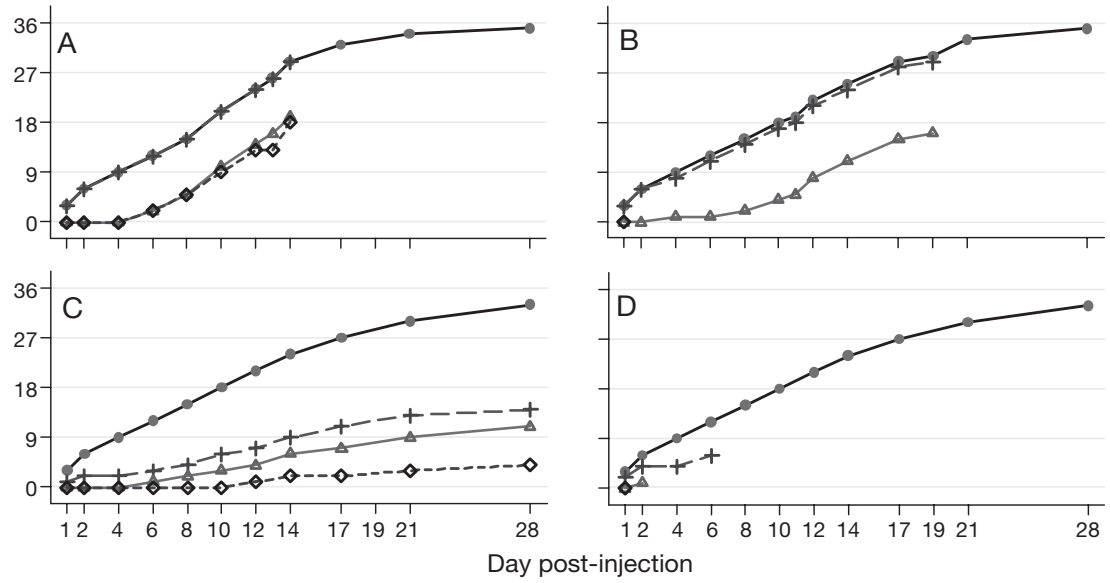

Fig. 4. *Aphanomyces invadans* challenge experiments. Number of fish sampled and infected by *A. invadans* for (A) three-spot gourami, (B) European catfish, (C) rainbow trout, and (D) European eel. Lines are cumulative number of fish sampled (●), positive by semi-nested PCR (+), with visible hyphae in GMS-stained sections (△) and with mycotic granuloma (◇). Symbols show sampling points. No infection found in any species at Day 35

and a few hyphae without any cellular response between muscle fibres. No fungal invasion, inflammatory response or mycotic granulomas could be seen in any other eels, including those in the control group. PCR amplicons of the expected size were obtained from 6 eels within the first 6 d of the trial (Table 2). After Day 6, no more positive PCR results were obtained from the eels (Fig. 4D). All individuals in the control group tested PCR-negative.

PCR assays

Choice of primers. Primer locations in the sequence of ITS1 and ITS2 regions of *Aphanomyces invadans* NJM9701 and in relation to other oomycetes are shown in Fig. 1. Primer BO73 diverges in the target area by at least 7 nucleotides (nt) from closely related oomycetes. Primers BO487 and BO639 differ by 3 and 5 nt from *A. astaci* (Fig. 1) and even more from all other oomycete species sequences available at the time of primer design. Primers BO487 and BO639 only diverge by 1 nt each from *A. frigidophilus*, of which the sequence became available at a later stage.

Limits of detection. The lowest amount of DNA extracted from *Aphanomyces invadans* mycelium detected by the semi-nested (Fig. 5A) and single round PCR assay (Fig. 5B) was 25 fg. In relation to data provided on the genome size of other oomycetes (Kamoun 2003), this corresponds to 0.1 to 1 genomic unit. When submitting DNA extracted from *A. invadans* zoospores to PCR, the detection limit of the semi-nested PCR assay was the equivalent of 0.025 spores (Fig. 6). The detection limit for spiked muscle tissue samples was 10 spores (Fig. 6).

Using primers Ainvad-2F/Ainvad-ITSR1 (Vandersea et al. 2006) the lowest amount of DNA detected was 2.5 pg, which corresponds to 10 to 100 genomic units (gel electrophoresed results not shown). When using primers ITS11/ITS23 (Phadee et al. 2004) minimum DNA amount detected was 2 ng, corresponding to 8000 to 80000 genomic units (gel electrophoresed results not shown).

Specificity of primers. The specificity of the semi-nested PCR assay with primers BO73/BO639 and BO487/BO639 was tested using nucleic acid templates of the organisms listed in Table 1. A single amplicon of 152 bp was observed in the second round PCR in reactions using DNA of *Aphanomyces invadans* strains as the template (Fig. 7). No positive results or non-specific products were visualised for any of the other organisms tested except when 10 ng or more of *A. frigidophilus* DNA were submitted to the semi-nested PCR assay (10 ng correspond to approx. 0.4 to 4×10^5 genomic units). This was due to a high homology of primers BO487 and BO639 (used in the second round PCR) with *A. frigidophilus* DNA (primer sequences only differ by 1 nt each from *A. frigidophilus* in target region). No cross-reactions were observed using the single round PCR assay (primers BO73 and BO639; Table 1).

All DNA extracts from the clinical study were retested using the single round PCR assay to confirm

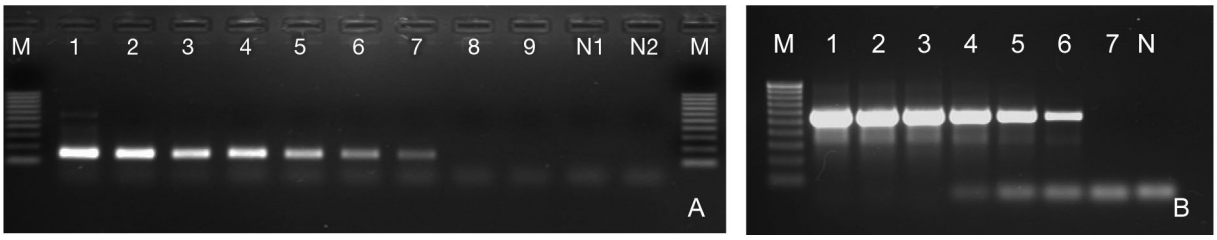

Fig. 5. Sensitivity of the semi-nested and single round (primers BO73 and BO639) PCR using genomic *Aphanomyces invadans* DNA. M, 100 bp marker. (A) Results of semi-nested PCR. Lane 1, 25 ng of genomic DNA; Lane 2, 2.5 ng; Lane 3, 250 pg; Lane 4, 25 pg; Lane 5, 2.5 pg; Lane 6, 250 fg; Lane 7, 25 fg; Lane 8, 2.5 fg; Lane 9, 250 ag; N1, negative control of first round PCR; N2, negative control of semi-nested PCR. (B) Results of single round PCR. Lane 1, 2.5 ng; Lane 2, 250 pg; Lane 3, 25 pg; Lane 4, 2.5 pg; Lane 5, 250 fg; Lane 6, 25 fg; Lane 7, 2.5 fg; N, negative control

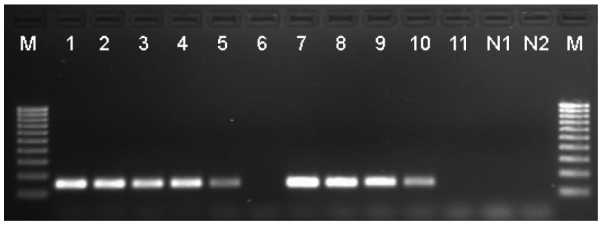

Fig. 6. Sensitivity of semi-nested PCR using DNA extracted from *Aphanomyces invadans* spores. M, 100 bp marker; N1, negative control of first round PCR; N2, negative control of semi-nested PCR; Lanes 1 to 7, serial dilution of DNA extracted from zoospores: Lane 1, 250 spores; Lane 2, 25 spores; Lane 3, 2.5 spores; Lane 4, 0.25 spores; Lane 5, 0.025 spore; Lane 6, 0.0025 spore; Lanes 7 to 11, 25 mg fish tissue spiked with spores prior to DNA extraction: Lane 7, 10 000 spores; Lane 8, 1000 spores; Lane 9, 100 spores; Lane 10, 10 spores; Lane 11, 1 spore

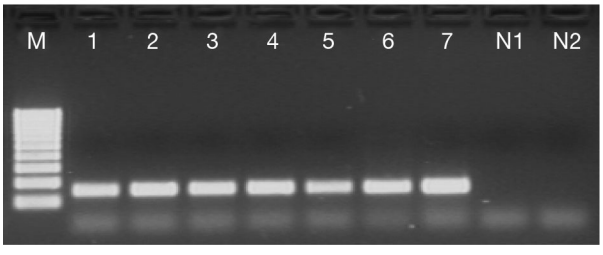

Fig. 7. Results of semi-nested PCR with *Aphanomyces invadans* DNA. M, 100 bp marker; N1, negative control of first round PCR; N2, negative control of semi-nested PCR; Lane 1, *A. invadans* NJM9701; Lane 2, *A. invadans* WIC; Lane 3, *A. invadans* PA7; Lane 4, *A. invadans* PA8; Lane 5, *A. invadans* UM3; Lane 6, *A. invadans* B99C; Lane 7, *A. invadans* T99G2

the PCR results obtained using the semi-nested assay. The DNA extracts had been stored for 2 yr at –20°C when retested. Of 29 samples previously PCR positive from three-spot gouramis, 28 tested positive, 5 out of 6 from European eel, 13 out of 14 from rainbow trout and all 29 previously PCR positive samples from European catfish were confirmed. The lack of confirmation in 3 samples is most likely due to DNA degradation over 2 yr.

Whereas no cross-reaction was observed when primers suggested by Vandersea et al. (2006) were applied, an amplicon of the size expected for *Aphanomyces invadans* was observed when primers ITS11/ITS23 (Phadee et al. 2004) were applied to 10 ng or more of *A. frigidophilus* DNA (primer ITS11 is fully homologous to *A. frigidophilus* in the target region and primer ITS23 deviates by only 1 nt).

In the control PCRs for non-*Aphanomyces invadans* organisms, an amplicon of the expected size was obtained using group- or species-specific primers. A summary of the results of the specificity tests is presented in Table 1.

DISCUSSION

Challenge experiments

We have demonstrated that European catfish produce typical macroscopical EUS ulcerative skin lesions when intramuscularly injected with secondary zoospores of *Aphanomyces invadans* strain NJM9701. However, mycotic granulomas encapsulating the penetrating hyphae typical for EUS were not observed in histological sections. Instead, from Day 10, a more diffuse inflammatory response was visible with an involvement of multinucleate giant cells. The cellular immune response observed in European catfish is therefore clearly distinct from the commonly observed host response consisting of mycotic granuloma formation. Similar observations were reported by Hawke et al. (2003), who described histopathological characteristics of naturally EUS infected channel catfish *Ictalurus punctatus* and black bullhead *Ameiurus melas,* suggesting a different pattern of cellular inflammatory response to *A. invadans*. In European catfish, giant cells do not seem to possess the ability to

Table 1. *Aphanomyces* spp. and other species used to test specificity of the PCR assays. Amplification and absence of amplification are shown as + and – respectively. If no result is shown for a primer set/DNA combination, the DNA has not been tested using these primers

Species	ID no.	BO73/ BO639	BO487/ BO639	ITS11/ ITS23	Ainvad-2F/ Ainvad-ITSR1
Aphanomyces invadans					
A. invadans	NJM9701	+	+	+	+
A. invadans	PA7	+	+	+	+
A. invadans	PA8	+	+	+	+
A. invadans	WIC	+	+	+	+
A. invadans	UM3	+	+	+	+
A. invadans	B99C	+	+	+	+
A. invadans	T99G2	+	+	+	+
Other *Aphanomyces* spp.					
A. astaci	S_v	–	–	–	–
A. astaci	D_1	–	–	–	–
A. astaci	K_v	–	–	–	–
A. astaci	P_c	–	–	–	–
A. brassicae	CBS121.80	–	–	–	–
A. cladogamus	CBS108.29	–	–	–	–
A. cochlioides	CBS477.71	–	–	–	–
A. euteiches		–	–	–	–
A. frigidophilus		–	(+)	(+)	–
A. helicoides	CBS210.82	–	–	–	–
A. iridis	CBS524.87	–	–	–	–
A. irregulare	CBS278.81	–	–	–	–
A. laevis	CBS107.52	–	–	–	–
***Saprolegnia* spp.**					
S. diclina		–	–	–	–
S. ferax	ATCC26116	–	–	–	–
S. furcata		–	–	–	–
S. litoralis		–	–	–	–
S. parasitica	CBS540.67	–	–	–	–
S. terrestris		–	–	–	–
Other oomycetes					
Achlya racemosa	CBS578.67	–	–	–	–
Pythium flevoense, female	CBS 232.72	–	–	–	–
Fungal species					
Aspergillus sp.		–	–	–	–
Fusarium solani		–	–	–	–
Candida albicans		–	–	–	–
Ectoparasitic protozoans					
Chilodonella sp.		–			
Ichthyobodo necator		–			
Ichthyophthirius multifiliis		–			
Trichodina sp.		–			
Bacterial species					
Aeromonas hydrophila	04045	–			
Aeromonas salmonicida salmonicida	04107	–			
Citrobacter freundii		–			
Edwardsiella tarda		–			
Escherichia coli	DSM1103	–			
Flavobacterium psychrophilum		–			
Listonella anguillarum	97007	–			
Mycobacterium fortuitum		–			
Mycobacterium marinum		–			
Pseudomonas aeruginosa	DSM1117	–			
Staphylococcus aureus	DSM2569	–			
Fish muscle tissue					
Anguilla anguilla		–			
Cyprinus carpio		–			
Oncorhynchus mykiss		–			
Silurus glanis		–			
Trichogaster trichopterus		–			

control the penetrating hyphae (this study). On the other hand, in common carp *Cyprinus carpio* which are known to be resistant to EUS, multinucleated giant cells play an important role in cellular defence mechanisms to eliminate the pathogen (Wada et al. 1996). However, the ability of multinucleate giant cells to eliminate the hyphae seems to vary between fish species and their relevance to the outcome of EUS infection is unclear (Johnson et al. 2004).

European eels tested here were not susceptible to *Aphanomyces invadans* infection. In spite of the high spore dose inoculated intramuscularly, no cutaneous lesions could be induced. Negative PCR results after Day 6 and the absence of any inflammatory response and hyphae in histopathological sections (except for 1 eel sampled on Day 2 p.i.), suggest that the eels were able to successfully eliminate *A. invadans* spores or hyphae in the early stage of infection; alternatively, the host may not have provided suitable conditions for germination of *A. invadans* spores and/or growth of *A. invadans* hyphae.

No ulcerative skin lesions could be induced in the rainbow trout tested in the present study. The outcome of our infection experiments largely correspond to results presented by Thompson et al. (1999), who used spore doses from 10^2 to 10^5 per rainbow trout for inoculation. With a spore dose of 10^3 spores, which is comparable to our study, those authors did not observe grossly visible ulcerative lesions. However, we detected hyphae in GMS-stained histological sections until Day 28 p.i.

The lack of positive detection of *Aphanomyces invadans* in gouramis 14 d p.i. and in European catfish 19 d p.i. (Fig. 4) suggests that these species have either developed clinical infection or managed to control the infection within this time. By contrast, positive detection of *A. invadans* was still observed in rainbow trout after 3 and 4 wk p.i. (Fig. 4), suggesting that the affected fish were unable to eliminate the pathogen within that time.

Water temperature is an important factor in the development of EUS. Generally, mortalities from EUS occur when water temperatures are comparatively low—either due to a sudden drop in temperature associated with massive rainfalls and/or in the cold season of the year (Chinabut et al. 1995, Lilley et al. 2002). So far, the majority of fish species affected by EUS are tropical species and the temperatures considered 'low' are around 25°C (Pathiratne & Jayasinghe 2001). The experiments with rainbow trout reported here were undertaken at the bottom end of the temperature range at which *A. invadans* has been reported to cause natural infection in fish and below the optimum temperature range for growth of *A. invadans in vitro* (Lilley & Roberts 1997). The temperature chosen was also slightly above the optimum temperature range for rainbow trout. The combination of suboptimal growth temperature for the pathogen and suboptimal functionality of the immune response in the host could explain why 3 wk p.i., rainbow trout were still found with active infection.

In snakehead, a reduced and delayed immune response has been observed when fish injected with *Aphanomyces invadans* spores were kept at 19°C, compared to 26 and 31°C (Chinabut et al. 1995). A temperature of 19°C is below the preferred range for this fish species and the authors attributed the delayed immune response to temperature.

Further challenge experiments of rainbow trout over longer time periods and a range of temperatures could be valuable in assessing the outcome of EUS infection in this species. This might help in establishing whether rainbow trout could potentially act as a carrier for the pathogen.

We initially tried to use goldfish as positive control fish, since they have been repeatedly found to be susceptible to *Aphanomyces invadans* infection (Chinabut & Roberts 1999, Phadee et al. 2004). However, despite repeated attempts to infect goldfish, we did not succeed. This might suggest differences in pathogenicity of *A. invadans* strains depending on host species, or differences in strain susceptibility among goldfish.

PCR methods

Analytical test sensitivity

To identify the detection limits of the various PCR assays compared in this study, cellular DNA extracted from pure culture of *Aphanomyces invadans* was used. The detection threshold of the single round and semi-nested PCR assay (this study) was 25 fg (0.1 to 1 genomic unit), whereas the assays described by Vandersea et al. (2006) (detection threshold: 2.5 pg; 10 to 100 genomic units) and Phadee et al. (2004) (detection threshold 2 ng; 8000 to 80000 genomic units) appeared less sensitive. For this study we adapted the PCR protocol described by Phadee et al. (2004) by increasing the number of PCR cycles from 25 to 40, since we had been unable to generate a PCR product at 25 cycles with DNA from *A. invadans* isolate NJM9701. We also adapted the primer concentration given by Vandersea et al. (2006) from 25 pM to 0.5 µM per primer. Phadee et al. (2004) reported an analytical test sensitivity of 250 fg when submitting pure fungal DNA of isolate NJM0204, whereas Vandersea et al. (2006) did not provide any data on test sensitivity.

Differences in the sensitivity between the various PCR protocols can partially be explained by the different number of amplification cycles. Phadee et al. (2004): 40 amplification cycles; Vandersea et al. (2006): 35 amplification cycles; compared to the present study: 50 (single round assay) and 20 + 30 cycles (semi-nested assay). However, the relatively low detection limit for the PCR protocol described by Phadee et al. (2004) is likely to be mainly due to a mismatch of primer and template. The sequence of the isolate used by the authors to determine the analytical test sensitivity is identical to the sequence of strain RF6 for the areas shown in Fig. 1. In the target region, *Aphanomyces invadans* strain RF6 (and strain NJM0204) provides a 100% match with primer ITS11. However, the sequence of strain NJM9701 deviates in the target area of primer ITS11. At the time of submission of this manuscript, 16 *A. invadans* sequences were available on GenBank, covering the area targeted by primer ITS11; of those, 6 sequences provide a 100% match with primer ITS11, whereas 10 sequences have an additional nucleotide in the target area of the primer. Corresponding checks were done for other primers used: Forward primer BO73 matches 100% with all 16 *A. invadans* sequences. For the area of the reverse primer BO639, sequence data were only available for 10 *A. invadans* isolates. Nine of those provide a 100% match, whereas 1 sequence appears to have an additional nucleotide (*A. invadans* isolate IMI836083, accession no. AF396684). The reverse primer presented by Vandersea et al. (2006) was 100% homologuos with all 16 *A. invadans* sequences. Sequence information in the area of the forward primer is only available for 2 *A. invadans* isolates, for which the forward primer also provided a 100% match. Vandersea et al. (2006) tested the specificity of their PCR on 3 *A. invadans* strains isolated in the USA from Atlantic menhaden. In the present study, we tested the primers described by Vandersea and colleagues on 6 additional strains, including strains from Southeast Asia, and with all of them an amplicon of the expected size was obtained.

Our present results suggest that it is important to test whether PCR primers are fully homologous with *Aphanomyces invadans* isolates from different geographical origins and species.

The detection threshold determined in tests using muscle tissue spiked with *Aphanomyces invadans* zoospores is likely to be the best indication of the detection limit of *A. invadans* in tissue samples. This was determined to be 10 zoospores (corresponding to 10 genomic units) for the semi-nested assay, which was reduced (by $10^{2.6}$) compared to pure genomic DNA.

Phadee reported the analytical test sensitivity of their assay for infected goldfish muscle tissue as 500 fg (35 PCR cycles). However, the method of establishing the amount of *Aphanomyces invadans* DNA in the tissue sample was not explained.

The test sensitivity required from a diagnostic test depends on its intended application. A PCR protocol with limited analytical test sensitivity may be sufficient for the detection of *Aphanomyces invadans* in clinical cases of suspected EUS, where fresh tissue material is available and intensive growth of *A. invadans* can be expected. However, if the intended application is the detection of potential carriers or the testing of samples which have undergone some degradation, a more sensitive assay may be more suitable. Further applications requiring a test with high analytical sensitivity might be tests of samples from the aquatic environment or other aquatic animals to investigate the still unresolved question of potential reservoirs of *A. invadans* in the period between EUS outbreaks.

The emergence of new Aphanomyces species, such as *Aphanomyces frigidophilus*, highlights the fact that it remains important to try to cultivate pathogens, even if cultivation is difficult, as is the case with *A. invadans*.

Test specificity

We have shown that the single round assay presented here (primers BO73 and BO639) and the primers described by Vandersea et al. (2006) specifically amplify the DNA of *Aphanomyces invadans* and not the DNA of any other species tested (Table 1). Given the potential cross-reaction with *A. frigidophilus* of the semi-nested PCR assay presented here, we recommend the single round assay (primers BO73 and BO639).

To date, *Aphanomyces frigidophilus* has only been reported in Arctic char eggs in Japan, white-clawed crayfish *Austropotamobius pallipes* in Spain (Ballesteros et al. 2006) and signal crayfish *Pacifastacus leniusculus* (B. Oidtmann & S. Geiger unpubl. data). Since *A. frigidophilus* may also act as a saprophyte on dermal lesions of fish as do other water molds (Roberts et al. 1993, Blazer et al. 1999), it is important to use an assay capable of excluding *A. frigidophilus.*

Confirmation that the amplified PCR product is indeed *Aphanomyces invadans* can be best achieved by sequencing the PCR product. The expected size of the PCR product amplified using the single round assay presented here is 564 bp with the amplified product covering part of the ITS1, the 5.8 ribosomal DNA and part of the ITS2 region. Several sequences of other closely related Aphanomyces and other oomycetes have been made publicly available for these areas and therefore sequencing of the PCR product will assist in confirming the identity of the template DNA. Sequence differences to other closely related oomycetes are likely to allow clear confirmation (12 nt difference from *A. frigidophilus,* 28 nt difference from *A. astaci* differs in the respective region). Although the PCR presented by Vandersea et al. (2006) appeared to be highly specific, the PCR product generated is shorter and covers a sequence region less used for sequencing and hence fewer sequences are available for comparison. Therefore, the single round assay presented here might be more suitable if confirmation via sequencing is applied.

Mode of infection

In the experiments presented here, fish were exposed to *Aphanomyces invadans* by intramuscular injection. Positive PCR results in the first days of sampling are probably attributable to remnants of the inoculation material. Furthermore, the amount of spores inoculated does not conform to a natural infection. Hence, the setup of experiments is unsuitable to show whether the PCR methods presented are suitable for the detection of *A. invadans* in the early stages of infection or low level infection. A model of infection which more closely mimics the natural infection process, such as bath challenge, would need to be conducted. Experimental induction of EUS by bath challenge in some known EUS-susceptible species has proven difficult (Lilley et al. 2001). Challenge experiments with strains of *A. invadans* from Southeast Asia have suggested that zoospores attach to the dermis and not the epidermis before invasion (Lilley et al. 1998). Skin injury is, therefore, considered to be required to provide a portal for infection. In contrast, Kiryu et al. (2003) found that zoospores of a USA strain of *A. invadans* were able to attach to and invade intact skin of Atlantic menhaden and induce EUS in bath challenged fish without inflicting skin damage prior to challenge. However, they also found a much higher incidence of ulcer formation in Atlantic menhaden to which skin damage had been inflicted prior to bath

challenge, compared to untraumatised fish. In epidemics in the wild, Atlantic menhaden has stood out as particularly susceptible to the disease. Further studies may examine whether fish susceptible by injection of the pathogen are similarly susceptible by bath challenge — either with or without previously applying some damage to the skin.

Samples from early stages of naturally infected fish would be valuable, but were not available to us since the disease has so far not occurred in Europe. However, the analysis of the sensitivity of the presented methods suggests that the assays are suitable for detection of low levels of infection and possibly to detect asymptomatic carriers.

Potential spread of EUS into Europe

In the past, occurrence of the EUS was limited to the Asian-Pacific region and the east coast of the USA. However, EUS has a clear tendency to spread to previously unaffected areas. Outbreaks in Southeast Asia were reported from more than 18 different countries over the last 35 yr (Lilley et al. 1998) and distribution of *Aphanomyces invadans* in the eastern USA is no longer restricted to the estuaries (Hawke et al. 2003). While natural outbreaks to date have been limited to latitudes between 35°N and 35°S (Lilley et al. 1998), a spread of the disease to temperate zones cannot be ruled out. For example, there are reports of EUS occurring in freshwater ponds in Louisiana in the winter months at a water temperature of 10 to 15°C (Hawke et al. 2003). Also, experimental infection of ayu *Plecoglossus altivelis* has been successful at 15 to 16°C (Wada et al. 1996). The above findings suggest that environmental conditions for *A. invadans* in Europe might be suitable at least during summer months. However, the potential risk of *A. invadans* is hard to assess since, at the moment, it is still unclear how natural infections are initiated. No reproductive structures such as sporangia or zoospores have been observed in EUS lesions (Roberts et al. 1993), so fish are considered as a terminate host with an alternate source of infection present in the environment (Vandersea et al. 2006).

Our knowledge on the susceptibility of fish species, native or introduced into Europe, to EUS is still very limited. Future studies should investigate the susceptibility of further species, especially those most likely to be affected due to their natural environment. This will assist in assessing the likely impact of an introduction of the pathogen into Europe.

In conclusion, we have shown that European catfish are highly susceptible, rainbow trout moderately susceptible and European eel largely resistant to infection by *Aphanomyces invadans* via intramuscular injection. The inflammatory response observed in European catfish by histopatholgy does not include mycotic granulomatosis. We have also shown that a PCR assay developed for this study is fully specific and highly sensitive for the detection of *A. invadans* in clinical samples. Two other published PCR assays were examined, of which one was found to be highly specific and sensitive.

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