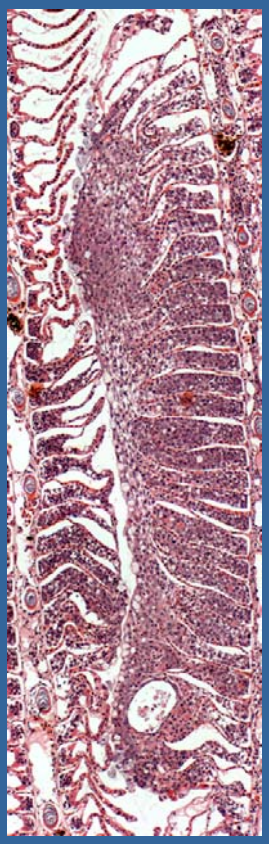
Final Report

Aquafin CRC - Atlantic Salmon Aquaculture Subprogram:
Establishment of challenge for AGD

Barbara Nowak, Neil Young, Richard Morrison, Michael Attard,
Benita Vincent and Phil Crosbie

June 2008

Aquafin CRC Project 3.4.1 (3)
(FRDC Project No. 2004/215)



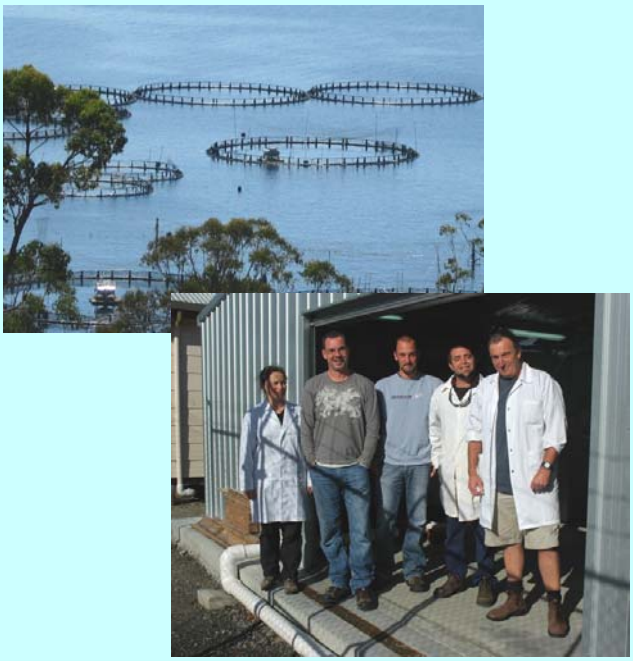


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Table of contents

2004/215 Aquafin CRC - Salmon Aquaculture Subprogram: Establishment of challenge for AGD

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OBJECTIVES:
1. Standardisation of AGD challenge models (research)
2. Use of challenge to appraise trial vaccines developed in the vaccine development project (essential service)
3. Provision of gill-associated and cultured amoebae to collaborators (essential service)
4. Cryopreservation of virulent amoebae (research)
5. Maintenance of infection tank (essential service)
6. Provision of freshwater salmon for experiments in other projects (essential service)

Non-Technical Summary

OUTCOMES ACHIEVED

This project has increased our knowledge of Amoebic Gill Disease, in particular about the pathogen and the dynamics of infection. We have described a new species of neoparamoeba, *Neoparamoeba perurans,* and showed that it has been consistently associated with AGD worldwide. Stocking density, acclimation to sea water and amoeba batch variability affected AGD infections. During this project challenge protocols were developed, which have been successfully used and their results correlated well with field challenge. This project provided crucial support for all AGD research through provision of amoebae and salmon for all AGD projects and running experimental challenges for trial vaccines.

The main objectives of this project were to provide essential service for AGD research. During this project we standardised existing AGD challenge protocol and developed a new in vivo gill attachment challenge assay. Both challenge protocols have been successfully applied in AGD research. Research on virulent amoebae resulted in a description of a new species, which consequently has been shown to be involved in all AGD cases worldwide. This discovery led to the development of new diagnostic tests, which are now available for confirmation of AGD infections and further research.

In conclusion, this project has not only provided essential support for all AGD research by supplying amoebae and salmon and running AGD challenges for the experimental vaccines, but also increased our knowledge and understanding of AGD.

KEYWORDS:
Amoebic Gill Disease, salmon, aquaculture

Acknowledgments

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Background

Commercial salmonid culture in Australia is among the country's top three aquaculture sectors. The salmonid aquaculture industry is an important player in the Tasmanian economy. Production of Atlantic salmon and ocean trout in Tasmania is approximately 22000 tonnes and accounts for 95% of Australia's annual salmonid production. The majority of Tasmanian Atlantic salmon production is from estuarine and marine grow-out cages in southeast Tasmania. At present the Tasmania salmonid growers are fortunate in that their stocks are free of many infectious diseases that hamper salmon culture in other parts of the world. However, over the last 15 - 20 years the production of this Atlatnic salmon in Tasmania has been increasingly affected by the occurrence of amoebic gill disease (AGD). AGD results in an ongoing and increasing drain on industry resources. AGD was first described by Munday (1986) and remains the most serious health issue facing salmon producers in Australia. The losses of stock and implementation of treatment regimes account for 10-20% of production costs (Munday et al 2001). The causative agent of AGD is *Neoparamoeba* spp., a widely distributed, free-living and parasitic marine amoeba. At present the only useful treatment is freshwater bathing of entire seacages once a particular level of infection determined on the basis of gross gill scores is achieved.

For the impact of AGD to be ameliorated, from a host-pathogen perspective two major areas require investigation. Firstly it is essential that more economic and effective treatment options are identified and secondly, experimental vaccines need to be developed and tested. This project is critical to the evaluation of alternative treatment options in the interim and to the eventual development of an efficacious vaccine in the longer term.

The project followed two paths: Firstly, performance of research and provision of services to other AGD projects comprised three key elements:
- development of a robust, well-defined challenge model
- use of the model to perform challenge tests, which were a major service component for the vaccine development project
- provision of the organism to collaborators, which was an important service component to all AGD projects.

Secondly, results flowing from this investigation contribute to the broader outcomes as stated by Aquafin CRC by:

1) “reducing the economic impact of disease in finfish farming”
2) “providing environmentally-friendly approaches to disease management”
3) “improving industry and government responsiveness to disease outbreaks”.

Need

The continued existence of Atlantic salmon farming in Tasmania is threatened by AGD. Production is expected to increase over the next few years and this will undoubtedly lead to an increase in the incidence of AGD. The AGD control method of freshwater bathing has increased in frequency with the production growth and this trend is expected to continue. There is an urgent need to reduce the impact of AGD on the salmon industry and maintain the industry viability in the future. Multidisciplinary teams have been assembled to achieve this outcome through a number of projects. The projects are complementary, and in some cases interdependent where progress in one are depends on progress in another. This was particularly the case with the service component of this project and the vaccine development program, where supply of infective material and a means of controlled testing of candidate vaccines were integral to the success of the latter. Vaccine development requires identification of specific antigens from the pathogen that will elicit a protective immune response in the host, hence the need for significant quantities of infective material. Similarly, success of further investigations of AGD treatments depends on supply of cells for initial screening of a battery of potential therapeutants in vitro before attempting further trials. The research component of this proposal, which is the development of a standard AGD challenge method, is essential for the success of these projects. We needed to be able to induce AGD in a consistent manner to appraise treatments and candidate vaccines before moving on to costly field trials.

Aquafin CRC Centre Commonwealth Agreement:
This proposal made an essential contribution towards the objective to reduce the economic impact of disease in finfish farming (Program 3 Health) and provide environmentally friendly approaches to disease management (Program 3 Health).

This project provided training for one postdoctoral fellow and one technician and contributed to consolidation of knowledge and capability for parasitology of aquatic animals, which was one of the priorities.

Objectives

1. Standardisation of AGD challenge models (research)
2. Use of challenge to appraise trial vaccines developed in the vaccine development project (essential service)
3. Provision of gill-associated and cultured amoebae to collaborators (essential service)
4. Cryopreservation of virulent amoebae (research)
5. Maintenance of infection tank (essential service)
6. Provision of freshwater salmon for experiments in other projects (essential service)

Methods

Objective 1. Standardisation of AGD challenge models (research)
(Attard et al 2006, Crosbie et al 2007, Crosbie et al in prep)

As a part of the amoebic gill disease challenge development several factors were investigated to standardise the challenge method. These included: variation in virulence amongst individual isolation batches of *N. perurans*, the effect of fish biomass and the effect of seawater acclimation on progression of amoebic gill disease in salmon. A short-term (2-3 d) challenge method was also developed to be used to assess the impact of any prospective treatments on the ability of *N. perurans* to attach to salmon gills and cause measurable pathology.

Variation in virulence (Crosbie et al 2007)
Three batches of *N. perurans* isolated on 3 separate occasions were used to infect fish in small scale infection experiments. Salmon were housed in 6 separate closed systems each consisting of 3 x 80 L tanks and a 80L sump, each tank held 4 fish (mean weight 60.7 g +/- 10.4 g) which were acclimated over 2 days and were then infected with *N. perurans* at 500 cells per L. All 3 inoculating batches of amoebae were collected, using the adherence method of Morrison et al (2004) at 2 day intervals, and inoculated to systems the following day. Prior to inoculation quadruplicate aliquots of each suspension were counted to ensure accurate numbers and cell densities adjusted to an infecting concentration of 500 cells per L. Each batch was then divided into 2 and used to inoculate duplicate systems. To allow an even dispersal of amoebae to each system the cells were added to a watering can containing 5 L of seawater then carefully added to individual tanks and sumps. Water quality parameters (salinity and ammonia) were monitored every second day and a 30% water exchange was necessary at 3 days post infection for each system. Each infection was allowed to run for 8 days, then all fish were euthanased with a lethal dose of anaesthetic and gills were removed and the second left hemibranch was processed for histology to confirm the association of amoebae with lesions. Amoebic gill disease severity was quantified by determining the percentage of filaments with lesions by histology of the second left hemibranch and by photographs of 6 hemibranchs per fish. Severity of AGD induced by separate batches of amoebae was then compared. A control group consisting of 12 salmon housed in a separate 300L tank showed no gross lesions. Data were analysed by ANOVA with SPSS software. There were no significant differences between duplicate systems infected with individual batches, and therefore data were pooled at this level.

Biomass investigation (Crosbie et al in prep)
Seawater acclimated salmon (approximately 100g) were placed into duplicate 4000L tanks at densities of either 50 or 150 fish per tank (densities of 5 and 15 kg per tank respectively). Temperature was maintained at 16°C. 24h after tank allocation all fish were challenged with *N. perurans* which had been isolated and inoculated to tanks as described for the variation in virulence experiment (above). For the duration of the experiment water quality was checked every 2-3 days and water exchanges carried as required. Moribund or dead fish were

removed from the tanks (those moribund were euthanased) and gills removed to confirm the presence of AGD by histology. The experiment was terminated after 38 days and surviving fish were euthanased and gill samples taken to confirm AGD status.

Seawater acclimation investigation (Attard et al 2006)
Different acclimation protocols were examined to identify the most appropriate seawater acclimation regime. Three groups of 23 fish (approx. 110 g) were infected with *N. perurans* after:

1. no incremental seawater acclimation
2. acclimation over 7 days (5 ppt per day)
3. acclimation over 14 days (5 ppt per day then held for a further 7 days)

All acclimations occurred in 300L tanks, then immediately prior to challenge with *N. perurans* fish were anaesthetised, tattooed with alcian blue to allow group identification, and then placed in 1 3000L tank for challenge with amoebae isolated and inoculated to tanks as described above. A separate 300L tank containing 5 fish representing each group was not challenged.

Short-term in vivo challenge model (Crosbie et al 2007)
This model was developed to test the impact of antibiotics on the ability of *N. perurans* to attach to salmon gills and cause AGD. Antibiotic treatment of inoculating amoebae is desirable to limit the number of potentially disease causing *Vibrio* spp., which had caused co-infections in some larger scale challenge trials. Amoebae were collected using the method of Morrison et al (2004) and after counting were divided equally into six 500 mL glass bottles (approximately 3.0×10^{-5} cells per bottle), then filled to 200 mL with 0.2 µm filtered sea water. Antibiotics oxolinic acid (20 µg mL^{-1}) and ampicillin (Sigma) at concentrations of 20 and 9.6 µg mL^{-1} respectively were added to three bottles only and then all six bottles were incubated for 17 h at 14 °C on an orbital mixer. Antibiotic concentrations used were chosen to reflect the highest concentration that was not detrimental to the amoebae, as previously determined empirically (data not shown). A 50 mL aliquot was then removed from each bottle, amoebae were concentrated by centrifugation (500 x *g* for 5 mins) then counted, and cell viability was confirmed by the ability to adhere to Petri dishes and by neutral red (0.33 % w/v) inclusion. Observation of the parasome (the endosymbiont) in the vast majority of adhered cells confirmed their identity as *Neoparamoeba* spp.

After a 17 h incubation period with antibiotics, *Vibrio* spp. numbers were estimated as colony forming units on thiosulphate citrate bile sucrose (TCBS) agar using the spread plate viable count method. A five hundred microliter aliquot was removed from each of two bottles (one with and one without antibiotic exposure) and serially diluted tenfold to 10^{-4} in sterile sea water, and then 100 µL of the each dilution was spread across the surface of TCBS agar plates and incubated at room temperature for 24h. Colonies were then counted and numbers expressed as colony forming units mL^{-1} after correction for the extent of the dilution and the volume of the inoculum.

Immediately prior to challenge, seawater-acclimated Atlantic salmon (naïve to AGD) of approximately 80 g (n = 36) were equally divided into 3 groups and

then placed into identical 320 L capacity recirculating systems each consisting of three 80 L tanks connected to an 80 L sump. Tanks in all 3 systems had been isolated from sumps and filled with 1 µm sea water to 35 L only and supplied with aeration before 4 fish were placed into each (i.e. 12 fish per system), 2 systems were then challenged with amoebae (8500 cells L^{-1}) either treated or not treated with antibiotics whilst the third system remained unexposed to amoebae. After 6 h all fish were removed from the challenge tanks and placed into 3 identical adjacent systems containing (1 µm filtered) sea water without additional amoebae. Water quality was maintained during this phase of the experiment by a daily 25 % sea water (1 µm filtered) exchange. Total ammonia levels remained below 2 mg L^{-1}, water temperatures were 15.5 °C and salinity constant at 35 ‰. After 72 h the experiment was terminated and all fish were euthanased by lethal anaesthesia (Aqui-S at 0.02% v/v). Gills were removed and placed into seawater Davidson's fixative for 17 h, then transferred to 70% ethanol for 2 h. The second left gill hemibranchs from all fish were then processed for routine histology. Percentages of gill filaments displaying typical early AGD lesions, i.e. regions of hyperplasia in association with amoebae (Adams and Nowak, 2003) were then determined.

2. Use of challenge to appraise trial vaccines developed in the vaccine development project (essential service)

Several challenges were performed for collaborators using protocols that improved during the course of the project (see Appendix 3). Trials were completed for CSIRO, who employed an expression library immunisation strategy and produced various mixtures of *N. perurans* cDNA [under Aquafin CRC projects 3.4.4 (FRDC 2002/251 - Development of a vaccine for amoebic gill disease: genomic and cDNA library screening for antigen discovery) and 3.4.4(2C) (FRDC 2004-217 - Development of an AGD vaccine: phase II (CSIRO component)] which were tested for protective ability against AGD. Initially these were carried out in replicated 4000L tanks at the Aquaculture Centre at the University of Tasmania, but later the purpose built challenge facility financed by the Tasmanian Salmon Growers Association and the Aquafin CRC was used.

3. Provision of gill-associated and cultured amoebae to collaborators (essentials service)

Gill-associated amoebae were routinely isolated, using the adherence method of Morrison et al (2004), for collaborators as required. For the majority of the project the yields of 4 isolations per week were forwarded to researchers at UTS. Various environmental and gill-derived strains of *Neoparamoeba pemaquidensis* and *N. branchiphila* were maintained on malt yeast agar plates being sub-cultured every 15-20d. When required in large numbers they were grown on multiple lawn plates then 3-5 days post plate inoculation cells were harvested by gentle flushing with sterile seawater. Initially both gill-derived amoebae and cultured *N. pemaquidensis* cells were required for the subtractive cDNA library constructed by researchers at CSIRO and they were also required by collaborators at UTS for monoclonal antibody production and antigen profiling studies.

4. Cryopreservation of virulent amoebae (research)
(Young et al 2007, Young et al 2008a, Young et al 2008b)

Virulent amoeba
Investigation into identity of the virulent amoeba was performed as described in Young et al 2007 and Young et al 2008. Briefly, two amphizoic amoebae *Neoparamoeba pemaquidensis* and *N. branchiphila* have been cultured from AGD-affected gills. To determine which of these amoebae is associated with AGD lesions we PCR amplified the 18S rRNA gene of non-cultured, gill-derived (NCGD) amoebae from AGD-affected Atlantic salmon (*Salmo salar* L.) using *N. pemaquidensis* and *N. branchiphila*-specific oligonucleotides. Variability in PCR amplification led to comparisons of 18S and 28S rRNA gene sequences from NCGD and clonal cultured, gill-derived (CCGD) *N. pemaquidensis* and *N. branchiphila*. Phylogenetic analyses inferred from either 18S or 28S rRNA gene sequences unambiguously segregated a clade consisting of NCGD amoebae from other members of the *Neoparamoeba* genus. Clade-specific oligonucleotide probes that hybridise 18S rRNA were designed, validated and used to probe gill tissue from AGD-affected Atlantic salmon as well as archival samples from AGD affected fish from Tasmania, USA, Scotland, Ireland, Spain and New Zealand. PCR protocols were developed for biopsy procedures.

Cryopreservation method
The standard method used, which is based on the American Type Culture Collection (ATCC) protocol for *Neoparamoeba* spp. cryopreservation, uses the cryoprotectant dimethylsulphoxide (DMSO) at 7.5%, equilibration time of 20 mins at room temperature, controlled cooling from ambient to approximately -40°C, then immersion in liquid nitrogen. When applying this protocol to *N. perurans* cells appear rounded in shape and are not viable post thaw. DMSO can be toxic at a range of concentrations to various protozoans but this toxicity may be ameliorated with the inclusion of blood serum in the freezing medium (Hubálek, 2003). Recent variations to methods and/or media used to cryopreserve *N. perurans* include:

1. Starvation for 4 days (incubated in sterile seawater with antibiotics)
2. Addition of salmon serum (10%)
3. Addition of heat-inactivated salmon serum (10%) and glucose (7.5%) as an extracellular cryoprotectant
4. Adjustment of DMSO concentration to 5% with salmon serum (10%)
5. DMSO (7.5%) and rabbit serum (10%), equilibration time 30 mins
6. DMSO (7.5%) and rabbit serum (5%), equilibration time 30 mins
7. Replacement of DMSO with glycerol (10%) and rabbit serum (10%), equilibration time 2 hours
8. Glycerol (5%) and rabbit serum (10%), equilibration time 2 hours

5. Maintenance of infection tank (essential service)

At present the only source of infective amoebae capable of initiating AGD is fish which have AGD, therefore an infection tank was maintained where salmon were replaced as they succumbed to AGD. In this way there was a constant source of virulent amoebae. The tank is a 4000L recirculating system with an external biofilter. Temperature is maintained at 15-18°C, salinity is 35 ppt and pH of 8.2, water quality is maintained with weekly 25-50% water exchanges. Densities very according to demand for amoebae but range from 25-60 fish.

6. Provision of freshwater salmon for experiments in other projects (essential service)

All salmon were procured from the commercial hatchery Salmon Enterprises Tasmania (Saltas). They were collected and transported to the University of Tasmania as required to supply the infection tank and for researchers working on all the projects listed in Objective 5.

Results/Discussion

Objective 1. Standardisation of AGD challenge models (research)

(Attard et al 2006, Crosbie et al 2007, Crosbie et al in prep)

Variation in virulence (Crosbie et al 2007)

All 3 batches of *N. perurans* caused AGD in fish infected after 8 days; only 2 from 72 fish displayed no typical AGD lesions within this period. The results however show a clear variation in virulence as determined from the percentage of filaments showing lesions (Figure 1). The sources of variation are the amoebae, the fish, the holding system or a combination of these factors. The isolation method relies on the active adherence of the amoebae to plastic and is assumed to select for viable amoebae. As amoebae are isolated from a pre-existing, long-term infection cells isolated at any one time are likely to vary in the stage of their reproductive cycle and/or life span. Any loss of viability in a proportion of cells after harvesting in one batch relative to another or, any differential rates of cell division between batches after inoculation would effectively alter the dose which could affect the timing of the onset of clinical AGD and severity of lesions over an 8 day period. Morrison et al. (2004) have shown a linear relationship between amoebae dose and severity of AGD, therefore variation in pathology seen here may be due to differences in amoebae survival or numbers immediately after inoculation because of one or more cell divisions. All fish were from the same cohort and were handled in an identical manner, but could be a source of individual variation, although the degree of replication (n = 24) for each batch should account for any individual variation. The holding systems are of identical design and conditions were monitored and kept uniform throughout the experiment.

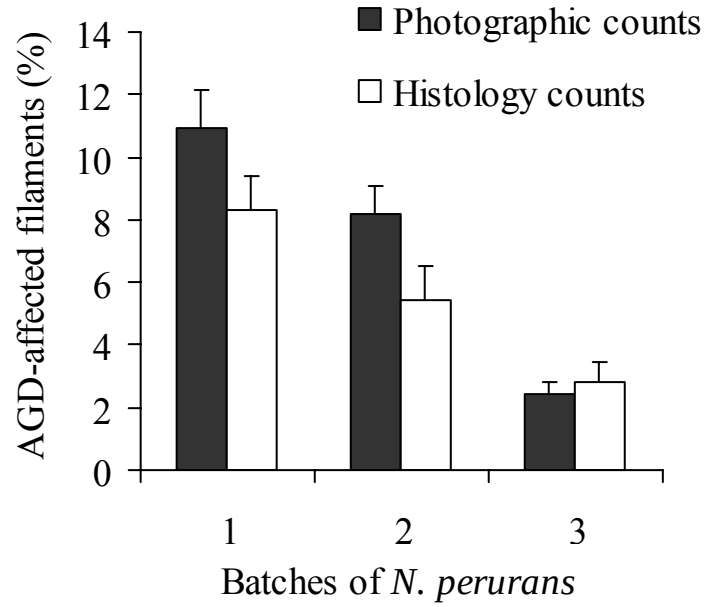

Figure 1. Quantification of AGD lesions on Atlantic salmon infected with 3 batches of *N. perurans* collected at 2 day intervals on 3 occasions. Values are means +/- SE, n = 24, superscripts denote significant differences.

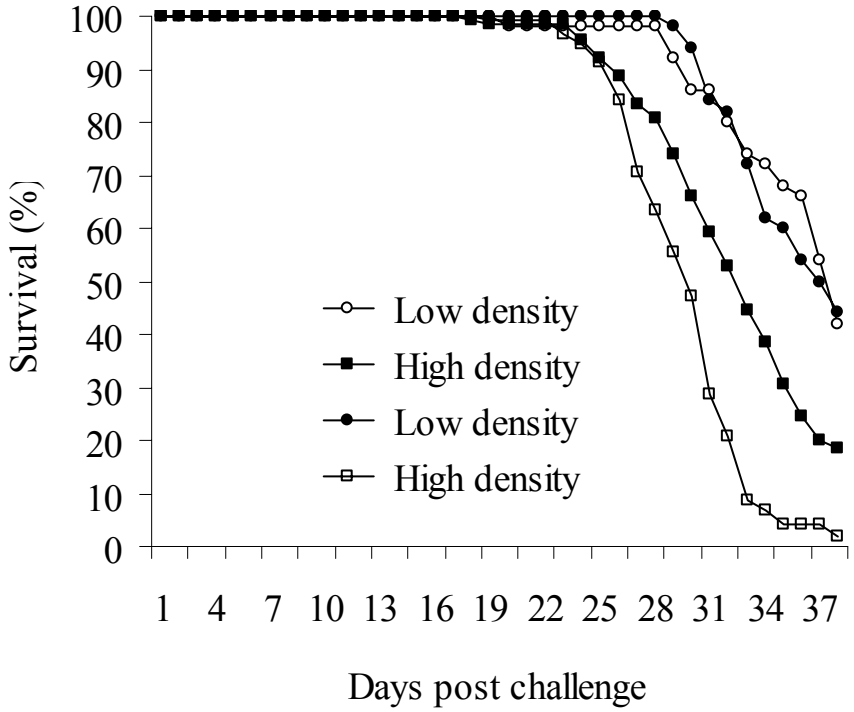

Figure 2. Survival curves for Atlantic salmon held in duplicate 4000L tanks at low (1.25 kg per m^3 i.e. 5kg per tank) and high (3.75 kg per m^3 i.e. 15kg per tank) after a 38 d challenge with *N. perurans*

Biomass investigation (Crosbie et al in prep)
Survival curves (Figure 2) showed a more rapid onset of morbidity in the high density tanks with AGD-related morbidity beginning on day 24 post challenge compared with day 30 for the low density tanks. Similarly, survival was greater in the low density tanks ranging from 42-44% compared with 2-18% in tanks with a higher biomass.

Seawater acclimation investigation (Attard et al 2006)
Survival up to 34 days post infection indicated that seawater acclimation time affected post-challenge mortality. All fish with no seawater acclimation (including 4 out of 5 from the unchallenged group) died within 4 days of introduction to seawater whereas survival for salmon subjected to the 7 and 14 acclimation periods was 52% and 17% respectively (Figure 3). Histological analyses confirmed the presence of AGD in the infection group and no fish in the control group displayed any clinical signs of AGD.

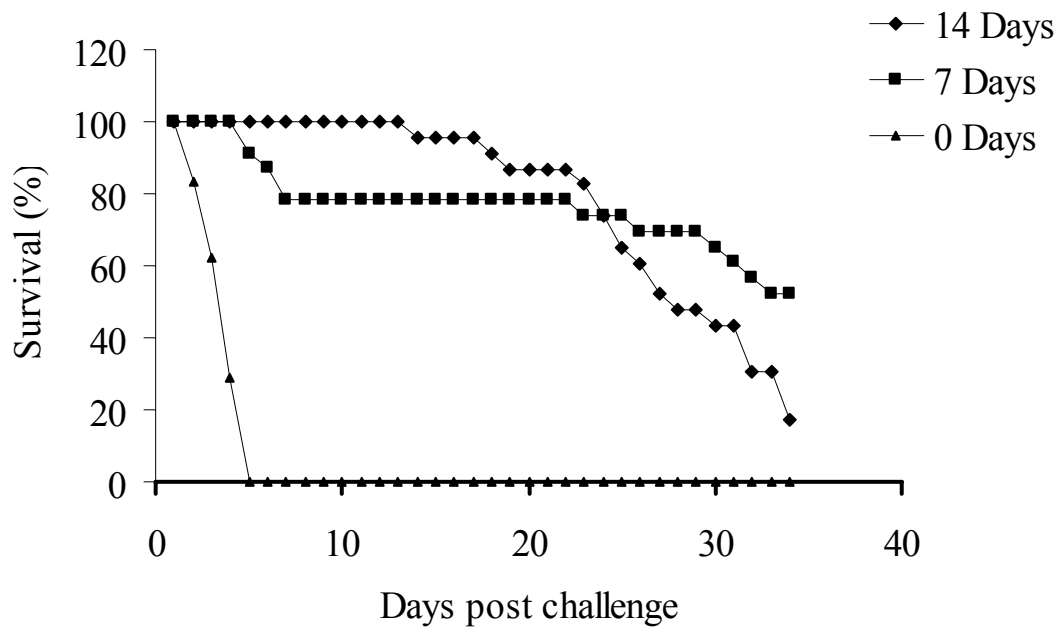

Figure 3. Survival curve for Atlantic salmon acclimated to seawater over 0, 7 or 14 days then challenged with *N. perurans*

Short-term in vivo challenge model (Crosbie et al 2007)
All salmon exposed to *N. perurans* showed histological signs of AGD (i.e. small, focal gill lesions with associated *N. perurans* trophozoites) after an initial exposure time of 6 h irrespective of antibiotic treatment of the infective inoculum, whereas gills from the control group did not (Figure 4)

There was no significant difference in the severity of AGD between groups as determined by the percentage of filaments with lesions (Figure 5). The antibiotic treatment of the *N. perurans* inoculum resulted in a 100 fold decrease in *Vibrio* spp. numbers as determined by growth on TCBS (Table 1).

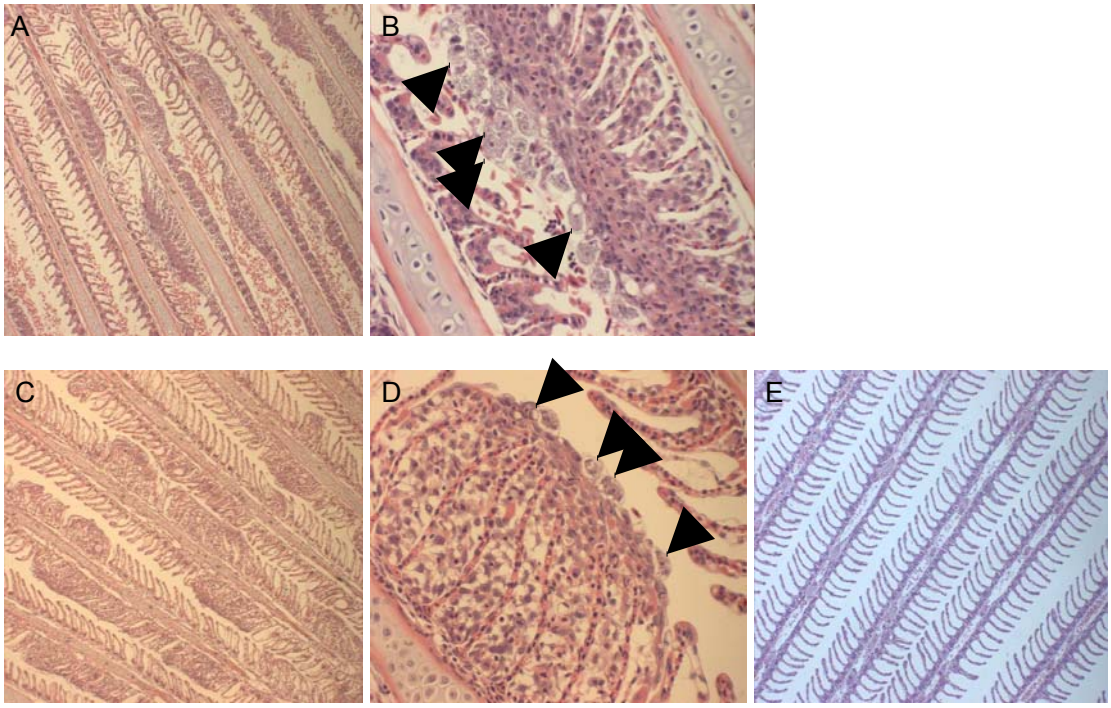

Figure 4. Histological sections of Atlantic salmon gills. (A) & (B) After exposure to *N*. *perurans* pre-incubated with antibiotics. (C) & (D) After exposure to *N*. *perurans* with no prior antibiotic treatment. (E) Negative control representative with no *N*. *perurans* exposure. Arrows indicate amoebae trophozoites in association with lesions. (A, C &E x 40 magnification; B & D x 200 magnification)

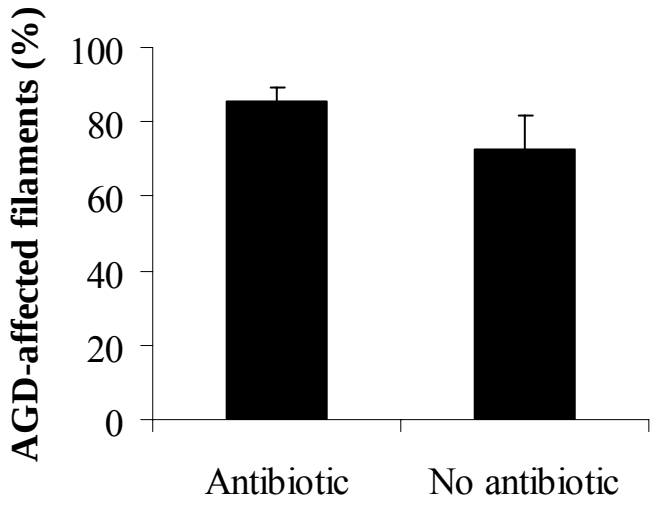

Figure 5. Percentage of Atlantic salmon gill filaments displaying AGD lesions 72h after challenge with *N. perurans* which had been pre-incubated with antibiotics (oxolinic acid and ampicillin at concentrations of 20 and 9.6 µg mL^{-1} respectively) or incubated in the same way with no antibiotic exposure. Values are means ± SE and there was no significant difference between groups.

Table 1. Total colony counts of *Vibrio* spp. in suspensions of *N. perurans* inoculated to TCBS agar plates after either 17h exposure to antibiotics (oxolinic acid and ampicillin at concentrations of 20 and 9.6 µg mL^{-1} respectively) or no exposure. Counts are means ± SE from 3 replicate plates. Total *Vibrio* spp. cells mL^{-1} are estimated from colony counts from TCBS plates with the lowest dilution inoculum

Dilution	Colony counts (mean ±SE, n=3)	
	N. perurans with antibiotic	*N. perurans* with no antibiotic
10^{-1}	15 ±0.8	Confluent growth
10^{-2}	2 ±0	168 ± 0.8
10^{-3}	0	23 ± 0.8
10^{-4}	0	1 ± 0.4
Total estimated *Vibrio* spp. (cells mL^{-1})	1.5×10^3	1.68×10^5

2. Use of challenge to appraise trial vaccines developed in the vaccine development project (essential service)

During this project a total of 7 vaccine/challenge experiments were carried out for CSIRO each in 4 replicate 4000L tanks and of approximately 3 months duration. The last 2 trials were completed in a new challenge facility. Two immunisation/challenge trials have been completed for collaborators at the University of Technology Sydney (UTS) which tested the protective ability of a high molecular weight protein fraction (HMWF) derived from *N. perurans* and/or Freund's adjuvant. These trials were performed in single 4000L tanks (due to limited supplies of the HMWF) and were of 4 months duration. The trials for UTS were carried out under Aquafin CRC project 3.4.4(2) (FRDC 2004/217) Development of an AGD vaccine: phase 11 (UTS).

3. Provision of gill-associated and cultured amoebae to collaborators (essential service)

Isolations of gill-derived amoebae yielded around 0.5-1.0 x 10^6 per event and over the course of the project approximately 4.82 x 10^8 were isolated and supplied to UTS. Another 12.0 x 10^6 were supplied to CSIRO. Lawn plates of cultured *N. pemaquidensis* yielded around 1.0 x 10^6 per agar plate and approximately 4.39 x 10^8 were supplied to UTS and 4.10 x 10^6 supplied to CSIRO.

4. Cryopreservation of virulent amoebae (research)

(Young et al 2007, Young et al 2008a, Young et al 2008b)

Virulent amoeba (Young et al 2007, Young et al 2008)

Briefly, the NCGD amoebae-specific probe bound to AGD-associated amoebae while neither *N. pemaquidensis* nor *N. branchiphila* were associated with AGD-lesions. Together, these data indicate that NCGD amoebae represent a new species, designated *N. perurans* and this is the predominant aetiological agent of AGD of Atlantic salmon cultured in Tasmania, Australia. In archival samples,

N. perurans was the only detectable amoeba, confirming that it has been the predominant aetiological agent of AGD in Tasmania since epizootics were first reported. *N. perurans* was also the exclusive agent of AGD in four host species across six countries. These data show that *N. perurans* is a cosmopolitan agent of AGD and therefore of significance to the global mariculture industry. An assay to detect *N. perurans* was developed (Young et al 2008b). The assay, which utilises PCR to amplify the *N. perurans* 18S rRNA gene, was shown to be specific and highly sensitive. *N. perurans* was detected in both gill samples and primary isolates of non-cultured gill-derived amoebae obtained during necropsy or biopsy from AGD-affected Atlantic salmon (*Salmo salar* L.). The PCR-based assay provides a simple, flexible tool that will be a useful addition to the diagnostic repertoire for AGD. It may also be used for the genotypic screening of trophozoites during culture and could facilitate further epidemiological and ecological studies of AGD.

Cryopreservation protocols

None of the above variations to the standard ATCC protocol resulted in significant survival post thaw. Viability after the addition of salmon serum ranged from 0 to 2%. Some cells appear viable based on neutral red uptake and adherence (see Figure 6) but the results are not clear. After the addition of rabbit serum (10%) to the DMSO (7.5%) between 5 and 30% took up neutral red (average 19.6% +/- SD16.1%), when the concentration of rabbit serum was reduced to 5% viability was between 2 and 4% (average 3.3% +/- SD 1.3%). However, the dispersion of the neutral red dye throughout the cytoplasm rather than being concentrated into vacuoles suggested passive uptake mechanisms. The adoption of a spherical shape and lack of pseudopods (Figure 6 B) indicate the cells are dead or dying based on previous observations. Similarly when appraising viability by adherence some cells appeared to adhere to plastic 9Figure 6D & E), but only a very small proportion. There was no viability evident when glycerol was used to replace DMSO as a cryoprotectant.

Attempts to introduce thawed cells into culture on malt yeast agar were not successful as flat amoeba such as *Vanella/Platyamoeba* spp. dominated the culture and appeared to be the only amoebae present. These species make up a very low proportion of the amoebae isolated from the gills and are not associated with AGD. *N. perurans* may be cryopreserved but may need a period in culture first. One *N. pemaquidensis* strain (library isolate NP 251002) was successfully recovered post freeze and thaw after it had been in laboratory culture for 3 months. It is routine to freeze and recover the cultured amoebae.

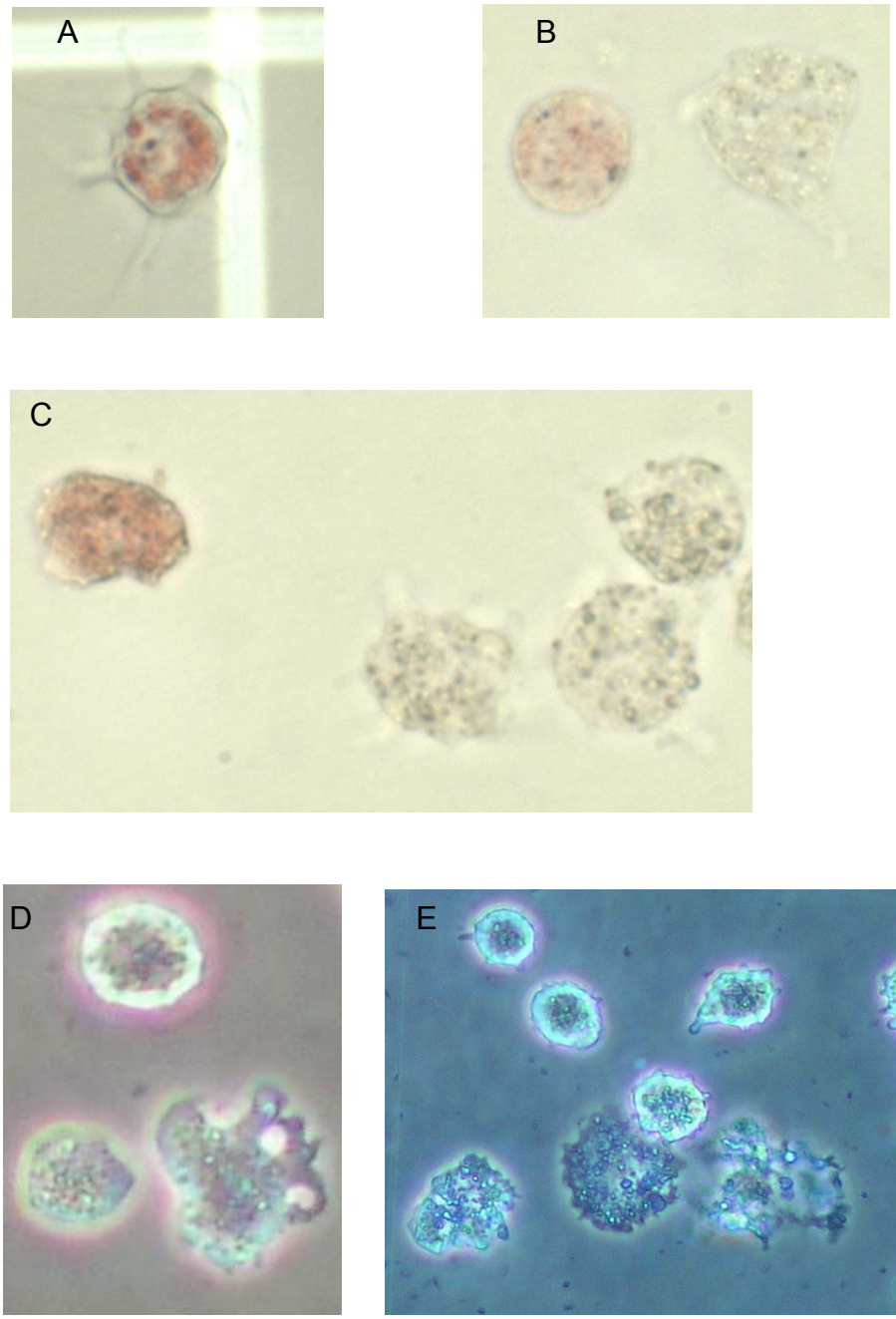

Figure 6. Viability images of *N. perurans* A. Living cell prior to cryopreservation (neutral red inclusion assay) B. & C. Cells post freeze/thaw after addition of rabbit serum at 10% to cryopreservation medium (neutral red inclusion assay). D & E. Attached and unattached cells post freeze/ thaw using above medium.

5. Maintenance of infection tank (essential service)

Annual turnover through this tank is between 1800-2000 salmon. Lowering the numbers of fish and temperature slows down the rate of morbidity in the tank which has been the source of all infectious amoebae for initiating AGD trials. The infection tank has supplied infectious material for other Aquafin CRC projects namely, 3.4.1(2) (FRDC 2004/213) Commercial AGD and salmon health, 3.4.2(2) (FRDC 2004/210) Use of immunomodulation to improve fish performance in Australian temperate water finfish culture and 3.4.4(2) (FRDC 2004/217) Development of an AGD vaccine: phase 11 (UTS).

6. Provision of freshwater salmon for experiments in other projects (essential service)

Approximately 6-7 transports were carried out each year of the project for delivery of a total of approximately 4000 salmon

Benefits and Adoptions

This project directly benefits the salmon industry by providing essential support for all AGD research, including testing of experimental vaccines. During this project we identified the species of *Neoparamoeba* responsible for AGD, developed new diagnostic methods for AGD, which have been already used to confirm outbreaks of AGD in previously unaffected areas overseas. These methods can also be applied in future epidemiological investigations. Challenge protocols are available for further AGD research.

Aquatic Animal Health research (human capital development) in Australia benefited from this project through training of one postdoctoral fellow and one technician.

This project has indirect benefit for other aquaculture sectors in Australia. It provides research methods and knowledge that can be applied to other fish farmed in marine pens.

Further Development

While this project has increased our understanding of Amoebic Gill Disease, it has also identified knowledge gaps. The new knowledge and methods developed can facilitate further epidemiological studies of AGD and investigations into ecology of *Neoparamoeba perurans*. In particular, further investigation of the presence of *Neoparamoeba perurans* in the environment and its association with AGD outbreaks are now possible. Culture and cryopreservation methods for *Neoparamoeba perurans* should be developed.

Results of this project have been widely disseminated throughout the salmon industry through industry meetings, workshops and through the Aquafin CRC and FRDC Salmon Aquaculture Subprogram conferences. The results can be exploited commercially by individual companies if they choose to adopt any of the suggested strategies.

Planned Outcomes

A collective outcome of the all AGD research, for which this project provided crucial support, was development of more effective control measures. In this project, we developed AGD challenge model, in vivo AGD attachment test. We enhanced our knowledge of AGD outbreaks and risk factors through better understanding of the effects of different factors such as stocking density or acclimation to salinity on infection. We significantly increased our knowledge of the pathogen through description of the new species *Neoparamoeba perurans* and showed that it is consistently associated with AGD outbreaks worldwide.

Conclusions

1. Standardisation of AGD challenge models (research)
AGD challenge model has been standarised. Fish biomass, sea water acclimation protocol and amoebae batch have been shown to affect AGD infection and have to be standarised. Short term in vivo attachment model has been developed and used.

2. Use of challenge to test trial vaccines developed in the vaccine development project (essential service)
We have successfully used challenge to test trial vaccines developed in the vaccine development project. The experimental model has been shown to represent field infections well (see continuing Project 3.4.4(3); FRDC 2007-234).

3. Provision of gill-associated and cultured amoebae to collaborators (essential service)
We have provided gill-associated and cultured amoebae to collaborators.

4. Cryopreservation of virulent amoebae (research)
We have shown that a new species, *Neoparamoeba perurans* has been consistently associated with AGD outbreaks worldwide. So far it has not been possible to culture this species. Direct cryopreservation of gill associated amoebae has not been possible. Further research is required to develop culture methods and cryopreservation methods for this species. We have developed diagnostic methods (PCR and in situ hybridisation) which are available for confirmation of AGD infections and further research.

5. Maintenance of infection tank (essential service)
We have maintained AGD infection tank.

6. Provision of freshwater salmon for experiments in other projects (essential service)
We have provided freshwater salmon for AGD experiments as required by other projects.

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Appendix 1

INTELLECTUAL PROPERTY

The intellectual property and valuable information arising from this report are:

1. Copyright in this report

Appendix 2

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Appendix 3

AGD CHALLENGE MODELS

A. *Neoparamoeba perurans* challenge protocol for Atlantic salmon in 4000L tanks in salmon shed

Endpoint: morbidity, or predetermined period post infection
Duration: 2-3 months,
Application: primarily vaccine efficacy testing

System:

- 4 x 4000L rathbun systems.
- Lighting within the room connected to individual timers and switches to allow hatchery light regimes to be mimicked at the School of Aquaculture aquatic centre.
- All tanks fitted with biofilters, foam fractionators and solids filtration tanks and UV lamps to disinfect circulating water
- Water temperature 15°C (maintained by heater/chiller unit).
- All systems fitted with sensors to detect equipment failure (i.e. heater/chiller unit and pumps).
- Fish biomass maximum 15 kg per tank.

Water quality:

Freshwater phase

- Whilst fish are held in freshwater all in-coming water will be conditioned in tanks in C-line to ensure effective dechlorination and temperature matching
- Water quality will be monitored every second day and water exchanges performed as required

Seawater phase

- All in-coming seawater will pass through nominal 02µm filtration and UV disinfection units.
- Seawater acclimation to occur in individual group tanks over 14 days at 5 ppt increments every 2 days.
- If required temperature acclimation will be 1°C per day up to 15°C.
- Once at 35ppt active biofilters will be placed into sumps and water will be monitored daily for nitrogenous compounds (i.e. ammonia, nitrite and nitrate) to ensure biofilter function.
- Once biofilters are established nitrogenous compound monitoring will be every second day for the duration of the experiment.
- Water will be exchanged as required to reduce nitrate build up and salinity monitored. If required salt will be added in the form of sea salt.

Fish:

- On arrival at the aquaculture centre at the University of Tasmania, fish will be transferred via a 150 mm pipe directed from the transport truck directly into individual tanks to avoid dip netting

- Fish will be fed a ration of 2% body weight per day (approximately 1% in morning and afternoon) during all phases.
    - commercial transfer diet (Skretting) during seawater acclimation.
    - normal on-growing diet after acclimation.

- All invasive handling procedures i.e. pit tagging/tattooing and injection vaccinating will occur in freshwater phase preferably in hatchery prior to relocation to the aquatic centre at the University of Tasmania.
- Where fish handling is required to administer treatment or collect samples anaesthesia will be used (Aqui-S dose as recommended by manufacturer).
- Fish will only contact smooth surfaces and all handlers will wear latex gloves
- Post-treatment fish will be returned directly to holding tanks and recovery tanks will not be used in order to minimise fish handling.
- Any moribund or dead fish will be removed from the system, grossly examined and any external and gill lesions noted.
- Any non-AGD related moribund or dead fish will be sent to the DPI Fish Health Unit at Mt Pleasant Laboratories for post mortem examination.
- After inoculation of systems with *N. perurans* gills of any fish removed from the systems will be fixed in seawater Davidson's fixative for later histological examination.

Challenge with *Neoparamoeba perurans*

- Approximately 14 days prior to initiation of an infection additional fish will be added to the infection tank to maximise the likelihood of harvesting enough amoebae to infect replicate tanks in a minimal number of isolation events
- *N. perurans* will be harvested from donor fish, as they succumb to AGD in the infection tank, using the adherence technique of Morrison et al (2004).
- When amoebae are attached to Petri dishes and unattached debris washed off, they will be overlaid with filtered seawater containing antibiotics (ampicillin and oxolinic acid at 9.6 and 20 µg/mL respectively) and incubated overnight (18°C) to reduce *Vibrio* sp. contamination of the inoculum.
- After harvesting and counting each batch of amoebae will be divided into 4 and inoculated into each tank to a concentration of 500 cells per L.
- Inocula will be suspended in 3-4L of filtered seawater and administered to individual tanks using a watering can to ensure even dispersal.
- All pumps will be switched off for 3-4 h after inoculation
- UV lamps will be switched off for 24 h after inoculation
- If insufficient amoebae are collected from a single isolation additional isolations will occur and amoebae added to all tanks cumulatively until the required dose is reached.
- Morbidity will be monitored and as fish show overt signs of AGD they will be removed from the tanks.
- At a specific level of morbidity the trial will be terminated and all surviving salmon will be euthanased and gills removed for quantitative gill histopathology.

Antibiotic Preparation

Ampicillin final concentration required is 9.6μg/ mL

Stock Solution Preparation

Weigh 0.1g of ampicillin

Add to 52 mL of distilled water (conc is 0.00192g/ mL) then use a syringe filter to sterilise

Add 5 mL of stock solution (i.e. 5 x 0.00192g = 0.0096g) to 1L of seawater (final conc is 0.0096g in 1000mL = 0.0000096g/mL = 9.6 μg/ mL)

Keep remaining 47mL of stock at 4°C for subsequent isolations

Oxolinic acid final concentration required is 20μg/ mL

Stock Solution Preparation

Weigh 0.1g of oxolinic acid

Add to 25mL of distilled water (add a few drops of saturated sodium hydroxide to dissolve-conc is 0.004g/ mL) (**Oxolinic acid stock solution**)

Add 5 mL of stock solution. (i.e. 5 x 0.004g = 0.02g) to the same L of seawater containing the ampicillin (final conc is 0.02 g in 1000mL = 0.00002g/mL = 20 μg/ mL)

Keep remaining 20mL of stock at 4°C for subsequent isolations

B. *Neoparamoeba perurans* challenge protocol for Atlantic salmon in 40-80 L tanks to quantify amoebae attachment

Endpoint: 72h post challenge

Application: test amoebae attachment inhibiting treatments

System:

- 6 x 320L recirculating systems each with 3 x 80L tanks and 80L sump.
- Systems housed in a quarantined light and temperature controlled room.
- No filtration units
- Fish biomass maximum 0.4 kg per tank.

Water quality:

Seawater phase

- All in-coming seawater will pass through a nominal 02μm filtration unit.
- 25% water exchanges will be carried out daily to reduce ammonia accumulation
- Ammonia levels will be monitored every 24h

Fish:

- Seawater acclimated salmon no bigger than 120 g
- Fish will starved for 48h prior to and not fed for the duration of the challenge
- Fish will be transferred to the small treatment tanks immediately prior to challenge
- Any moribund or dead fish will be removed from the system, grossly examined and any external and gill lesions noted.

Challenge with *N. perurans:*

- The challenge will occur in 2 phases in different tanks
    - Initial amoebae attachment to gill phase (80L tank filled to 35L only and static)
    - Development of gill pathology (80L tank filled to capacity and recirculating through sump)
- *N. perurans* will be harvested from donor fish, as they succumb to AGD in the infection tank, using the adherence technique of Morrison et al (2004).
- After harvesting and counting amoebae will be equally distributed to the treatment tanks (35L, static) at a concentration of 8500 cells per L.
- After 6 h fish will be transferred to clean systems filled to capacity (80L , recirculating) and pathology allowed to progress for 72h
- All fish will then be euthanased and gills removed for quantitative gill histopathology.

Appendix 4

PRESENTATIONS RESULTING FROM THIS PROJECT

Adams, M, Crosbie, P, Morrison, R & Nowak, B (2004) An introduction to amoebic gill disease of Atlantic salmon (*Salmo salar* L.). 2nd FRDC Aquatic Animal Health Program Scientific Conference, 26-28 July, Cairns.

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Appendix 5

PUBLICATIONS ARISING FROM THIS PROJECT

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3. Embar-Gopinath, S., Crosbie, P. B.B., Nowak, B. (2005) Use of potassium permanganate to disinfect Atlantic salmon (*Salmon salar*) gills. Bulletin of the European Association of Fish Pathologists, **25**, 291-296.

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7. Crosbie, P.B.B., Adams, M.B., Attard, M., Nowak, B.F. (2007) Development of an in vivo assay to assess attachment of *Neoparamoeba* sp. (an amphizoic gymnamoeba) to the gills of Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases, 30, 187-190.

8. Young, N.D., Crosbie, P.B.B., Adams, M.B., Nowak, B.F., Morrison, R.N. (2007) *Neoparamoeba perurans* s. sp. an agent of amoebic gill disease in Atlantic salmon (*Salmo salar* L.) International Journal for Parasitology, 37, 1469-1481.

9. Young, N.D., Dyková, I., Snekvik, K., Nowak, B.F., Morrison, R.N. (2008) *Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill disease. Diseases of Aquatic Organisms, 78, 217-223.

10. Young, N.D., Dyková, I., Nowak, B.F., Morrison, R.N. (2008) Development of a diagnostic PCR to detect *Neoparamoeba perurans*, agent of amoebic gill disease (AGD). Journal of Fish Diseases, 31, 285-295.

11. Embar -Gopinath, S., Crosbie, P., Nowak, B.F. (2008) A culture dependent 16S RNA gene-based approach to identify bacteria associated with amoebic gill disease in Atlantic salmon, Bulletin of European Association of Fish Pathologists, 28, 27-34.

Appendix 6

ATTACHED PAPER

Young, N.D., Crosbie, P.B.B., Adams, M.B., Nowak, B.F., Morrison, R.N. (2007) *Neoparamoeba perurans* n. sp. an agent of amoebic gill disease in Atlantic salmon (*Salmo salar* L.) International Journal for Parasitology, 37, 1469-1481.

Research Paper

Neoparamoeba perurans n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar* L.)

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Abstract

Amoebic gill disease (AGD) is a potentially fatal disease of some marine fish. Two amphizoic amoebae *Neoparamoeba pemaquidensis* and *N. branchiphila* have been cultured from AGD-affected fish yet it is not known if one or both are aetiological agents. Here, we PCR amplified the 18S rRNA gene of non-cultured, gill-derived (NCGD) amoebae from AGD-affected Atlantic salmon (*Salmo salar* L.) using *N. pemaquidensis* and *N. branchiphila*-specific oligonucleotides. Variability in PCR amplification led to comparisons of 18S (small subunit) and 28S (large subunit) rRNA gene sequences from NCGD and clonal cultured, gill-derived (CCGD) *N. pemaquidensis* and *N. branchiphila*. Phylogenetic analyses inferred from either 18S or 28S rRNA gene sequences unambiguously segregated a lineage consisting of NCGD amoebae from other members of the *Neoparamoeba* genus. Species-specific oligonucleotide probes that hybridise 18S rRNA were designed, validated and used to probe gill tissue from AGD-affected Atlantic salmon. The NCGD amoebae-specific probe bound AGD-associated amoebae while neither *N. pemaquidensis* nor *N. branchiphila* were associated with AGD-lesions. Together, these data indicate that NCGD amoebae are a new species, designated *N. perurans* n.sp. and this is the predominant aetiological agent of AGD of Atlantic salmon cultured in Tasmania, Australia.

Keywords: amoebic gill disease; *Neoparamoeba pemaquidensis*; *branchiphila*; *perurans*; *Salmo salar*; *in situ* hybridisation; rRNA; 18S; 28S

1. Introduction

Amoebae of the Vexilliferidae and Paramoebidae families are ubiquitous within marine and estuarine environments (Page, 1983; Page, 1987). Phylogenetically these families converge to create the so-called "PV lineage" (Peglar et al., 2003). *Neoparamoeba* spp. Page, 1987 (Lobosea) belong to the Vexilliferidae family and are small, lobose amoebae that form dactylopodiate subpseudopodia in their locomotive form. They lack the well organised cell-surface structures of other Vexilliferids such as hexagonal glycostyles (*Vexillifera* Schaeffer, 1926) or surface scales (*Korotnevella* Schaeffer, 1926) and possess one or more intracellular perinuclear bodies, known as 'parasomes'. These 'parasomes' are described as *Perkinsiella amoebae*-like organisms (PLOs) and are eukaryotic endosymbionts, phylogenetically related to flagellated, parasitic marine protozoans of the *Ichthyobodo* Pinto, 1928 genus (Dyková et al., 2003).

Neoparamoeba is an ecologically important group since it contains amoebae that are reportedly amphizoic. Initially, *N. pemaquidensis* (Page, 1970) was considered the single aetiological agent of amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*, L. 1758) (Kent et al., 1988; Roubal et al., 1989) and this conclusion was based upon morphological features, primarily the presence of one or more 'parasomes' and the lack of surface scales (Kent et al., 1988; Roubal et al., 1989). While morphological characteristics distinguish *Neoparamoeba* from other Vexilliferids, attempts to demarcate members of the *Neoparamoeba* genus using morphological characteristics alone have been unsuccessful (Dyková et al., 2000; Dyková et al., 2005). Therefore, the initial interpretation of *N. pemaquidensis* as the aetiological agent of AGD was presumptive. Immunological detection of *Neoparamoeba* using anti-*N. pemaquidensis* antiserum was successful and supported the role of *N. pemaquidensis* in AGD aetiology

(Howard and Carson, 1993). However the anti-*N. pemaquidensis* antiserum was later shown to bind non-specifically to other marine amoebae (Morrison et al., 2005).

Recently, a new species of *Neoparamoeba*, *N. branchiphila* Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Machackova and Dvořáková, 2005 was cultured from the gills of AGD-affected fish (Dyková et al., 2005). This amoeba was characterised using a combination of morphological and molecular phylogenetic analyses inferred from 18S (small subunit) rRNA gene sequences (Fiala and Dyková, 2003; Dyková et al., 2005) and were clearly differentiated from *N. pemaquidensis* and *N. aestuarina* (Page, 1970). These data also resolved inter-specific relationships within the *Neoparamoeba* group (Fiala and Dyková, 2003; Dyková et al., 2005; Mullen et al., 2005), with the segregation of phylogenetic lineages suggesting that AGD may be a disease of mixed aetiology. This led to the development of species-specific diagnostic tools, based upon 18S rRNA gene amplification by PCR to study disease aetiology where *Neoparamoeba* were the presumptive pathogens (Fiala and Dyková, 2003; Wong et al., 2004; Dyková et al., 2005; Mullen et al., 2005). Indeed, PCR amplification of DNA isolated from amoebae cultured from gill tissues of AGD-affected fish was consistent with the proposition that both *N. pemaquidensis* and *N. branchiphila* may be associated with AGD (Wong et al., 2004; Dyková et al., 2005). However, attempts to determine the pathogenicity of either species by re-infecting fish using clonal cultured, gill-derived (CCGD) strains have been universally unsuccessful (Kent et al., 1988; Howard and Carson, 1993; Morrison et al., 2005; B. Vincent pers. comm.). While the presence of *N. pemaquidensis* and *N. branchiphila* on the gills of AGD-affected fish is unequivocal (Fiala and Dyková, 2003; Wong et al., 2004; Dyková et al., 2005), their role in AGD aetiology is yet to be confirmed formally.

Here, we used a PCR-based approach to examine the abundance of *N. pemaquidensis* and *N. branchiphila* in amoebae preparations directly isolated from the gills of AGD-affected Atlantic salmon. Persistent variability in PCR amplification led to phylogenetic analyses of 18S and 28S rRNA gene sequences obtained from non-cultured gill-derived (NCGD) amoebae. Evidence is presented to support the creation of a new phylogenetically distinct lineage of *Neoparamoeba*, exclusive to NCGD amoebae. Using *in situ* hybridisation (ISH) with *Neoparamoeba* species-specific probes we verified that the only detectable amoebae directly associated with AGD lesions in Atlantic salmon in Tasmania all belonged to this new phylogenetic lineage. This undermines the putative role of *N. pemaquidensis* and *N. branchiphila* in AGD.

2. Materials and Methods

2.1. Acquisition of clonal, cultured gill-derived (CCGD) and non-cultured gill-derived (NCGD) amoebae

AGD-affected Atlantic salmon, *Salmo salar* were obtained from a recirculation tank-based population of fish (D1) maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). This recirculation tank was originally populated with AGD- affected Atlantic salmon from the Huon Estuary, Tasmania, Australia (Huon Aquaculture Company) in October, 2001 and the disease has been propagated by cohabitation with naïve fish. Farm-reared AGD-affected Atlantic salmon were obtained from the Huon Estuary, Tasmania, Australia (Huon Aquaculture Company). Fish were anaesthetised (50 mg L^{-1} Aqui-S NZ Ltd, Lower Hutt, New Zealand) and assessed for AGD lesions as previously described (Munday et al., 2001). Fish presumptively diagnosed with AGD were euthanized (100 mg L^{-1} Aqui-S) and

amoebae were directly isolated from gill tissues as previously described (Morrison et al., 2004) herein termed NCGD amoebae.

Clonal cultures of amoeba strains were obtained from a culture collection held at the School of Aquaculture, University of Tasmania (Table 1). Previous identification of strains was based on phylogenetic analyses and 18S rRNA gene-specific PCR (Wong et al., 2004; Dyková et al., 2005). Amoebae culture and harvesting procedures followed those previously described (Dyková et al., 2000; Dyková et al., 2005). Harvested and purified trophozoites were maintained for no longer than 30 min in phosphate buffered saline, pH 7.4 (PBS) until further processing.

2.2. DNA extraction and assessment of NCGD amoebae using 18S rRNA gene PCR

Aliquots of between 1-5 × 10^5 amoebae were centrifuged (10,000 × *g*, 1 min) and the supernatant removed. The cell pellet was stored at -80°C or processed immediately. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Doncaster, Victoria, Australia) as per the manufacturer's instructions.

The presence of *N. pemaquidensis* and *N. branchiphila* in NCGD amoebae primary isolates was confirmed with 18S rRNA gene PCR using species-specific oligonucleotides as previously described (Wong et al., 2004; Dyková et al., 2005). Where amplification was unsuccessful, PCR was repeated using 35 cycles. PCR amplification efficiency of template DNA was assessed with universal 18S rRNA gene oligonucleotides [18e and 18i (Hillis and Dixon, 1991)] as previously described (Mullen et al., 2005). All PCR reactions were initiated with 20 ng of purified DNA template. Control templates included genomic DNA from Atlantic salmon, cultured *N. pemaquidensis* and *N. branchiphila* strains and no template controls. PCR reactions were electrophoresed through 1 to 2% agarose/TBE.

2.3. Amplification and sequencing of 18S and 28S rRNA

The PCR amplification of the full-length 18S rRNA gene from amoebae was performed as previously described (Dyková et al., 2005). PCR amplification of a portion of the 28S rRNA gene from amoebae including divergent domains D1 to D3 and the conserved core region upstream of domain D3 was performed using universal oligonucleotides as previously described [28 F and 1438 R (Bergholtz et al., 2005)]. Amplification of the 28S rRNA gene was performed in volumes of 25 μL containing 1 U of Platinum *Taq* (Invitrogen, Mount Waverley, Victoria, Australia), 1 × Platinum *Taq* PCR buffer, 200 μmol of each deoxynucleotide triphosphate (dNTP; dATP, dCTP, dGTP, and dTTP), 1.5 mmol of $MgCl_2$ and 10 pmol of each oligonucleotide. PCR cycle conditions were 94°C for 3 min; 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, for 30 cycles; and 72°C for 10 min. Control templates included genomic DNA from Atlantic salmon, cultured *N. pemaquidensis* and *N. branchiphila* strains and no template controls.

PCR products were ligated into pGEM-T easy plasmid vector according to manufacturer's instructions (Promega, Annandale, Australia). After transformation into *Escherichia coli* Castellani & Chalmers, 1919 strain DH10β, positive clones were identified by blue-white colour selection (BlueTech, Mirador DNA Design, Montreal, Quebec, Canada) followed by PCR. Clones (herein termed molecular clones) were inoculated into Luria broth and plasmid DNA was purified (MiniPrep, Qiagen). Nucleotide sequencing was performed using either the DTCS Quick Start Dye Terminator Kit (Beckman Coulter, Fullerton, California, USA) or ABI Prism BigDye Terminator (version 3.1) Cycle Sequencing Kit (Applied Biosystems (ABI), Scoresby, Victoria, Australia) following the manufacturer's instructions. Sequencing reactions were initiated using plasmid DNA template and the insert amplified using M13 forward or reverse oligonucleotides. Samples were analysed on a CEQ 8000 sequencer

(Beckman Coulter) or ABI 3730xl DNA analyser (ABI). The 18S and 28S rRNA gene sequences analysed in this study were deposited in GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) with the accession numbers shown in Fig. 3 and 4 respectively.

2.4. Construction and support for Neoparamoeba *phylogeny using the 18S and 28S rRNA genes*

The 18S rRNA gene sequences from seven molecular clones from five independent NCGD amoebae primary isolates, 36 strains of *Neoparamoeba* obtained from GenBank and an outgroup containing *Korotnevella stella* Goodkov, 1988 (GenBank accession number: AY183893), *Korotnevella hemistylolepis* O'Kelly et al., 2001 (AY121850), *Vexillifera armata* Page, 1979 (AY183891), *Vannella anglica* Page, 1980 (AF099101) and *Vannella aberdonica* Page, 1980 (AY121853) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2). The outgroup contained species that were morphologically distinct from *Neoparamoeba* species but closely-related based on phylogenetic analyses of the 18S rRNA gene (Peglar et al., 2003; Fiala et al., 2003). The alignment was checked manually using BioEdit (Hall, 1999) and has been submitted to the European Molecular Biology Laboratory (EMBL)-Align database (accession number ALIGN_001117). Genetic distances among 18S rRNA gene sequences were calculated as mean character differences using PAUP*, version 4.0b10 (Swofford, 2001). Phylogenetic tree searches using the aligned 18S rRNA gene sequences were conducted using three methods of analysis. Maximum parsimony analyses (MP) were performed in PAUP*, version 4.0b10 using the heuristic search with tree-bisection-reconnection (TBR) branch swapping, the ACCTRAN option and 10 random-taxon addition iterations. Gaps were treated as missing data. Clade reliability was estimated using bootstrap resampling (Felsenstein, 1985) with 1000 replicates. The

Akaike Information Criteria (AIC) test in Modeltest version 3.7 (Posada and Crandall, 1998) selected the general time reversible model of evolution with gamma distribution and a proportion of invariable sites (GTR + Γ +I) as the most appropriate likelihood model for further phylogenetic analyses. Tree searches by maximum likelihood (ML) analysis were performed in PAUP*, version 4.0b10 using the heuristic process with TBR branch swapping. Estimates of the α-parameter (0.5044), frequency of base proportions (A = 0.2858, C = 0.1600, G = 0.2228 and T = 0.3314), the substitution rate model matrix ([A-C] = 1.2941, [A-G] = 8.1120, [A-T] = 2.5793, [C-G] = 0.6096, [C-T] = 10.2555, and [G-T] = 1.0000) and proportion of invariable sites (0.2984) determined using the AIC test were fixed for the analysis. Clade reliability for the most parsimonious ML tree was estimated using bootstrap resampling with 1000 replicates generated by SEQBOOT in PHYLIP Version 3.66 (Felsenstein, 1989). ML analysis of the 1000 bootstrap replicates was performed with PHYML (Guindon and Gascuel, 2003) with the GTR + Γ +I model settings previously described. The ML tree obtained in PAUP*4b10 was used as the starting tree. Bayesian phylogenetic inference (BI) was determined using Markov chain Monte Carlo (MCMC) analysis in MrBayes, version 3.1.2 (Ronquist and Huelsenbeck, 2003; Huelsenbeck and Ronquist, 2005). The likelihood parameters that were set for BI were based on the GTR + Γ +I model. Three million generations of MCMC analysis were performed and trees were recorded every 100^{th} generation. At this point, the standard deviation of split frequencies was < 0.01 and the potential scale reduction factor (PSRF) approached one. Consensus trees were generated using the 50% majority rule criterion on bootstrap replicate trees generated with MP and ML analyses and the final 75% of trees generated by BI.

Partial 28S rRNA gene sequences from six independent NCGD amoebae primary isolates, four strains of culture-purified *N. pemaquidensis*, three strains of *N.*

branchiphila and *P. hoguae* (Table 1) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2). *P. hoguae* was selected as an outgroup as it is morphologically distinct from *Neoparamoeba* species but closely-related based on phylogenetic analysis of the 18S rRNA gene (Peglar et al., 2003). The partial 28S rRNA gene alignment was submitted to the EMBL-Align database (accession number ALIGN_001118). Genetic distances among 28S rRNA gene sequences were calculated as described above. The same methods for phylogenetic analyses and the selection of likelihood models were used as described above for the 18S rRNA gene sequence alignment. The GTR + Γ +I likelihood model was selected for ML analysis and BI. Fixed parameter estimates using the AIC test for ML analysis were the α-parameter (0.4756), frequency of base proportions (A = 0.3044, C = 0.1533, G = 0.2415 and T = 0.3008), the substitution rate model matrix ([A-C] = 0.6297, [A-G] = 10.6129, [A-T] = 3.6016, [C-G] = 1.2679, [C-T] = 13.6820, and [G-T] = 1.0000) and proportion of invariable sites (0.3685). BI using MCMC analysis was performed for 5×10^5 generations and trees were recorded every 100^{th} generation. At this point, the standard deviation of split frequencies was < 0.01 and PSRF approached one. Consensus trees were generated as described above for the 18S rRNA gene sequence alignment.

2.5. Design and validation of Neoparamoeba *species-specific rRNA oligonucleotide probes and their application on AGD-affected Atlantic salmon gill tissue*

Oligonucleotide probes that hybridise to 18S rRNA from each clade of *Neoparamoeba* were designed where suitable base mis-matches were observed in aligned sequences. The probes were designed following established guidelines (Hugenholtz et al., 2001), synthesised and 5' end-labelled with digoxygenin (DIG) (Thermo Electron, Hamburg, Germany).

The specificity of each probe was validated using *in situ* hybridisation with representative *Neoparamoeba* strains (Table 1) that were embedded within a single paraffin block (termed a *Neoparamoeba* array). Approximately 0.5-1 × 10^6 NCGD or CCGD amoebae were centrifuged at 500 × *g* for 5 min at 4°C and the supernatant removed. Further centrifugations were performed in a similar manner. Amoebae were resuspended in 30 mL of 1 × PBS, centrifuged and the supernatant removed. The cell pellet was resuspended in 10 mL of seawater Davidson's fixative (SWD) and fixed for 30 min. Fixed amoebae were centrifuged and the supernatant removed. Amoebae were sequentially washed with 70% ethanol and 3× with PBS. The cells were finally resuspended in 100 µL of PBS, heated to 65°C for 5 min then mixed in a 0.2 mL PCR tube with 100 µL of premelted 2% agarose/PBS. Once set, the agarose blocks were dehydrated in 70% ethanol for 2 h and all strains were embedded within a single mould. Atlantic salmon skeletal muscle fixed in a similar manner was added to the array as a host rRNA control.

Gill tissues from AGD-affected Atlantic salmon from tank and field-based populations were obtained for *in situ* hybridisation. Atlantic salmon presumptively diagnosed with AGD were euthanized (100 mg L^{-1} Aqui-S), the second left gill arch removed, placed in SWD for 24 h and processed for routine histology. Gills were sectioned (5 µm) and stained with haematoxylin and eosin (H & E) following routine histological procedures. After confirming that amoebae were attached to AGD lesions using light microscopy, tissues were further processed for *in situ* hybridisation.

Protocols were identical for hybridising the species-specific oligonucleotide probes to the rRNA within the *Neoparamoeba* array and AGD-affected gill tissues. Tissue sections (7 µm) were placed onto coated glass slides (Polysine, Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. Unless specified, all washes

were performed at room temperature (RT). Sections were dewaxed, rehydrated and sequentially washed for 2× 5 min with diethyl pyrocarbonate (Sigma-Aldrich, Castle Hill, New South Wales, Australia)-treated PBS (DEPC-PBS), 10 min with DEPC-PBS containing 100 mM glycine, 15 min with DEPC-PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 2× 5 min with DEPC-PBS. Sections were then permeabilised with 5 μg mL^{-1} RNase-free proteinase K (Amresco, Solon, Ohio, U.S.A.) at 37°C for 30 min, post-fixed for 5 min in DEPC-PBS containing 4% paraformaldehyde (ProSciTech, Townsville, Queensland, Australia) at 4°C then washed in DEPC-PBS for 2× 5 min. Sections were acetylated in 0.1 M triethanolamine (Sigma-Aldrich) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Fluka, Castle Hill, New South Wales, Australia) for 10 min on a rocking platform. Sections were then overlayed with 80 μL prehybridisation buffer [2× saline sodium citrate (SSC), 1× Denhardt's solution, 10% dextran sulphate (Sigma-Aldrich), 50 mM phosphate buffer, pH 7.0, 50 mM DDT, 500 μg mL^{-1} denatured and sheared cod DNA and a volume of deionised formamide (dF) (Sigma-Aldrich) specific for each probe (18S universal probe 61% dF; *N. pemaquidensis* probe 46% dF; *N. branchiphila* probe 37% dF and NCGD amoebae probe 56% dF)], a coverslip was added and slides were incubated in a humid chamber at 37°C for 2 h. Coverslips were removed by immersing sections in 2× SSC for 5 min. Sections were then overlayed with 80 μL hybridisation buffer with a probe (prehybridisation buffer and 4 ng μL^{-1} probe) or without a probe (no probe controls), a coverslip was added and slides were incubated in a humid chamber at 37°C for 17 h. Coverslips were again removed in 2× SSC then the slides were sequentially washed on a shaking platform at 37°C in 2× SSC for 2× 15 min, 1× SSC for 2× 15 min and 0.25× SSC for 2× 15 min. DIG-labelled probe detection was performed using a BCIP/NBT immunological method. Slides were washed on a shaking platform in tris buffered

saline (TBS) (100 mM Tris-HCl, pH 7.5 and 150 mM NaCl) for 20 min. Sections were then covered with blocking solution (TBS containing 0.1% Triton X-100 and 2% normal sheep serum) and incubated for 30 min. The blocking solution was decanted and slides were covered with TBS containing 0.1% Triton X-100, 1% sheep serum and a 1:500 dilution of sheep anti-DIG-alkaline phosphatase (Roche, Kew, Victoria, Australia) for 2 h in a humid chamber. Slides were washed in TBS for 20 min on a shaking platform then incubated for 10 min with TBS-$MgCl_2$ (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5). The TBS-$MgCl_2$ was decanted and sections were overlayed with a premixed BCIP/NBT solution (Sigma-Aldrich) and incubated for up to 2 h. The reaction was monitored under a light microscope and stopped by briefly washing slides in TE buffer (10 mM Tris-HCl, pH 8.1 and 1 mM EDTA). Sections were counterstained for 5 min in 0.1% nuclear fast red (Sigma-Aldrich), dehydrated and mounted (VectaMount™, Vector Laboratories, Burlingame, California, U.S.A).

3. Results

3.1. Confirmation of the presence of Neoparamoeba *species in Atlantic salmon NCGD amoebae primary isolates*

To determine whether *N. pemaquidensis* or *N. branchiphila* were represented within gill-derived amoebae preparations obtained directly from AGD-affected Atlantic salmon, template DNA was amplified using species-specific oligonucleotides (Fig. 1A and B, upper). Either *N. pemaquidensis* or *N. branchiphila* were PCR-amplified however amplification was qualitatively variable and PCR product yield was comparatively lower than products generated from the CCGD strains of either species. Furthermore, PCR products were weakly amplified from only two of the four amoebae primary isolates examined using *N. branchiphila*-specific oligonucleotides (Fig. 1A, upper). When *N. pemaquidensis*-specific oligonucleotides were used with template

DNA from the same amoebae primary isolates, PCR products were successfully amplified but some variability occurred (Fig. 1B upper). Equivalent concentrations of template DNA from cultured strains of *N. pemaquidensis* and *N. branchiphila* consistently amplified PCR products using their respective oligonucleotides with qualitatively more DNA yield than from NCGD amoebae preparations (Fig. 1A and B, upper). PCR inhibition may result from differences in culture and isolation techniques (see review Wilson, 1997). All templates were uniformly PCR amplified using universal 18S rRNA gene oligonucleotides, indicating that PCR inhibition did not contribute to the observed variability in PCR amplification (Fig. 1A and B, lower).

3.2. Examination of the species-specificity of current Neoparamoeba *species PCR oligonucleotides*

Neoparamoeba-specific oligonucleotides amplify regions of the 18S rRNA gene. Current *Neoparamoeba* oligonucleotide sequences were compared to their respective annealing sites on the 18S rRNA gene sequences from CCGD and NCGD amoebae as well as Atlantic salmon. PCR amplification and sequencing of the entire 18S rRNA gene of seven molecular clones from five independent primary isolates of NCGD amoebae generated sequences ranging in length from 2044 to 2132 bp with G+C contents between 39.8 and 40.5 %. Consensus 18S rRNA gene sequences were generated for the NCGD amoebae from AGD-affected fish, all described *Neoparamoeba* and Atlantic salmon using mixed base nomenclature standards (NC-IUB, 1985). The consensus sequences were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2) and the specificity of the *N. pemaquidensis* and *N. branchiphila* oligonucleotide annealing sites were assessed. *N. branchiphila* oligonucleotides aligned specifically with *N. branchiphila* (data not shown) however the *N. pemaquidensis* oligonucleotides partially matched sequences from NCGD amoebae

(Fig. 2). Of particular note is the infidelity between bases at the 3' end of the sense and antisense oligonucleotides and the template rRNA gene sequences which may partially inhibit PCR amplification.

3.3. The phylogenetic relationship of NCGD amoebae to Neoparamoeba *based on 18S rRNA gene sequences*

As we observed variability in the 18S rRNA gene sequences from NCGD amoebae along the annealing sites of diagnostic oligonucleotides, we speculated that these sequences may be phylogenetically distinct from other known *Neoparamoeba*. The 18S rRNA gene alignment consisted of 2243 nucleotide sites and of these, 627 were parsimony-informative. The nucleotide similarity values among the 18S rRNA gene sequences are summarised as the range of percent similarity among the phylogenetic lineages examined (Table 2). The ML and BI strict consensus trees yielded three distinct lineages within the *Neoparamoeba* genus (clades A, B, & C. Fig. 3A). Strains of *N. pemaquidensis* and *N. aestuarina* were unable to be separated and clustered within a large monophyletic group (clade A). There was strong support (76-85%) for *N. branchiphila* strains as a distinct monophyletic group (clade B) while the 18S rRNA gene sequences from NCGD amoebae isolated from tank (NCGD-D1) and field-based (NCGD-HAC) populations of AGD-affected Atlantics salmon were phylogenetically similar. Together, these sequences from NCGD amoebae primary isolates formed a monophyletic group (clade C) that was positioned basal or ancestral to the other described *Neoparamoeba* (100% support). The MP analysis of the 18S rRNA gene sequence alignment yielded a strict consensus tree that was similar to the BI and ML analysis (Fig. 3B). Strains of *N. pemaquidensis* and *N. aestuarina* clustered within a similar monophyletic group (clade A) although the clustering among *N. pemaquidensis* strains varied from the tree topology generated by BI and ML analyses. Strains of *N.*

branchiphila formed a monophyletic group (clade B, 91% support), positioned as the more divergent of the *Neoparamoeba*. NCGD amoebae isolated from tank (NCGD-D1) and field-based (NCGD-HAC) populations of AGD-affected Atlantic salmon were phylogenetically indistinguishable and formed a well-supported monophyletic group (clade C, 93% support).

3.4. Support for a new phylogenetic lineage of Neoparamoeba *using partial 28S rRNA gene sequence*

To provide additional support for the grouping of phylogenetic lineages inferred from the 18S rRNA genes, partial 28S rRNA gene sequences from representatives of clades A, B, & C described in Fig. 3 were PCR-amplified and analysed. A total of four strains representative of clade A, three strains representative of clade B, six primary isolates representative of clade C and one strain of *P. hoguae* were assessed (Table 1). Sequences ranged in length from 1615 to 1686 bp with G+C contents between 38.0 and 41.5%. The 28S rRNA gene alignment consisted of 1750 nucleotide sites and of these, 250 were parsimony-informative. The nucleotide similarity values among the 28S rRNA gene sequences are summarised as the range of percent similarity among the phylogenetic lineages examined (Table 2). Phylogenetic analyses of the 28S rRNA gene sequence alignment generated strict consensus trees using BI, MP and ML analyses that converged on identical tree topologies (Fig. 4). There was strong support (83-100%) for the exclusion of NCGD amoebae sequences from those representatives of clades A and B in Fig. 3, consistent with the preliminary analyses that indicated NCGD amoebae represent a lineage distinct from known *Neoparamoeba*. Representative strains of clade A and B were clustered in well-supported clades (97-100%) as described in Fig. 3. There was only modest support (54-61%) for the grouping of clades A and B as sister clades with clade C as the more divergent group, consistent with Fig. 3A.

3.5. *Design and validation of* Neoparamoeba *species-specific oligonucleotide probes for* in situ *hybridisation*

To identify which phylogenetic lineage(s) of *Neoparamoeba* are associated with AGD, oligonucleotide probes that specifically hybridise rRNA from *N. pemaquidensis*, *N. branchiphila* or the new NCGD amoebae phylogenetic lineage were designed (Fig. 5A). To ensure the suitability of fixed tissues for comparative *in situ* hybridisation, a universal 18S rRNA probe (18S probe) was initially hybridised to all tissue sections. The 18S probe hybridised to all amoebae across the *Neoparamoeba* array confirming the integrity of their rRNA (Fig. 5B). The specificity of each probe was validated using representative strains from *N. pemaquidensis*, *N. branchiphila* and NCGD amoebae (Fig. 5B). Species-specific probes hybridised to their respective representative strain of *Neoparamoeba* with no cross-hybridisation detected. Non-specific signal was not observed on the no-probe control sections (data not shown).

3.6. Description of AGD aetiology by hybridising species-specific oligonucleotide probes to amoebae associated with AGD-lesions

The role of the three phylogenetic lineages of *Neoparamoeba* described in this study (clades A, B and C, Fig.3) in AGD aetiology was assessed by hybridising species-specific probes to amoebae associated with typical AGD lesions. All serially sectioned gill arches from three tank and four field-based Atlantic salmon presented with typical AGD lesions as previously described (reviewed by Munday et al., 2001). Sections of gill filaments presented with hyperplasia of epithelia-like cells resulting in lamellar fusion. Amoebae with at least one intracellular PLOs were adjacent to these lesions (Fig. 6). The universal 18S probe hybridised evenly to rRNA in gill tissues and amoebae trophozoites, confirming the rRNA integrity in tissues across all sections (data not shown). In all tank and field-based AGD-affected Atlantic salmon gill tissues, the

NCGD amoebae-specific probe hybridised with amoebae associated with typical AGD-lesions and unambiguously associated the new phylogenetic lineage (clade C) with AGD (Fig. 6). The *N. pemaquidensis* and *N. branchiphila* probes did not hybridise with sectioned amoebae on the gill arches and non-specific signal was not observed on the no-probe control sections (data not shown).

3.7. Description of Neoparamoeba perurans *n. sp. (Lobosea; Vexilliferidae)*

3.7.1. Description

N. perurans n. sp. morphology corresponds to previous descriptions of *Neoparamoeba* species (Page, 1987; Dyková et al., 2005). Amoebae with digitiform pseudopodia when free floating, and mamilliform pseudopdia when adhered. Trophozoites 41-56 μm in adhered form. One or more *Perkinsiella amoebae*-like organisms (5.3-8.0 μm) adjacent to nucleus (3.3-6.0 μm) and cell-surface microscales absent. Histologically, visible nucleus and parasome present in trophozoites associated with host gill tissue. Cytoplasm vacuolated. Member of an exclusive phylogenetic cluster within the *Neoparamoeba* genus based on 18S and 28S rRNA gene sequenced from this species (GenBank accession numbers EF216898-EF216918).

3.7.2. Host

Type host Atlantic salmon, *Salmo salar* L. 1758. (Salmoniformes: Salmonidae). Trophozoites attached to gills adjacent to hyperplastic epithelia-like cells. Atlantic salmon reared in recirculating tank system infected with non-cultured, gill-derived amoebae by cohabitation with AGD-affected Atlantic salmon. Atlantic salmon reared in sea-cages obtained during AGD epizootic. In both cases, *N. perurans* n. sp. was the only amoeba identified in association with gill lesions.

3.7.3. Locality

Confirmed cases of *N. perurans* n. sp. infections are from the D'Entrecasteaux Channel, Tasmania, Australia.

3.7.4. Type material

Type material consisting of frozen and fixed *N. perurans* n. sp. derived from host gill tissue are held in the collection of the School of Aquaculture, University of Tasmania, Australia. H&E stained histological sections of Atlantic salmon gill tissue, confirmed by *in situ* hybridisation to be infected with *N. perurans* n. sp., were deposited in the collection of the Queensland Museum, Brisbane, Australia. Eighteen syntypes were deposited as six serial tissue sections from each of three fish (Fish 1: G464935-G464940, Fish 2: G464941-G464946, Fish 3: G464947-G464952). The 18S and 28S rRNA gene sequences of gill-derived amoebae were deposited in GenBank (accession numbers EF216898-EF216918).

3.7.5. Etymology

This species is named after the Latin word for inflame, representing the inflammation associated with attachment of amoebae to gill lamellae.

4. Discussion

In earlier studies, numerous strains of *N. pemaquidensis* and *N. branchiphila* were successfully cultured from AGD-affected fish (Fiala and Dyková, 2003; Dyková et al., 2005; Dyková et al., 2007). The morphological similarities of these cultured strains with trophozoites associated with AGD gill lesions led to the belief that both species could be causal in AGD (Wong et al., 2004; Dyková et al., 2005) however the parasite-disease nexus has never been directly corroborated. In this study, our initial objective was to address the aetiological role of *N. pemaquidensis* and *N. branchiphila* in AGD. Through a molecular approach, a new phylogenetically distinct lineage of *Neoparamoeba* was identified and shown to be the exclusive aetiological agent of AGD in all samples

examined. Therefore, we describe this new lineage as *Neoparamoeba perurans* n. sp. on the basis of both phylogenetic and virulence-related phenotypic divergence from other *Neoparamoeba* species.

Amoebae first assumed to be *N. pemaquidensis* and *N. branchiphila* were found in preparations of amoebae directly isolated from AGD-affected Atlantic salmon by PCR. However, amplification of NCGD amoebae DNA by PCR using *N. pemaquidensis* and *N. branchiphila*-specific oligonucleotides yielded comparatively lower amplicons, suggesting these were not the predominant species. This observation prompted the sequencing of the 18S rRNA gene from NCGD amoebae and the subsequent recognition that there was variation in NCGD amoebae sequences initially within the *N. pemaquidensis* and *N. branchiphila* oligonucleotide annealing sites. Thus the PCR results for *N. pemaquidensis* may have been confounded by the possibility that the *N. pemaquidensis* oligonucleotides partially amplify more than one species of *Neoparamoeba*. While species-specific PCR amplification may provide a rapid diagnostic tool to confirm the presence of *Neoparamoeba* in DNA preparations (Wong et al., 2004; Dyková et al., 2005), it is strictly dependent on oligonucleotide specificity. Until the *N. pemaquidensis* oligonucleotides are validated, particularly with respect to the amplification of the NCGD amoebae rRNA gene, their utility is questionable and further use without redesign is cautioned.

Qualitative assessment of *N. pemaquidensis* and *N. branchiphila* in amoebae preparations from AGD-affected fish using PCR led to the sequencing of 18S rRNA genes from NCGD amoebae isolates. These sequences were used to phylogenetically assess NCGD amoebae and closely-related taxa. NCGD amoebae sequences clustered

together with sequences from amoebae of the *Neoparamoeba* genus, conforming with the taxonomic classification of NCGD amoebae assigned using morphological characters (Morrison et al., 2004; Morrison et al., 2005). However no further morphometric evaluation of NCGD amoebae was performed given that sub-generic discrimination of *Neoparamoeba* using morphological features is unreliable (Dyková et al., 2005). Phylogenetic analyses of the 18S rRNA gene generated a monophyletic group exclusive to NCGD amoebae sequences. In addition, analyses of the 28S rRNA gene from representatives of the three phylogenetically distinguishable lineages were consistent with this preliminary inferred taxonomic classification where NCGD were excluded from other *Neoparamoeba* sequences.

During phylogenetic analyses, *N. pemaquidensis* and *N. aestuarina* sequences were not resolved into monophyletic groups as previously described (Fiala and Dyková, 2003; Dyková et al., 2005). The segregation of *N. pemaquidensis* and *N. aestuarina* based on a comparison of their 18S rRNA genes has already proven difficult. Following the inclusion of additional 18S rRNA gene sequences of *Neoparamoeba* and related taxa, the phylogenetic relationship between the *N. pemaquidensis* strain AVG 8194 and the *N. aestuarina* group became ambiguous (Dyková et al., 2007). On a broader scale, when *Neoparamoeba* 18S rRNA gene sequences were compared with similar sequences from a wide selection of protist taxa there was diminishing support for the separation of *N. pemaquidensis* and *N. aestuarina* (Fiala and Dyková, 2003; Peglar et al., 2003). It was suggested that the inability to distinguish *N. pemaquidensis* from *N. aestuarina* was due to the elimination of phylogenetically informative sites from the analyses after they could not be aligned with the non-*Neoparamoeba* species (Peglar et al., 2003). Here, the inability to distinguish previously described lineages of *Neoparamoeba* using the 18S

rRNA gene is possibly an artefact of poor resolution among closely-related species. While the highly conserved nature of 18S rRNA genes across the animal kingdom allows the comparison of divergent taxa (Hillis and Dixon, 1991), it also limits the power to discriminate between closely-related species (Adam et al., 2000). For example, the relationship between strains of the *Acanthamoeba* Volkonsky, 1931 genus remained unresolved using comparisons of the 18S rRNA gene (Stothard et al., 1998). Alternative regions of the rRNA gene with higher rates of variability between closely related taxa such as the 28S and internal transcribed spacer 1 (ITS1) regions have been used to distinguish inter- and intra-specific relations (for example Adam et al., 2000; Hansen et al., 2000; Bergholtz et al., 2005; Sato et al., 2005; Hosoi-Tanabe et al., 2006; Köhsler et al., 2006). In the present study, partial 28S rRNA gene sequences spanning the phylogenetically informative D1 to D3 domains were compared. Sequencing the partial 28S rRNA gene from additional cultured *Neoparamoeba* strains, NCGD amoebae primary isolates and strains of closely related taxa may be more useful to resolve the phylogeny of the entire *Neoparamoeba* lineage.

Given that there now was indirect evidence that up to three species of *Neoparamoeba* were associated with AGD-affected fish, we sought to resolve which of these species were responsible for AGD. Previously, it was suggested that an ISH-based test for histological sections would be the most suitable tool for prospective as well as retrospective AGD aetiological studies (Dyková et al., 2005). Therefore during our study, we adopted this approach whilst fulfilling the sequence-based guidelines for microbial disease causation (Fredricks and Relman, 1996). Using ISH, *N. perurans* n. sp. but not *N. pemaquidensis* and *N. branchiphila* was unambiguously identified as the predominant pathogenic amoeba adjacent to the gill-lesions of AGD-affected Atlantic

salmon in Tasmania. As neither *N. pemaquidensis* nor *N. branchiphila* have been demonstrably associated with AGD gill lesions, or shown to induce AGD (Kent et al., 1988; Howard and Carson, 1993; Morrison et al., 2005; B. Vincent pers. comm.) there remains a contrasting representation of *Neoparamoeba* species from *in vitro* and *in vivo* studies. Whether there is selection of *Neoparamoeba* species during continuous *in vitro* culture is unknown but warrants further investigation.

Since the representation of *Neoparamoeba* species differs between *in vitro* culture and *in vivo* infection, it was pertinent to investigate whether the species composition could alter when virulent *Neoparamoeba* were maintained using cohabitation of fish in a recirculation tank for approximately five years. Data presented here clearly show congruent 18S and 28S rRNA gene sequences of NCGD amoebae isolated from tank and field-based populations of Atlantic salmon. In addition, *N. perurans* n. sp. was the only detectable species associated with AGD lesions in samples obtained from both populations of fish. This indicates that experimentally-induced AGD in tank-based infectivity trials reflect the AGD aetiology observed in field-based populations of AGD-affected Atlantic salmon in Tasmania and that *N. perurans* n. sp. may have been the predominant species of amoeba responsible for the experimental induction of AGD in previously published studies (for example Zilberg et al., 2001; Bridle et al., 2003; Adams and Nowak, 2004; Gross et al., 2004; Morrison et al., 2004; Embar-Gopinath et al., 2005; Gross et al., 2005; Morrison et al., 2005; Bridle et al., 2006; Morrison et al., 2006; Vincent et al., 2006).

In summary, a molecular-based approach led to the discovery of *N. perurans* n. sp. that was linked to cellular pathology in AGD-affected Atlantic salmon. This provides compelling evidence of causation, consistent with the sequence-based identification of

microbial pathogens guidelines (Fredricks and Relman, 1996). The spatial and temporal distribution of *Neoparamoeba* in the context of AGD is unknown; however data presented here highlight the need to incorporate culture-independent methods in future studies.

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Table 1. Marine amoebae included in either phylogenetic analyses using partial sequences of the 28S rRNA gene or included in a *Neoparamoeba* tissue array.

Species	*C ID*[a]	*Origin*[b]	*Accession Number*[c]
Neoparamoeba pemaquidensis	PA027*	Dover, G.	AF371967
	NP251002*	Infection Tank, University of Tasmania, G	AY714351
	WTUTS*	Infection Tank, University of Tasmania, G	AY714361
	GILLNOR1*	D'Entrecasteaux Channel, Bruny Island, G	AY714352
Neoparamoeba branchiphila	NRSS*	Infection Tank, University of Tasmania, G	AY714367
	ST4N*	Huon Estuary, Dover, G	AY714365
	SEDMHI*	Macquarie Harbour, S	AY714366
Paraflabellula hoguae	NETC3	Huon Estuary, Dover, N	AY277797

[a]Clonal culture identification tags from a collection held at the School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia.
[b]Region of Tasmania, Australia from which original isolates were derived and whether they were cultured from gill (G). sea-cage net (N) or sediment (S) samples
[c]GenBank accession numbers (http://www.ncbi.nlm.nih.gov/)
*Amoebae included within the *Neoparamoeba* tissue array

Table 2. Percent similarity among the aligned *Neoparamoeba* 18S or 28S rRNA gene sequences used for phylogenetic analyses derived from mean character differences [a]

	N. pemaquidensis	*N. aestuarina*	*N. branchiphila*	*N. perurans*	Outgroup [b]
N. pemaquidensis	96.6 – 100 **95.8 - 98.4**	93.9 - 96.3	91.1 - 92.6 **89.8 - 91.1**	92.9 - 94.2 **90.8 - 92.3**	79.4 - 88.9 **73.9 - 74.1**
N. aestuarina		95.4 - 99.2	90.31 - 92.0	91.7 - 92.7	79.8 - 88.5
N. branchiphila			96.5 - 98.9 **98.1 - 98.3**	91.2 - 92.0 **89.8 - 90.8**	79.5 - 88.6 **73.3 - 73.5**
N. perurans				98.3 - 99.3 **97.3 - 99.6**	80.2 - 88.8 **74.2 - 74.5**
Outgroup [b]					79.5 - 93.3 -

[a] the range of percent sequence similarities are displayed for the 18S and 28S (bold text) rRNA gene sequences among the *Neoparamoeba* species examined

[b] the outgroup for the 18S rRNA gene sequences alignment included *V. armata*, *K. hemistylolepis*, *K. stella*, *V. aberdonica*, and *V. anglica*. The outgroup for the 28S rRNA gene sequence alignment was *P. hoguae*

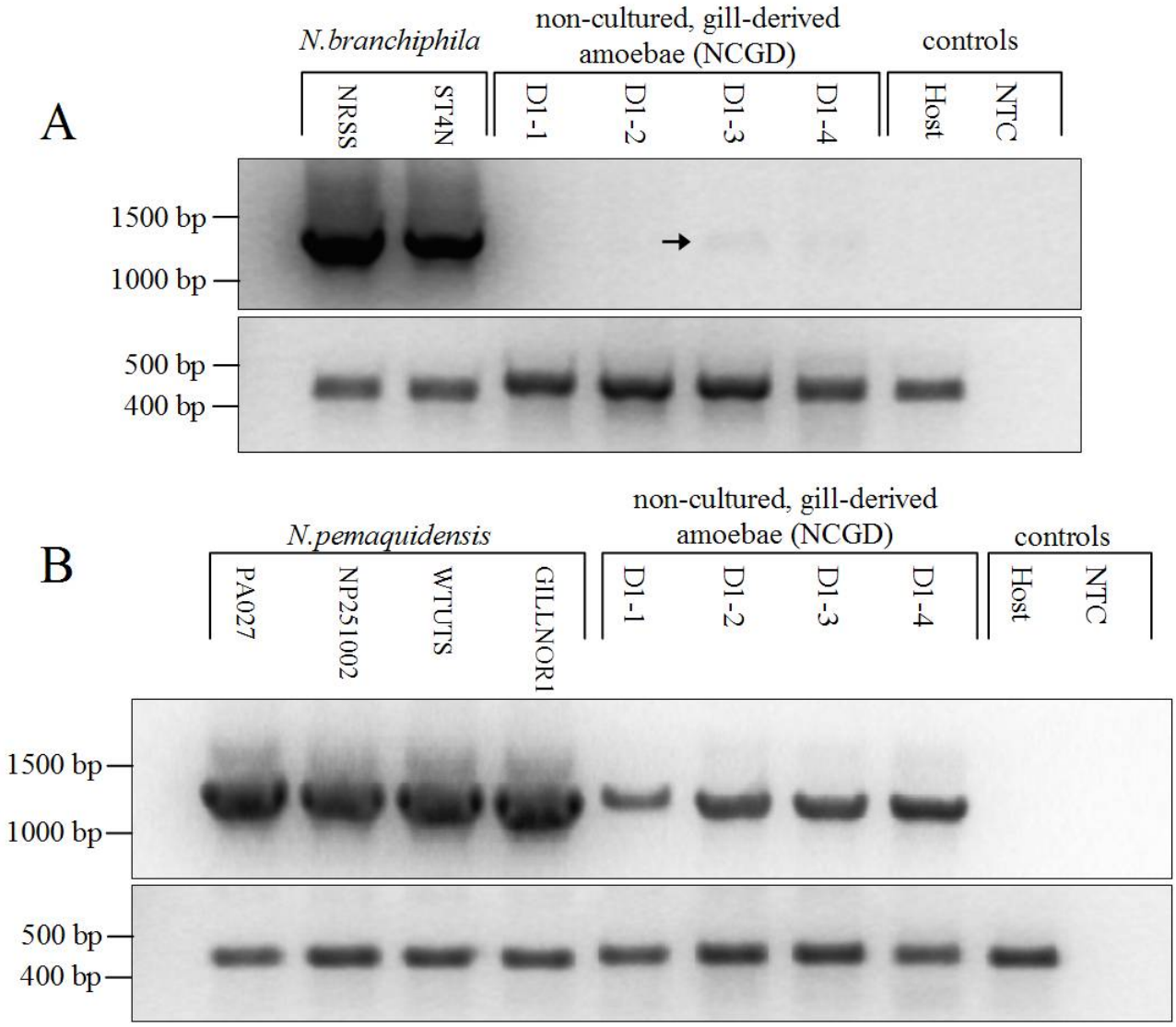

Fig. 1. Variability in PCR amplification of the 18S rRNA gene from non-cultured, gill-derived (NCGD) amoebae using *Neoparamoeba* species-specific oligonucleotides. (A upper) PCR amplification using *N. branchiphila*-specific oligonucleotides. Amoebae DNA templates included *N. branchiphila* strains NRSS and ST4N, four NCGD amoebae primary isolations from AGD-affected Atlantic salmon, *Salmo salar* L. (D1-1 to D1-4), control Atlantic salmon DNA (Host) and no template control (NTC). Arrow shows faint PCR amplicon from NCGD amoebae D1-3 and D1-4. (B upper) PCR amplification using *N. pemaquidensis*-specific oligonucleotides. Amoebae templates included *N. pemaquidensis* strains PA027, NP251002, WTUTS and GILLNOR1, the same NCGD amoebae templates described above (D1-1 to D1-4), control Atlantic salmon DNA (Host) and no template control (NTC). (A and B lower) PCR amplification of identical templates described above using universal 18S rRNA gene oligonucleotides.

	N. pemaquidensis sense (5' to 3')	*N. pemaquidensis* antisense (3' to 5')
	----CATCTCCTTACTAGACTTTCATG----	----CGGGT-AGAGCGAGTTTGTTGTG----
N. pemaquidensis	HGTH.....RTTR	CTYTY....-.....RAGG
N. branchiphila	TATCTT.....WY.....CTATTR	CTCTTTT..G.RRATR.....C....GAGA
N. aestuarina	TRYW.....Y.RATG	YTYT....D-R..RY.....WKKY.GGGA
NCGD amoebae	TGTA.T.....CTATYA	CTYTY....G.A.AT.....R..GAGA
Salmo salar	GCCC.TG.CT..CGGCGCC.CC..---GATG	CTCTT....G.A.AT.....GAGA

Fig. 2. *Neoparamoeba pemaquidensis* sense (fPA -Hxe23a1) and antisense (rPA -Hx49, reverse complimented) oligonucleotides described by Wong et al. (2004) aligned against consensus 18S rRNA gene sequences for known *Neoparamoeba* species, NCGD amoebae isolated from AGD-affected Atlantic salmon, *Salmo salar* and host 18S rRNA. Mixed bases are shown using mixed base nomenclature standards (NC-IUB, 1985). Consensus in bases is represented in the alignment as a dot (.) while a gap in the sequence is represented by a dash (-).

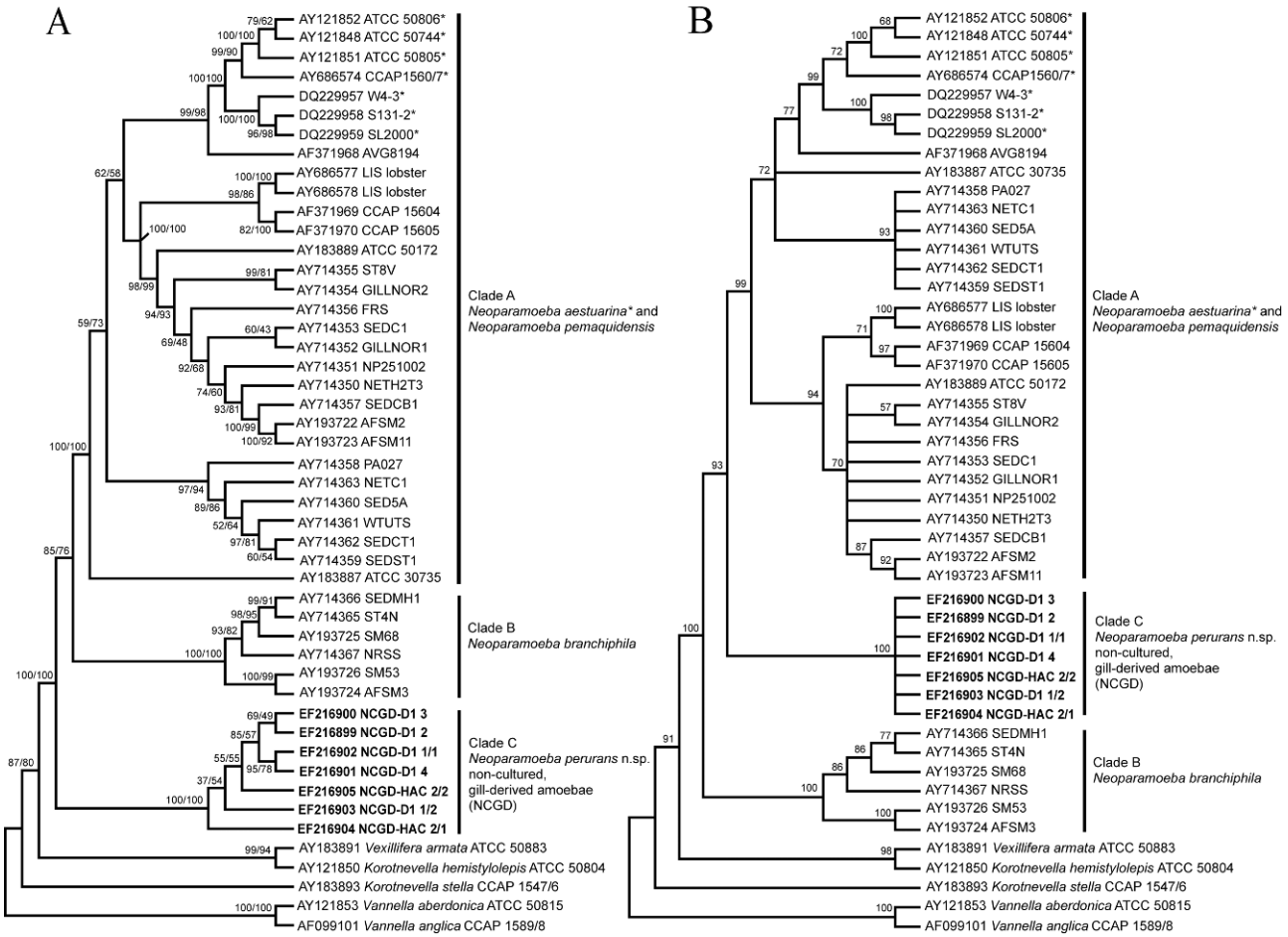

Fig. 3. Two strict consensus trees resulting from the phylogenetic analysis of *Neoparamoeba* 18S rRNA gene sequences recognise a new phylogenetic lineage of *Neoparamoeba* derived from the gills of AGD-affected Atlantic salmon, *Salmo salar*. Phylogeny of *Neoparamoeba* was inferred from the 18S rRNA gene sequences using maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic inference (BI). BI and ML analyses yielded equally parsimonious consensus trees that distinguished three *Neoparamoeba* clades (Fig. 3A). MP analysis supported the division of *Neoparamoeba* into the same three clades however the strict consensus tree differed in topology (Fig. 3B). Clade A is composed of strains of *N. pemaquidensis* and *N. aestuarina* (denoted by an asterix). Clade B is composed of strains of *N. branchiphila*. Clade C is composed of new 18S rRNA gene sequences (in bold) of non-cultured, gill-derived amoebae (*Neoparamoeba perurans* n. sp.) from AGD-affected Atlantic salmon. Values indicated on the branches represent bootstrap support (Fig 3A: BI/ML). The GenBank accession number of each sequence is shown.

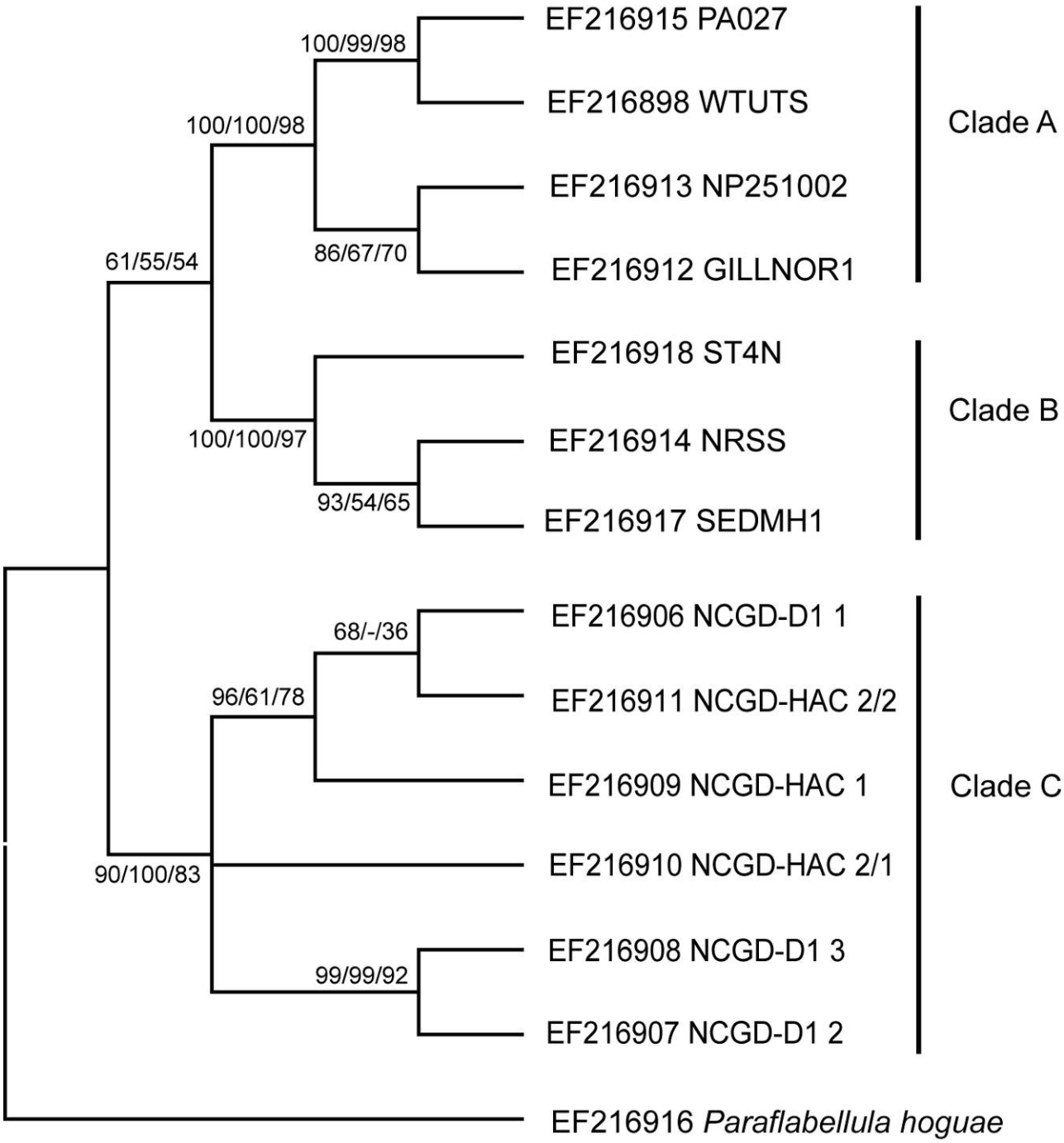

Fig. 4. Strict consensus tree resulting from the phylogenetic analysis of partial 28S rRNA gene sequences supports the addition of a new phylogenetic lineage to the *Neoparamoeba* genus. Phylogeny of the *Neoparamoeba* lineage of amoebae inferred from 28S rRNA gene sequences using maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic inference (BI). Values indicated on the branches represent bootstrap support (BI/MP/ML). Representatives of clades A, B and C described in Fig. 3 formed three monophyletic groups and are also designated clades A, B and C in this Figure. The GenBank accession number of each sequence is shown.

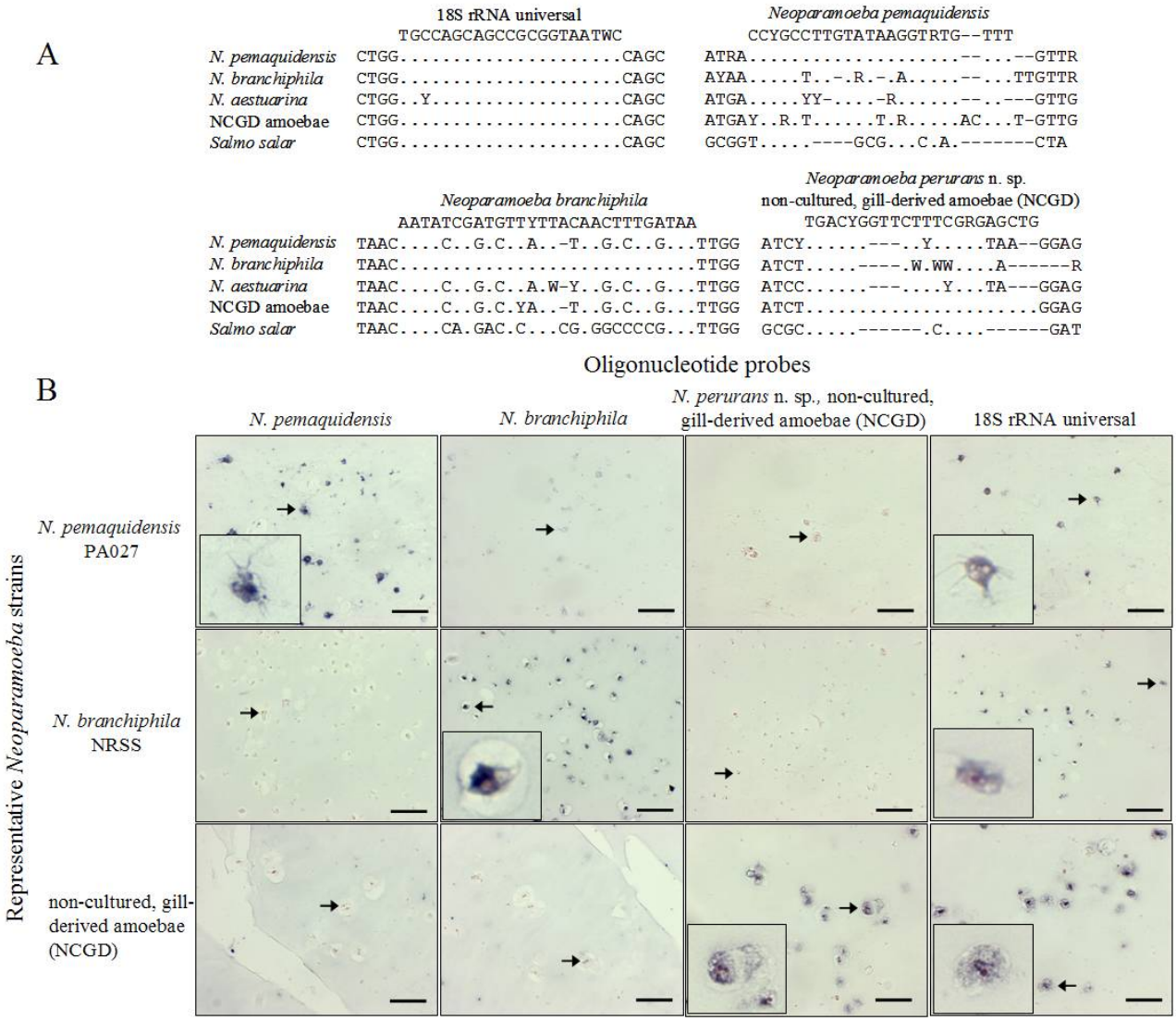

Fig. 5. Species–specificity of 18S rRNA targeted oligonucleotide probes demonstrated by sequence alignment and *in situ* hybridisation using a *Neoparamoeba* array. (A) Probes aligned to consensus 18S rRNA gene sequences of known *Neoparamoeba* species, non-cultured, gill-derived (NCGD) amoebae isolated from AGD-affected Atlantic salmon, *Salmo salar* L. and Atlantic salmon. Mixed bases are shown using mixed base nomenclature standards (NC-IUB, 1985). Consensus in bases is represented in the alignments as a dot (.) while a gap in the sequence is represented by a dash (-). Probes are presented in the reverse complement orientation to align with the genomic sequences. (B) *In situ* hybridisation showing the species-specificity of probes using a *Neoparamoeba* array including representatives from clades A, B and C described in Fig. 3 and 4. *N. pemaquidensis* (PA027), *N. branchiphila* (NRSS) and NCGD amoebae from AGD-affected Atlantic salmon are shown. Arrows highlight amoebae and probe-positive cells are magnified within the inserts. Scale bars represent 50 µm.

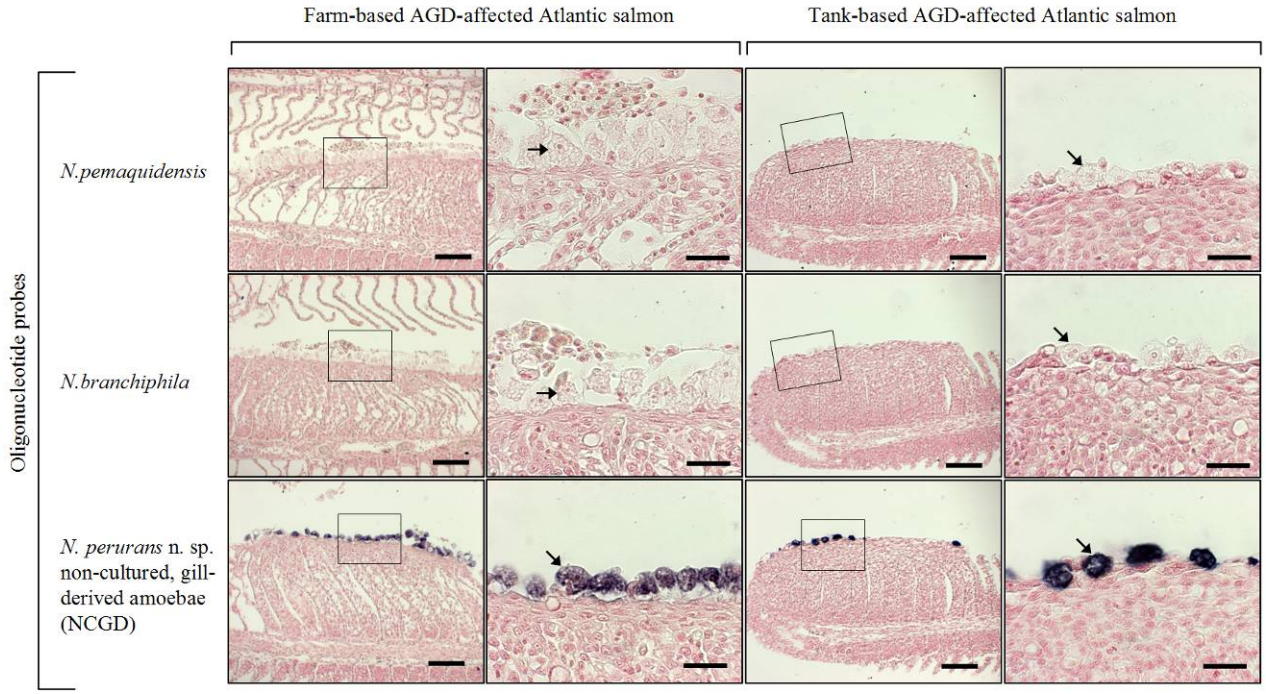

Fig. 6. A fourth phylogenetic lineage of *Neoparamoeba* is associated with gill lesions from AGD-affected Atlantic salmon, *Salmo salar*. Species-specific oligonucleotide probes that hybridise to 18S rRNA of *Neoparamoeba pemaquidensis*, *N. branchiphila* and non-cultured, gill-derived (NCGD) amoebae (*N. perurans* n.sp.) were used to probe gill tissue from tank and field-based AGD-affected Atlantic salmon. Images are representative of *in situ* hybridisation experiments using a total of three tank-based and four farm-based AGD-affected fish (ie. n = 7 fish). Serial sections of gill filaments with typical AGD lesions with amoebae attached to the lesion surface (arrows). Inset boxes are magnified in the adjacent image. Low magnification scale bar represents 100 µm, high magnification represents 30 µm. Non-specific signal was not observed on the no-probe control sections (data not shown).