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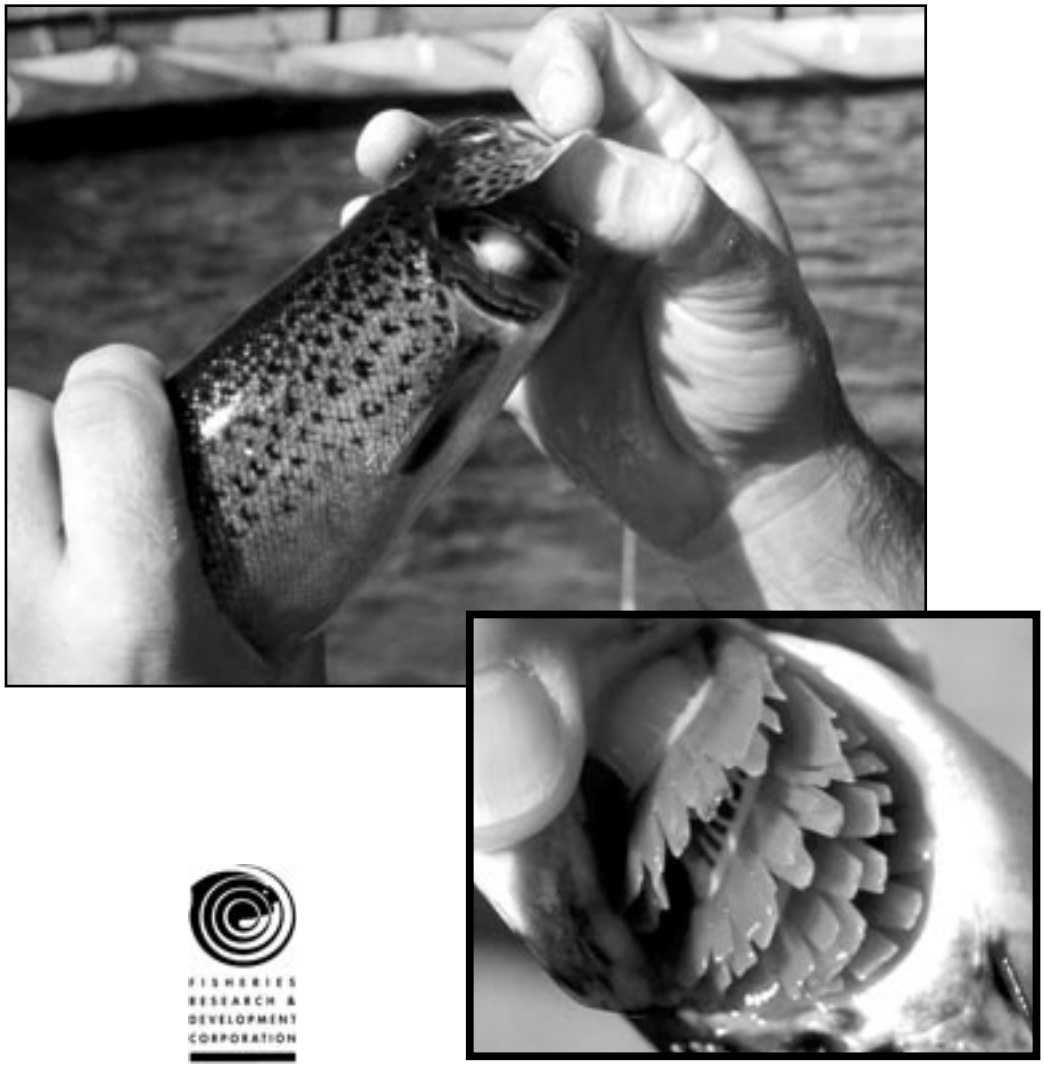
Detection and abundance of Paramoeba species in the environment

Nicholas G. Elliott, Frank Wong and Jeremy Carson



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CSIRO MARINE RESEARCH

Tasmanian Aquaculture & Fisheries Institute
University of Tasmania

Detection and abundance of Paramoeba species in the environment

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1. NON-TECHNICAL SUMMARY

98/209 Detection and abundance of *Paramoeba* species in the environment

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OBJECTIVES:

- Obtain a library of paramoeba isolates and a range of amoebae from infected Atlantic salmon and the environment, sequence regions of the small-subunit ribosomal DNA to identify DNA sequences specific to *Paramoeba* species.
- Using DNA sequences specific to *Paramoeba* species, develop and test a diagnostic assay procedure based on PCR (polymerase chain reaction) technology.
- Using the diagnostic assay developed, identify the major sources and reservoirs of *Paramoeba* in the environment, in and adjacent to fish farms.

NON TECHNICAL SUMMARY:

- Amoebic gill disease (AGD) remains the major disease associated with sea-cage culture of Atlantic salmon in Tasmania. AGD is associated with a *Paramoeba* species infecting the gills. Current treatment involves multiple freshwater bathes for all fish. This treatment has a significant financial impact on the industry, a cost estimated to be equivalent to over 10% of the industry GVP. Prevalence of the disease and costs associated with the freshwater treatment are increasing.

- While *Paramoeba* sp. had been identified as the causative agent for AGD, relatively little is known of the biology and life cycle of the organism; including, prior to this project, the species identity of the pathogen. Likewise the source of the infecting *Paramoeba* in the environment is unknown. A selective and sensitive method for detecting the particular *Paramoeba* species in the environment was required to assist with the management of AGD.

- The complete DNA sequence of the 18S rDNA gene was obtained for four cultures of *Paramoeba* sp. originally isolated from the gills of Tasmanian Atlantic salmon. This sequence data (ca. 2,100 base pairs) was compared with that obtained for other amoebic organisms associated with the gills of local Atlantic salmon. In addition, comparative sequence data was obtained for cultures of four known *Paramoeba* species from reference collections at the American Type Culture Collection (ATCC) and the Culture Collection of Algae and Protozoa UK (CCAP). The latter reference material included the index strain of *Neoparamoeba pemaquidensis* previously implicated as the pathogen of AGD in coho salmon in the USA; all other strains originated from free-living isolates from coastal marine waters. Sequence data were also obtained from isolates of *Paramoeba* sp. affecting Atlantic salmon in Ireland.

- DNA sequence analyses confirmed that Tasmanian AGD-*Paramoeba* isolates are identical to reference isolates of *Neoparamoeba pemaquidensis* Page 1970 (Sarcomastigophora: Paramoebidae) and are believed to be the same species.

- Analyses also showed that *N. pemaquidensis* is a widely distributed marine species, both as a free-living marine organism and associated with AGD. Isolates from Australia, Ireland, Wales, and the USA shared 98-99% DNA sequence similarity over 2104 base pairs of the 18S rDNA gene.

- PCR (polymerase chain reaction) amplification primers that are specific to *N. pemaquidensis* were developed, optimised and tested. Optimisation included not only PCR conditions and detection limits, but also DNA extraction protocols for environmental samples (seawater, sediment and biological material).

- *N. pemaquidensis* cells were detected in seawater and biological samples using the PCR technique. No systematic environmental survey was performed due to limited sampling and analysis time following validation of the technique. Contamination of reference samples and ensuring good quality DNA from complex environmental samples were the main constraints experienced.

- The PCR technique provides the most specific detection and identification of AGD-associated paramoeba cells from environmental samples to date as it relies on the unique DNA signature of the target organism. Immunological based methods (e.g. IFAT) are inappropriate for screening crude environmental samples due to their uncertain specificity (may cross-react with other species). As DNA remains constant regardless of life cycle stage it remains detectable by PCR amplification at all life cycle stages. Culture enrichment of environmental samples prior to PCR amplification allows verification of the detection of viable (live) cells.

- A specific DNA based detection method for the causative agent of AGD is now available. Detailed spatial and temporal surveys of farm and non-farm sites using this specific method are now required to identify short and long term sources and reservoirs of the *Paramoeba*. Such information will allow a better understanding of both the intra- and inter- farm and annual variation, and of the life cycle of the organism leading to improved management and treatment of AGD.

OUTCOME ACHIEVED

A positive identification and improved ability to detect the causative agent of AGD through the development of a specific analytical procedure. The new assay procedure will assist researchers to investigate the epidemiology of Amoebic Gill Disease through planned research in the Salmon Health Project of the CRC for Sustainable Aquaculture of Finfish. Research will lead to improved management strategies for AGD, so reducing management costs and increasing the profitability and sustainability of salmon farming in Australia.

KEYWORDS: *Neoparamoeba pemaquidensis*, Atlantic salmon, *Salmo salar*, aquaculture, species identification, AGD, PCR amplification, DNA extraction

2. BACKGROUND

Amoebic gill disease (AGD) is the major disease of Atlantic salmon (*Salmo salar*) aquaculture in Tasmania. Current treatment is by bathing fish in freshwater to inactivate and/or remove adhering amoebae. This procedure is both expensive and time consuming and may adversely stress fish making them more susceptible to opportunistic infections. Reducing the impact of AGD is a high priority for the salmon industry as its incidence has been increasing in recent years.

AGD was first diagnosed in salmon smolt in southeast Tasmania in late 1985. The causative agent, an amoeboid marine protist of the genus *Paramoeba*, and the basic infection process and histopathology was subsequently determined (Foster and Percival, 1988). Amoebae of the *Paramoeba* genus are characterised by the presence of DNA-positive, membrane bound inclusions (known as parasomes) located adjacent to the cell nucleus (Cann and Page, 1982; Roubal *et al*., 1989). Morphological and ultrastructural studies of *Paramoeba* isolates from AGD infected salmon showed that the Tasmanian parasite most closely resembles the species *Neoparamoeba pemaquidensis* Page 1970 (Roubal *et al*., 1989). This species is commonly isolated as a free-living marine species (Page, 1973), but has also been implicated as the pathogen of gill disease in sea-farmed coho salmon, *Oncorhynchus kisutch* on the west coast of the USA (Kent *et al*., 1988). Association of *N. pemaquidensis* with a salmonid gill disease of similar aetiology to the Tasmanian AGD further supported a similarity with the Tasmanian parasite. Some discrepancies in the gross morphology and physiology observed between the Tasmanian AGD isolates and the index isolate of *N. pemaquidensis* from the USA have precluded a definitive species ascription. However, there was strong evidence to suggest that the species was *N. pemaquidensis* (Roubal *et al*. 1989; Howard and Carson, 1992). Possible morphological and physiological differences due to different environmental, host or culture conditions and life cycle stages highlight some limitations of using morphological criteria to compare and identify similar protistan isolates. For instance, *Paramoeba* cells cultured *in vitro* had modified ultrastructure and were smaller in size than cells attached to gills of host fish (Roubal *et al*., 1989), and floating cells in liquid culture exhibited a different morphotype to those on solid substrates (Cann and Page, 1982).

From the time of its identification as a parasomal amoeba to the present study (a period of more than a decade), little attempt has been made to systematically characterise the Tasmanian AGD parasite. In a series of studies, Howard and Carson (1992, 1993, 1994, 1995) were able to establish unequivocally that a species of paramoeba was the pathogen of AGD and developed and validated a rapid diagnostic test based on the immunofluorescent antibody technique (IFAT) for confirming the presence of paramoebae on fish gills. While there was good evidence that the agent of AGD was probably *N. pemaquidensis*, uncertainty remained about its true identity and it remained taxonomically unspeciated.

To date, little is known of the ecology of *Paramoeba* sp. within the coastal environments supporting salmon farming in Tasmania. This is a significant hindrance to better defining the possible factors (both environmental and ecological) involved in outbreaks of AGD and subsequently, the development of appropriate management strategies to minimise impacts to the industry. Proper identification and systematic

classification of the parasite and determining its relationship to taxonomically similar marine protists are obvious and important steps toward further ecological studies of *Paramoeba* and epidemiological analyses of AGD.

Early AGD research included the development of an immunological diagnostic method (IFAT), and this is used for routine diagnosis of AGD. However this method is only suitable for disease diagnosis. The method has a well defined but narrow range of application and its performance with environmental samples with respect to specificity would be highly questionable. Development of a DNA-based diagnostic method with the inherent greater assurance of specificity is preferable to attempting the re-engineering of the current polyclonal antibody. DNA based techniques are also well suited to testing complex, heterogenous environmental samples which can be problematic for many immunological techniques.

In this study we use sequence analysis of the small subunit (SSU) or 18S ribosomal RNA gene (rDNA) for systematic identification of AGD-associated *Paramoeba* isolates. Comparative analysis of 18S rDNA sequences determines the phylogeny or evolutionary relatedness between organisms or isolates, and is recognised as the most consistent and comprehensive approach to systematic classification using molecular data (Medlin *et al*., 1988; Sogin and Silberman, 1998). The sequence data is then used to develop a selective and sensitive method for detecting *Paramoeba* in the environment. This will allow the identification of reservoirs likely to act as sources of infection for farmed fish.

Paramoeba species have also been associated with gill disease outbreaks in teleosts in western North America, New Zealand, Chile, Spain and Ireland. Due to the severity of AGD in Tasmania, Australian R&D leads the world in attempts to understand and control the disease; this position is further enhanced by this project.

3. NEED

Amoebic gill disease remains the major disease affecting the Atlantic salmon industry in Tasmania. *Paramoeba* sp. has been identified as the causative agent but relatively little is known of its biology and life cycle, prohibiting the development of cost-effective management practices to contain the effects of the disease. AGD is estimated to cost the industry close to $A10m per annum, a value equivalent to over 10% of the industry GVP. Prevalence of the disease and costs associated with the freshwater bathing treatment are increasing. This trend is likely to continue without improved strategies to reduce or contain AGD.

The infecting source of *Paramoeba* sp. in the environment is currently not known. If the infecting source could be identified, appropriate management strategies could be developed to minimise infection of sea-farmed fish by reducing the numbers of *Paramoeba* sp. in the environment or by minimising contact between host and the pathogen. Knowledge of the location of the infecting sources of *Paramoeba* sp. would reduce the detrimental effects of AGD on farming Atlantic salmon and significantly reduce the current high cost of controlling the disease.

To be able to locate the source of the *Paramoeba*, firstly its actual identity and similarity to related species needs to be established, and secondly, a species-specific identification method is required. Protocols then need to be developed to enable analysis of complex environmental samples.

4. OBJECTIVES

1. Obtain a library of paramoeba isolates and a range of amoebae from infected Atlantic salmon and the environment, sequence regions of the small-subunit ribosomal DNA to identify DNA sequences specific to *Paramoeba* species.

2. Using DNA sequences specific to *Paramoeba* species, develop and test a diagnostic assay procedure based on PCR (polymerase chain reaction) technology.

3. Using the diagnostic assay developed, identify the major sources and reservoirs of *Paramoeba* in the environment, in and adjacent to fish farms.

5. MOLECULAR IDENTIFICATION OF *PARAMOEBA* SP.

5.1 Methods

5.1.1 *Paramoeba* Strains and Isolates

5.1.1.1 Reference *Paramoeba* and related strains

Type and reference cultures of representative *Paramoeba*, *Neoparamoeba*, and *Pseudoparamoeba* strains used in this study (Table 5.1) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and the Culture Collection of Algae and Protozoa (CCAP), Ambleside, Cumbria, UK.

5.1.1.2 *Paramoeba* sp. isolates associated with AGD in Atlantic salmon

Four isolates of *Paramoeba* sp. culture purified from AGD infected gills of Atlantic salmon commercially farmed in south-east Tasmania were used for analysis in this study (Table 5.1). These isolates were sourced from the Fish Health Unit (FHU), Department of Primary Industry, Water and Environment (DPIWE), Mt Pleasant Laboratories, Tasmania.

Ethanol (70% v/v) fixed cells from a *Paramoeba* sp. isolate originating from an AGD outbreak in sea-farmed Atlantic salmon in Ireland in 1995 was also included in our analysis (Table 5.1; Palmer *et al*., 1997).

5.1.1.3 Miscellaneous amoeba isolates

Miscellaneous amoeba cultures isolated from AGD infected Atlantic salmon gills (from AGD infection studies) were obtained from D. Zilberg (School of Aquaculture, University of Tasmania, Launceston, Tasmania). These isolates had been cultured as presumptive *Paramoeba* based on observations under light microscopy and *in-vitro* culture, but had not been subjected to further identification (D. Zilberg, pers. communication, 1999). DNA analysis was used to characterize and identify several of these isolates (Table 5.1).

Table 5.1. *Paramoeba* sp. isolates and related strains used for DNA analyses in this study

Species	Strain	Source	Origin
Reference strains:			
Neoparamoeba pemaquidensis[a]	ATCC 50172	gills, AGD[c]	WA, USA
	ATCC30735	seawater[d]	VA, USA
	CCAP1560/4	seawater	Wales, UK
	CCAP1560/5	seawater	Wales, UK
Neoparamoeba aestuarina[a]	CCAP 1560/7	seawater	Portugal
Paramoeba eilhardi	CCAP 1560/2	seawater	France
Pseudoparamoeba pagei[b]	CCAP 1566/1	seawater	England, UK
Tasmanian isolates:			
Paramoeba sp.	PA 027	gills, AGD	Tasmania, Australia
	PA 010	gills, AGD	Tasmania, Australia
	PA011	gills, AGD	Tasmania, Australia
	PA111	gills, AGD	Tasmania, Australia
Irish isolate:			
Paramoeba sp.	AVG 8194	gills, AGD	Ireland, UK
Miscellaneous isolates:			
unknown	UP 1/6	gills, AGD	Tasmania, Australia
unknown	UP3a/4	gills, AGD	Tasmania, Australia
unknown	UP4a/2	gills, AGD	Tasmania, Australia
unknown	UP4b/5	gills, AGD	Tasmania, Australia

[a]The genus nomenclature "*Neoparamoeba*" is currently used for these species in place of "*Paramoeba*" by ATCC and CCAP culture collections, as proposed by Page (1987).

[b]*Pseudoparamoeba pagei* is a non-parasomal species (Cann and Page, 1982).

[c]ATCC 50172 is the only AGD associated strain isolated from coho salmon, *Oncorhynchus kisutch* (Kent *et al*., 1988); all other AGD strains listed were isolated from Atlantic salmon, *Salmo salar*.

[d]Seawater isolates were originally cultured from free-living cells in the environment.

5.1.2 Culture maintenance and cell preparation

Freshly grown cells of the four Tasmanian *Paramoeba* sp. isolates (Table 5.1) were obtained from *in-vitro* cultures maintained by T. Wagner of the FHU, DPIWE. Purified *Paramoeba* sp. isolates were sub-cultured on malt-yeast-seawater (MYS) agar plates incubated at 20°C (Roubal *et al*., 1989). All cultures were grown monoxenically with *Stenotrophomonas maltophilia* bacterium.

The reference *Neoparamoeba* and *Pseudoparamoeba* cultures (Table 5.1) were grown xenically at the FHU, DPIWE with supplied bacterial cultures according to suppliers' instructions. In most cases, paramoeba cells were harvested from agar cultures following incubation for greater than a week to obtain sufficient cell numbers. Amoeba cells were harvested for DNA extraction by adding 2 to 3 mL filter-sterilized seawater or sterile phosphate buffered saline (PBS; Appendix 3) to each culture plate and scraping the agar surface into suspension using a sterile spreader. Cell suspensions from 2 to 3 plates were pooled and transferred into sterile capped tubes. Where possible, cells were subjected to DNA extraction immediately following harvest from plates. Excess suspensions were stored at minus 20°C.

Paramoeba eilhardi was cultured according to supplier's (CCAP) instructions in a modified Føyns-Erdschreiber broth medium. In general, cells of *P. eilhardi* were slow growing and required prolonged incubation to obtain a detectable cell concentration. Cells were harvested from culture broths by low speed centrifugation at 500 x *g* for 30 min and resuspended in 1 to 3 mL PBS.

5.1.3 Isolation of Cellular DNA

DNA isolation followed standard protocols utilizing enzymatic cell lysis (Ausubel *et al*., 1998). Harvested paramoeba cell suspensions were mixed by vortexing and 600 µL subjected to centrifugation at 1000 x *g* for 15 min. The pellet was washed by thorough resuspension in 700 µL ice-cold sterile PBS followed by centrifugation at 12000 x *g* for 5 min. Cells were resuspended in 600 µL lysis buffer (Appendix 3) and incubated at 65°C for at least 2 h. Lysed cells were vortexed before addition of 100 µL of 5 M NaCl and 80µL of 10% (w/v) hexadecyltrimethylammonium bromide (CTAB) solution in 0.7 M NaCl (Ausubel *et al*., 1998). The suspension was re-incubated at 65°C for a further 30 min.

DNA was extracted by addition of an equal volume of chloroform/isoamyl alcohol (IAA) (24:1) to the suspension and thoroughly mixing for 3 min before centrifugation at 12000 x *g* for 10 min. The aqueous (top) layer was transferred to a fresh tube and subjected to two further extraction steps of equal volumes of phenol/chloroform/IAA (25:24:1) and chloroform/IAA (24:1) respectively. Isopropanol was added to 0.6 sample volume and DNA allowed to precipitate overnight at minus 20°C. DNA was pelleted by centrifugation at 17000 x *g* for 30 min and washed twice by addition of 700 µL cold 70% (v/v) ethanol and re-spinning for 5 min. The resultant pellet was dried in a vacuum centrifuge and resuspended in 100 µL distilled water. DNA was

allowed to go into solution at 4°C overnight and used directly as template for polymerase chain reaction (PCR).

5.1.4 Amplification and Sequencing of 18S Ribosomal DNA (rDNA)

5.1.4.1 Universal 18S rDNA primers

PCR amplification and DNA cycle sequencing were performed using universal oligonucleotide primers complementary to conserved regions of the eukaryotic 18S rDNA (Table 5.2). Primers were commercially synthesized by Genset Pacific Pty. Ltd., Lismore, Australia.

5.1.4.2 Polymerase chain reaction (PCR) of 18S rDNA

PCRs were prepared in 0.2 mL thin-walled reaction tubes (Scientific Specialties Inc., Lodi, CA, USA) each containing 50 μL PCR reaction mix. Amplitaq Gold™ DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) was the enzyme of choice for PCR. Each reaction mix consisted of 5μL 10X PCR Gold™ buffer, 5 μL (2.5 mM) of 25 mM$MgCl_2$ solution, 1 μL (0.2mM) of 10 mM nucleotide solution (dNTP; Promega, Madison, WI, USA), 1.5 μL (0.3 μM) each of 10μM Med.18Sf and Med.18Sr primers respectively, 0.2 μL (1 U) of DNA polymerase, and 30.8 μL of RO-deionised (18 Mohm) water (MilliQ, Millipore Corp., Bedford, MA). All PCR reagents with the exceptions of dNTP and primer solutions were supplied with the Amplitaq Gold™ DNA polymerase. Five μL of extracted cellular DNA (Section 5.1.2) was added to the reaction mix as PCR template. A negative control reaction containing PCR mix with no DNA template added was included in every PCR preparation and subjected to the same amplification conditions.

PCR conditions consisted of an initial denaturation at 95°C for 10 min followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, and 68°C for 1.5 min. The initial DNA denaturation step was required for activation of the Amplitaq Gold™ DNA polymerase enzyme. A final 10 min extension step at 72°C was included following completion of the thermocycling steps. PCRs were performed using a GeneAmp® System 9700 thermocycler (PE Applied Biosystems).

Following PCR, a 5 μL aliquot of each reaction was mixed with 2 μL gel loading buffer (Appendix 3), and loaded into respective wells of a 2% (w/v) agarose gel (GibcoBRL, Life Technologies, Rockville, MD, USA) in 1X TBE buffer (Appendix 3). One kilobase (kb) DNA ladder (New England Biolabs, Beverly, MA, USA or GibcoBRL) was used as the molecular size standard. Electrophoresis was performed at 100 V for 45 min, the gel stained with 0.5μg/mL ethidium bromide, and DNA visualized under UV transillumination. Where required, the remaining 45 μL of PCR sample was retained for DNA sequencing.

Table 5.2. Universal oligonucleotide primers used for PCR and sequencing of the 18S rRNA gene (rDNA)

Primer[a]	Sequence (5'-3')	Size	Reference
Med.18Sf	AACCTGGTTGATCCTGCCAGT	21-mer	Medlin *et al*., 1988
Med.18Sr	TGATCCTTCYGCAGGTTCACCTAC	24-mer	"
Hill.18Sf	CTGGTTGATCCTGCCAGT	18-mer	Hillis and Dixon, 1991
Hill.18Sr	CGGTAGTAGCGACGGGCGGTGTG	23-mer	"
Elw.555f[b]	GTGCCAGCAGCCGCGG	16-mer	Elwood *et al*., 1985
Elw.892f	CAGAGGTGAAATTCT	15-mer	"
Elw.1704f	TGTACACACCGCCCGTC	17-mer	"
Elw.571r	ACCGCGGCTGCTGGC	15-mer	"
Elw.906r	AGAATTTCACCTCTG	15-mer	"
Elw.1277r	CGGCCATGCACCACC	15-mer	"
PA.1300f	CGATAACGAGCGAGACC	17-mer	this study

[a]Primer designations were assigned by this study and were not necessarily used in the original references (f , forward primers annealing to the coding strand of the target double stranded DNA; r, reverse primers annealing to the non-coding strand).

[b]Nucleotide positions used to label the primers were taken from Elwood *et al*. (1985) and were based on the 18S rDNA of *Dictyostelium discoideum*. These positions do not represent the actual annealing positions of genes sequenced in this study.

5.1.4.3 Direct DNA sequencing of PCR product

PCR amplified DNA was purified for sequencing using spin columns from the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. DNA concentration of the purified PCR product was determined in ng/µl using the GeneQuant*pro* RNA/DNA calculator (AmershamPharmacia Biotech, Cambridge, England).

DNA sequencing was performed using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNApolymerase (PE Applied Biosystems). Each sequencing reaction consisted of approximately 70 to 90 ng purified PCR product DNA, 4 µL ABI BigDye™ Terminator Ready Reaction Mix (half recommended volume was used), 3.2 pmol sequencing primer, and an appropriate volume of MilliQ water to achieve a 10 µL total reaction volume in a 0.2 mL thin-walled PCR tube. Samples were subjected to cycle sequencing in a GeneAmp® System 9700 thermocycler using cycling parameters recommended by the manufacturer. Sequencing products were precipitated using 3 M sodium acetate and 95% (v/v) ethanol according to sequencing kit instructions. DNA sequence data was obtained by running the samples in a sequencing gel in an ABI Prism™ 377 DNA sequencer (PE Applied Biosystems) for 10 h.

Both coding and non-coding strands of the 18S rDNA sequence were independently sequenced using a battery of forward and reverse primers respectively (Table 5.2). The integrity of sequences obtained from complementary DNA strands was checked against each other for each amoeba strain. Overlapping sequences obtained from different primers were also compared. Sequence discrepancies were checked by repeat sequencing of the DNA region in question using the appropriate primers.

5.1.5 Analyses of 18S rDNA Sequences

Analysis of DNA sequence data was performed using the Sequence Navigator™ ver. 1.0.1 program (PE Applied Biosystems). Sequences generated in this study were subjected to a BLAST sequence similarity search via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to confirm that 18S rDNA sequences were obtained and to search for potentially related taxa. Sequences from this study were aligned with each other, and with 18S rDNA sequences of selected eukaryotic taxa available from the GenBank sequence database (Table 5.3). This database was accessed through the Entrez search and retrieval system of NCBI. Only unambiguously homologous nucleotide positions were aligned and used for further analyses. Sequence alignments were performed manually (by eye) with the aid of the comparative alignment algorithm and the Clustal V multiple alignment algorithm incorporated within Sequence Navigator™. Sequence (primary structure) integrity was also checked for conformity with the 18S rDNA secondary folding structure (Van de Peer *et al*., 1999).

Aligned complete and partial 18S rDNA sequences were applied to the phylogenetic analyses of *N. pemaquidensis*, which infer inter- (global) and intra-specific relationships with other eukaryotic taxa based on the extent of evolutionary divergence between the respective genes. A phylogenetic tree representing global taxonomic relationships was constructed using the DNAPARS maximum parsimony program incorporated within the PHYLIP (ver. 3.5c) phylogeny inference package distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA (Felsenstein, 1989). Estimates of evolutionary distances between closely related sequences were made with Kimura two-parameter correction for multiple substitutions within the DNADIST program of PHYLIP.

Table 5.3. Eukaryotic taxa used for phylogenetic analysis and their associated GenBank 18S rDNA sequence accession numbers

Species	Common name or taxonomic grouping	18S rDNA GenBank Accession No.
Phreatamoeba balamuthi	Protist, amoeboid	L23799
Haplosporidium nelsoni	Protist, haplosporidian	X74131
Paramecium tetraurelia	Protist, ciliate	X03772
Plasmodium falciparum	Protist, apicomplexan	M19172
Babesia bovis	Protist, apicomplexan	L19077
Alexandrium fundyense	Protist, dinoflagellate	U09048
Porphyra miniata	Protist, red algae	L26200
Glaucocystis nostochinearum	Protist, glaucophyte	X70803
Skeletonema costatum	Protist, diatom	X85395
Fucus gardneri	Protist, brown algae	X53987
Emiliana huxleyi	Protist, haptophyte	M87327
Chlamydomonas reinhardtii	Green algae	M32703
Chlorella vulgaris	Green algae	X13688
Pleurastrum terrestre	Green algae	Z28973
Zea mays	Plant (maize)	K02202
Hartmannella vermiformis	Protist, gymnamoebia	X75515
Acanthamoeba castellanii	Protist, gymnamoebia	U07413
Acanthamoeba palestinensis	Protist, gymnamoebia	L09599
Rosette agent	Protist, choanoflagellate-like	L29455
Pneumocystis carinii	Fungi	X12708
Artemia salina	Metazoan, brine shrimp	X01723
Xenopus laevis	Metazoan, amphibian (frog)	X04025
Mus musculus	Metazoan, mammalian (mouse)	X00686

5.2 Results

5.2.1 Cellular DNA Extraction

High molecular weight cellular DNA was successfully extracted from all amoeba isolates (Table 5.1), including the ethanol preserved cells of *Paramoeba* sp. strain AVG 8194 from Ireland. Quality and yield of extracted DNA varied and depended on the density of cells in the harvested suspensions. Co-incubated bacteria contributed a large proportion of the cellular DNA in the extracts.

Cultures of *N. aestuarina* were supplied with relatively fast growing, agarolytic bacteria. The high proportion of bacteria in the samples, the presence of bacterial extracellular products, and contaminating agarose from the growth medium prohibited efficient isolation of *N. aestuarina* DNA, which subsequently affected downstream PCR procedures.

5.2.2 PCR Amplification of Paramoeba 18S rDNA

For most isolates in Table 5.1, PCR amplification of the entire 18S rRNA gene was achieved using the primer pair Med.18Sf and Med.18Sr (Table 5.2). These primers anneal to conserved sequences at the 5' and 3' termini of the gene respectively. PCR amplification using these primers and DNA extracted from *Paramoeba* sp. isolate PA027 produced an18S rDNA amplicon of approximately 2.1 kb in size (Figure 5.1). Respective 18S rDNA amplicons from three other Tasmanian *Paramoeba* sp. isolates, the Irish isolate, *N. pemaquidensis* strains from the USA and UK, and the *Pseudoparamoeba pagei* strain were similar in molecular size to that of PA 027. However, amplified 18S rDNA from unidentified amoebae (UP isolates) were slightly smaller in size at approximately 1.8 kb (Figure 5.1). Low concentrations of smaller, non-specific fragments present in some of the amplified samples were easily distinguishable from the expected 18S rDNA product by molecular size. These non-specific fragments did not affect downstream sequencing since specific 18S rDNA primers were used.

PCR amplification with primers Med.18Sf and Med.18Sr repeatedly failed to produce a detectable 18S rDNA product with cellular DNA prepared from cultures of *P. eilhardi* CCAP 1560/2 and *N. aestuarina* CCAP 1560/7. 18S rDNA was either not amplified in these extracts or was present at undetectable concentrations. Alternative PCR primer combinations that anneal to other sequence regions of the 18S rDNA were attempted with these strains. Amplification of *N. aestuarina* 18S rDNA was achieved using a nested two-step PCR procedure. Nested PCR involves the use of a second pair of primers in a secondary PCR to amplify a sequence fragment internal to a primary PCR product (using primers Med.18Sf and Med.18Sr), thus increasing the sensitivity of amplification with low starting concentrations of target DNA. Respective secondary primer pairs successfully used on primary-amplified 18S rDNA from *N. aestuarina* included Hill.18Sf/Elw.1277r, Elw.555f/Hill.18Sr, and Elw.555f/Elw.1277r (Figure 5.2).

Attempts to amplify 18S rDNA from *P. eilhardi* were unsuccessful using either standard or nested PCR procedures. The *P. eilhardi* cultures obtained in this study were believed to contain insufficient cell numbers for effective DNA extraction.

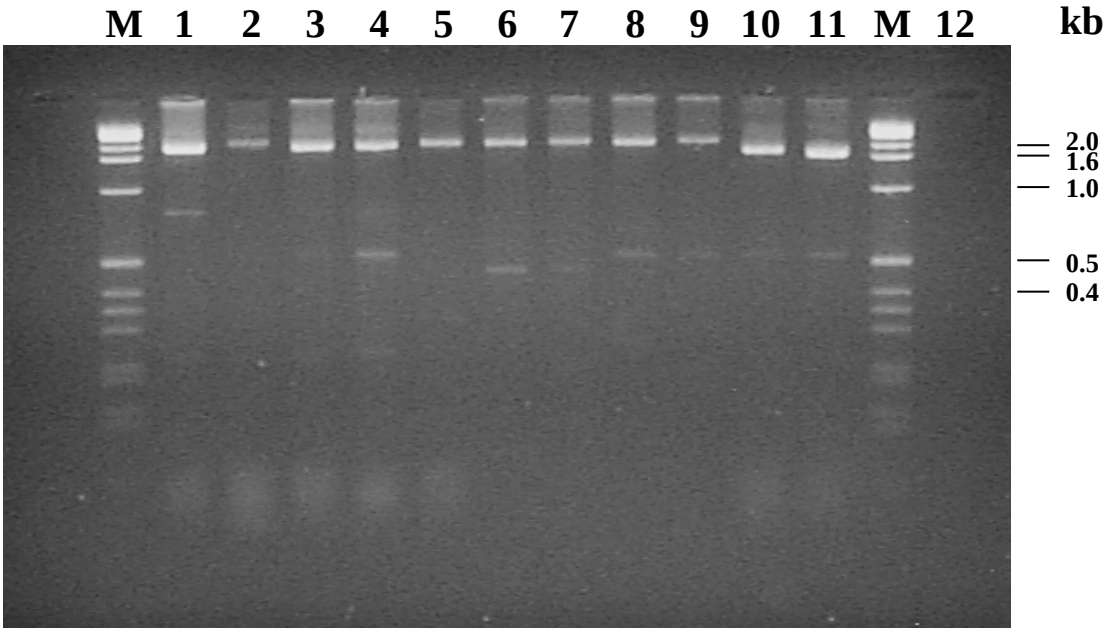

Figure 5.1. Representative 18S rDNA amplification products obtained from PCR using universal primers Med.18Sf and Med.18Sr. Lane1, *Pseudoparamoeba pagei* CCAP 1566/1; lanes 2 to 4, Tasmanian *Paramoeba* sp. PA 027, PA 010, and PA 011; lane 5, Irish *Paramoeba* sp. AVG 8194; lanes 6 to 9, *N. pemaquidensis* ATCC 30735, ATCC 50172, CCAP 1560/4, and CCAP 1560/5; lanes 10 and 11, Tasmanian unidentified amoeba isolates UP 1/6 and UP 4a/2; lane 12, negative control; and lane M, GibcoBRL DNA marker with fragment sizes indicated in kilobases (kb). The majority of 18S rDNA PCR products were ~2.1 kb in size, although 18S rDNA of unidentified gill amoebae were smaller at ~1.8 kb.

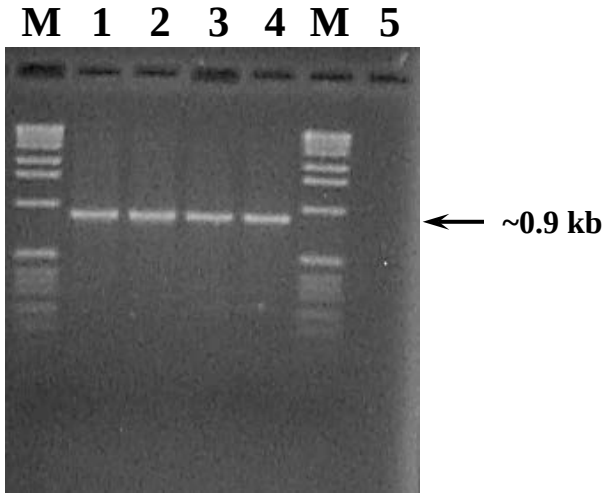

Figure 5.2. 18S rDNA amplification products of *N. aestuarina* CCAP 1560/7 from secondary PCR using nested primers Elw.555f and Elw.1277r. Lanes 1 to 4, PCR of four separate cellular DNA extracts from CCAP 1560/7 cultures; lane 5, negative control; and lane M, GibcoBRL DNA marker. The position of the ~0.9 kb PCR products obtained is indicated by arrow.

5.2.3 18S rDNA Sequence Analyses

5.2.3.1 18S rDNA sequences of *Neoparamoeba* and relatives

Complete 18S rDNA sequences were obtained for four AGD isolates of *Paramoeba* sp. from Tasmanian sea-farmed Atlantic salmon using direct sequencing of PCR amplified gene products. In each case, 2104 base pairs (bp) of the 18S rDNA sequence were obtained for comparative analysis. All four isolates possessed identical sequences and were considered to belong to the same strain. PA 027 had been used as the standard reference Tasmanian *Paramoeba* sp. strain in immunodetection and other AGD studies (Tan, 2000), and was also used as the representative isolate for sequence analyses in the present study. The complete 18S rDNA sequence of 2104 bp was also obtained for the *Paramoeba* sp. AGD isolate from Ireland. Initial BLAST screening of the entire unaligned sequence obtained for each *Paramoeba* sp. isolate confirmed homology with the eukaryotic 18S rDNA molecule, but did not reveal a close relationship with any protistan taxa in the existing databases.

Complete sequences (2104 bp) were obtained for the four *N. pemaquidensis* reference strains from respective culture collections in the UK and USA. Strains CCAP 1560/4 and CCAP 1560/5 had identical sequences and the latter strain was chosen to represent both free-living marine isolates originating from the same location in Wales, UK. In contrast, two strains from the USA (ATCC strains) had different and geographically separate origins. These isolates possessed highly similar but non-identical sequences and were considered to be distinctly separate strains of *N. pemaquidensis* (see below).

Near complete 18S rDNA sequence comprising 1901 bp was obtained for the Portuguese *N. aestuarina* reference strain CCAP 1560/7. The unaligned 18S rDNA sequences obtained for *N. pemaquidensis* and *N. aestuarina* strains were not closely related to any other protistan taxa when subjected to BLAST analyses, and represent new and unique sequence data for these species.

Four miscellaneous unidentified amoebae (UP isolates) isolated from Atlantic salmon gills in Tasmania had identical 18S rDNA sequences. The complete 18S rDNA sequences of these isolates each comprised 1873 bp, and are represented by isolate UP 1/6. The 18S rDNA of this amoeba has fewer bases than *Paramoeba* sp. and *N. pemaquidensis*, as was previously shown by specific PCR amplification of the gene. BLAST analysis of the sequence obtained for the unknown gill-associated amoeba was able to reveal its taxonomic identity. The sequence was almost identical (99.9% sequence similarity) to the published complete 18S rDNA sequence of the non-parasomal amoeba *Paraflabellula hoguae* (Amaral-Zettler *et al*., 2000), with only two base differences between them.

Partial 18S rDNA sequence of 1352 bp was obtained for *Pseudoparamoeba pagei* reference strain CCAP 1566/1. BLAST analysis of the unaligned 18S rDNA sequence from this organism revealed that it was not homologous to *Paramoeba* sp., *N. pemaquidensis*, or to any protistan taxa in the existing sequence databases. Sequence data from this non-parasomal species was not used for further analysis in this study.

5.2.3.2 Sequence homogeneity among *Neoparamoeba* strains

The respective 18S rDNA sequences of *Paramoeba* sp. isolates from Tasmania and Ireland, and the reference strains of *N. pemaquidensis* from the UK and USA, share high levels of homology (98.1 to 99.0% sequence similarities) (Table 5.4). These levels of sequence similarity show that isolates associated with AGD in sea-farmed Atlantic salmon and previously identified as *Paramoeba* sp., in fact belong to the same species *N. pemaquidensis*. The low estimates of evolutionary base substitutions between the respective sequences further highlight the high degree of relatedness among strains of this species (Table 5.4), despite having been isolated from several geographically distant countries and from different sources of origin.

Table 5.4. Sequence divergence and evolutionary distance estimates between strains of *Paramoeba* sp. and *N. pemaquidensis* over 2104 bp of the18S rDNA[a]

	Paramoeba sp.		*Neoparamoeba pemaquidensis*		
Isolates[b] (Origin)	PA 027 (Tasmania)	AVG 8194 (Ireland)	ATCC 50172 (USA)	ATCC 30735 (USA)	CCAP 1560/5 (Wales)
PA 027 (Tasmania)		0.125	0.173	0.105	0.159
AVG 8194 (Ireland)	**98.8**		0.193	0.115	0.183
ATCC 50172 (USA)	**98.3**	**98.1**		0.178	0.086
ATCC 30735 (USA)	**99.0**	**98.9**	**98.2**		0.164
CCAP 1560/5 (Wales)	**98.3**	**98.1**	**99.0**	**98.3**	

[a]Percentage (%) similarity values between strains are shown in the lower triangle (in bold). Evolutionary distance values are given as the estimated number of base substitutions per every 10 bases and shown in the upper triangle.

[b]Refer to Table 5.1 for details on the sources of origin of the respective strains.

Comparative analysis of 18S rDNA sequences shows that the species *N. aestuarina* is closely related to *N. pemaquidensis*. Comparison of 1901 bp of the 18S rDNA obtained for *N. aestuarina* strain CCAP 1560/7revealed 95.3 to 95.7% sequence similarities with analogous aligned sequences of the various *N. pemaquidensis* strains (including the *Paramoeba* sp. isolates). Estimated evolutionary distances between the two species ranged from 0.397 to 0.425 base substitutions per every 10 bases. However, this level of sequence divergence allowed *N. aestuarina* to be distinguished from the *N. pemaquidensis* group, supporting the status of two separate and distinct species.

The complete 18S rDNA sequences for *N. pemaquidensis* Tasmanian strain PA 027, Irish strain AVG 8194 and reference strains CCAP1560/4, CCAP 1560/5, ATCC 50172, and ATCC 30735 were deposited into the GenBank sequence database under the accession numbers AF371967 through to AF371972 respectively. Near complete sequence for *N. aestuarina* strain CCAP 1560/7 was also deposited into GenBank with the accession number AF371973.

5.2.3.3 Phylogeny of *N. pemaquidensis* and global relationships

The inter-specific relationships of *N. pemaquidensis* with taxa representing the major eukaryotic lineages were determined using maximum parsimony analysis of 18S rDNA sequences. Alignment of 18SrDNA sequences from the different taxa identified 1449 non-ambiguously aligned sites (homologous base positions), which were used for phylogenetic analysis. The species *N. pemaquidensis* is not closely related to other protistan taxa, including other genera within the protozoan subclass Gymnamoebia. However, the different members of this amoeboid subclass appeared to share a broader phylogenetic relationship. In the analysis performed in this study, this broader cluster included the genera *Acanthamoeba*, *Neoparamoeba*, *Hartmannella*, and *Paraflabellula* (Figure 5.3).

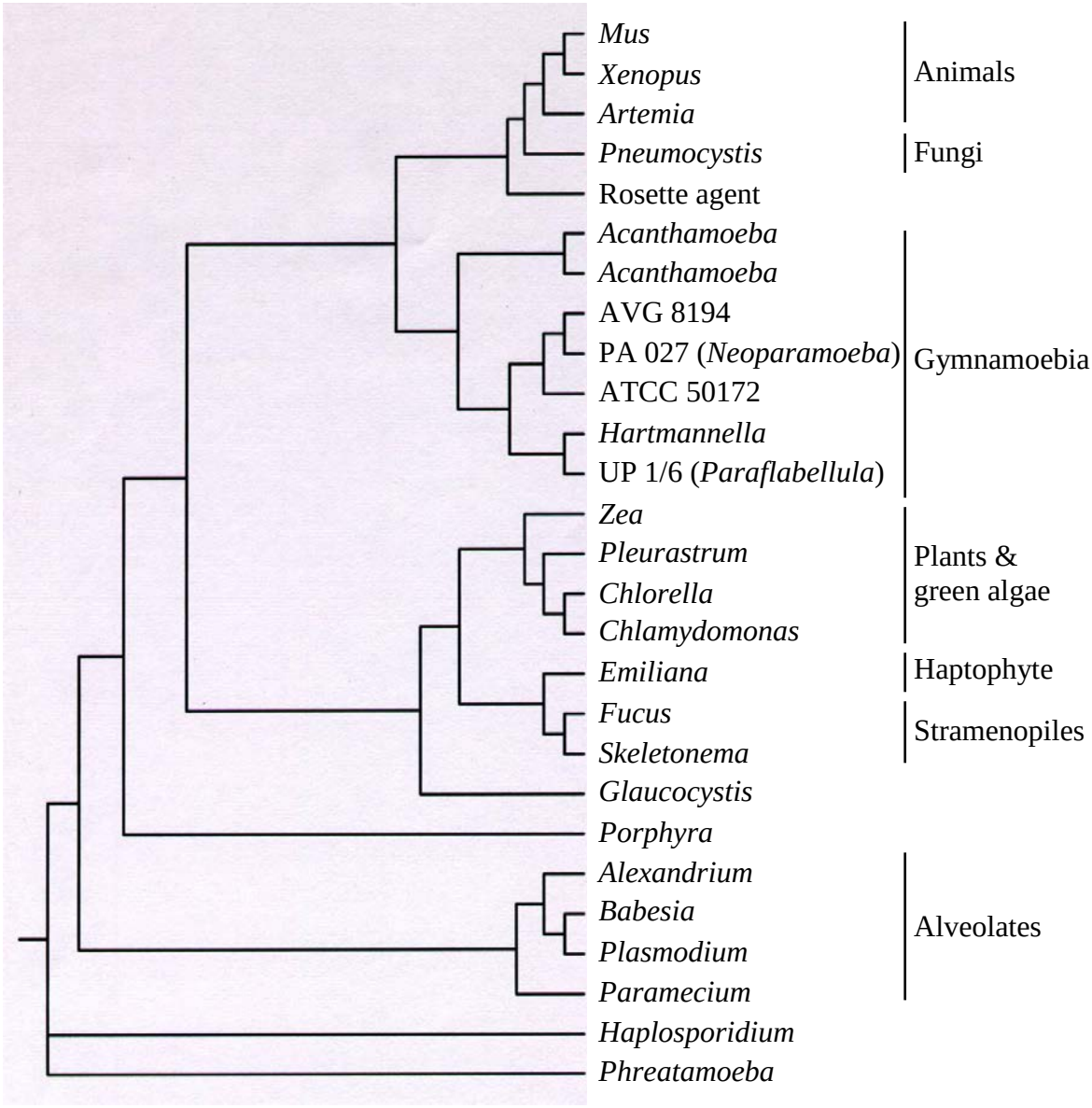

Figure 5.3. Unrooted phylogenetic tree showing the relationships of *N. pemaquidensis* with selected taxa representing the eukaryotic lineages listed on the far right. This schematic tree represents only branch order relationships. Branch lengths are not drawn to scale and do not represent evolutionary distances. Strain AVG 8194 was isolated from the gills of Irish Atlantic salmon, PA 027 from the gills of Tasmanian Atlantic salmon and ATCC 50172 from the gills of coho salmon from the USA.

6. MOLECULAR DETECTION OF *NEOPARAMOEBA PEMAQUIDENSIS*

6.1 Methods

6.1.1 PCR Detection of *Neoparamoeba pemaquidensis*

6.1.1.1 Development of specific PCR primers

Complete 18S rDNA sequences (*ca*. 2.1 kb) obtained for *N. pemaquidensis* (Section 5.0) were examined for suitable annealing sites for the development of species-specific PCR primers. Suitable target regions consisted of sequences that are identical among the various *N. pemaquidensis* strains and isolates, but contain sufficient nucleotide differences to distinguish *N. pemaquidensis* from other species. Deduced primer sequences were examined for possible complementarity with the 18S rDNA of other representative taxa used for the phylogenetic analysis in Section 5.0. Potential primer sequences and potential PCR primer pairs (sets) were also checked to minimize self-complementarity. Eight PCR primers targeting different regions of the 18S rDNA of *N. pemaquidensis* including free living and AGD isolates, were synthesized for evaluation (Table 6.1).

Table 6.1. Presumptive *N. pemaquidensis* specific primers tested in this study

Primer	Sequence (5'-3')	Size
fPA-Hxe10	GCCATCTTTCGGGGTGGA	18-mer
fPA-Hxe23a1	CATCTCCTTACTAGACTTTCATG	23-mer
fPA-Hxe23b1	GTGAGTGATGAGTAGACCTACTGG	24-mer
fPA-Hxe23c1	CTTCGGAGGTTGGTTCTGC	19-mer
fPA-Hxe23d1	CGGAGGTTGGTTCTGCTTAGATTCAG	26-mer
fPA-Hxe23y1	GGAACAGGACACGTATTCTAG	21-mer
fPA-Hx43a1	GTGATGCAAATGATTACATCCG	22-mer
rPA-Hx49	CACAACAAACTCGCTCTACCCG	22-mer

6.1.1.2 Preliminary primer testing and PCR optimization

Each of the seven forward primers (prefixed f, Table 6.1) was respectively paired with the rPA-Hx49 reverse primer (i.e. seven primer sets) and used in a standard PCR protocol to test for amplification of *N. pemaquidensis* 18S rDNA. PCRs were essentially prepared as described in Section 5.1.3.2. The standard reaction mix consisted of 5μL 10X PCRGold™ buffer, 5 μL (2.5 mM) of 25 mM$MgCl_2$ solution, 1 μL (0.2mM) of 10 mM dNTP solution, 0.5 μL (0.1μM) each of 10 μM forward and reverse primers respectively, 0.2 μL (1 U) of Amplitaq Gold™ DNApolymerase, and 34.8 μL of MilliQ water. Three μL of 10 ng/μL extracted cellular DNA (Section 5.1.2) was included as PCR template. Amplification conditions consisted of an initial denaturation at 95°C for 10 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and

72°C for 1.5 min; and a final extension at 72°C for 10 min. Either the *N. pemaquidensis* reference strain ATCC 50172 or the Tasmanian AGD strain PA027 was used as positive control in specific PCR tests. *Pseudoparamoeba pagei* strain CCAP 1566/1 was used as a negative DNA control. A negative control reaction without template DNA was also included in each PCR preparation. PCR products were visualized as previously described (Section 5.1.3.2).

Primers fPA-Hxe10, fPA-Hxe23b1 and fPA-Hx43a1, together with rPA-Hx49 were arbitrarily selected for further optimization towards a *N. pemaquidensis* specific PCR detection protocol. In order to increase the potential sensitivity of PCR detection, optimization procedures for each new primer pair included the respective determination of optimal primer and $MgCl_2$ concentrations. Amplification efficiency was tested in a series of independent reactions containing serially diluted concentrations of primer or $MgCl_2$ in an otherwise standard reaction mix. Equal concentrations of both primers (forward and reverse) were used in the same reaction mix.

6.1.1.3 Determination of PCR detection limits for DNA

The sensitivity of PCR detection of *N. pemaquidensis* DNA using several different primer sets was tested on serially diluted cellular DNA from strain PA 027. A 10-fold dilution series ranging from 100 ng through to 0.001 ng (1 pg) of PA027 DNA in sterile water was prepared as template for PCR. Each template dilution was subjected to amplification in separate reactions. Duplicate PCRs were performed for each different template concentration. Amplification reactions were performed using optimal PCR conditions previously determined.

6.1.1.4 Specificity testing of *N. pemaquidensis* specific PCR

Specificity of a particular primer set for the target organism was screened by PCR amplification of cellular DNA extracts from various *N. pemaquidensis* strains and non-target organisms (Table 5.1). In addition, respective primer sequences were subjected to random BLAST searches to determine if homologous or complementary sequences were present in the existing sequence databases. In particular, sequence complementarity to 18S rDNA sequences of other taxonomic representatives of the Gymnamoebia including *Acanthamoeba* species (Gast *et al*., 1996), *Hartmannella vermiformis* (Weekers *et al*., 1994), and *Vannella anglica* (Sims *et al*., 1999) was assessed.

6.2 Results

6.2.1 PCR Amplification Using Specific Primers

When screened using the standard PCR protocol, all seven presumptive *N. pemaquidensis* specific primer sets produced a specific amplification product from cellular DNA prepared from *N. pemaquidensis* ATCC 50172. In all cases, a single amplicon of the expected molecular size was produced indicating that the respective primers specifically amplified the targeted 18S rDNA region from *N. pemaquidensis* genomic DNA. Figure 6.1 shows the 18S rDNA amplification products produced by the fPA-Hxe23a1/rPA-Hx49, fPA-Hxe23b1/rPA-Hx49, fPA-Hxe23c1/rPA-Hx49, fPA-Hxe23d1/rPA-Hx49 and fPA-Hxe23y1/rPA-Hx49 primer sets. The expected molecular sizes of the specific PCR products produced by these primer sets are 1315 bp, 1250 bp, 1204 bp, 1201 bp and 1074 bp respectively. PCR amplification of *N. pemaquidensis* 18S rDNA using the primer sets fPA-Hxe10/rPA-Hx49 and fPA-Hx43a1/rPA-Hx49also produced expected amplification products of 1795 bp and 491 bp respectively (Figures 6.2 and 6.3).

Although extensive specificity testing was not performed on all of the primers, none produced an amplification product from *Pseudoparamoeba pagei* DNA under the PCR conditions used. This suggested that the primers had no apparent non-specific affinity for the conserved (universal) regions of the 18S rDNA or for other non-targeted DNA regions. As such, all of the developed primer sets possessed at least some level of specificity for *N. pemaquidensis*.

6.2.1.1 Optimization of primer and $MgCl_2$ concentrations

The effect of varying PCR primer concentrations on DNA amplification efficiency is represented in Figure 6.2, using the fPA-Hxe10/rPA-Hx49 primer set. The optimal concentration of each primer in a PCR reaction mix for amplification of extracted cellular DNA was found to be 0.1μM regardless of the primer set used. This primer concentration was used in subsequent PCR reactions.

The effects of serially adjusted $MgCl_2$ concentrations on DNA amplification efficiency using primer sets fPA-Hxe10/rPA-Hx49 and fPA-43a1/rPA-Hx49 are presented in Figure 6.3. All primer sets were found to optimally amplify extracted *N. pemaquidensis* DNA at a$MgCl_2$ concentration of 2.0 mM per reaction. This concentration was used in subsequent PCR preparations.

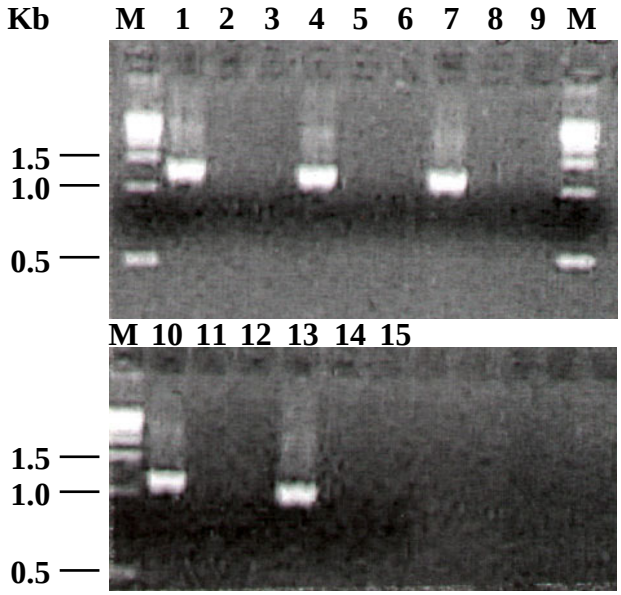

Figure 6.1. PCR amplification using a range of presumptive *N. pemaquidensis* specific primer sets. Lanes 1 to 3, fPA-Hxe23a1/rPA-Hx49 primer set; lanes 4 to 6, fPA-Hxe23b1/rPA-Hx49 primer set; lanes 7 to 9, fPA-Hxe23c1/rPA-Hx49 primer set; lanes 10 to 12, fPA-Hxe23d1/rPA-Hx49 primer set; lanes 13 to 15, fPA-Hxe23y1/rPA-Hx49 primer set; and lanes M, New England Biolabs 1 kb DNA ladder with fragment sizes indicated in kb. The three lanes corresponding to each different primer set are separate reactions using *N. pemaquidensis* ATCC 50172 template, *Pseudoparamoeba pagei* CCAP 1566/1 DNA template, and a negative control without DNA template respectively.

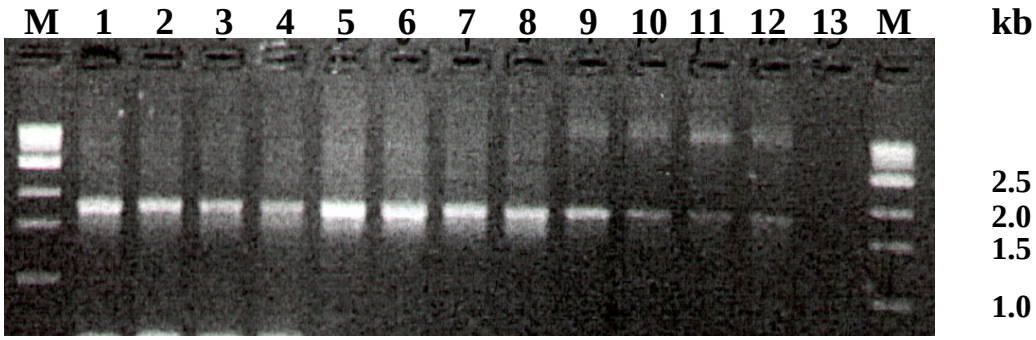

Figure 6.2. Optimization of primer concentrations for PCR using the presumptive *N. pemaquidensis* specific primer set fPA-Hxe10/rPA-Hx49. Cellular DNA templates were from *N. pemaquidensis* USA strain ATCC 50172 (lanes 1 to 4), UK strain CCAP 1560/5 (lanes 5 to 8) and Tasmanian strain PA027 (lanes 9 to 12) respectively. The four lanes corresponding to each *N. pemaquidensis* strain are separate reactions using 0.1 μM, 0.2 μM, 0.3 μM, and 0.4 μM primer concentrations respectively. Equal concentrations were used for both primers in each reaction. Lane 13, negative control using 0.1 μM primers; and lane M, New England Biolabs 1 kb DNA ladder. Position of the expected ~1.8 kb PCR product is indicated by arrow.

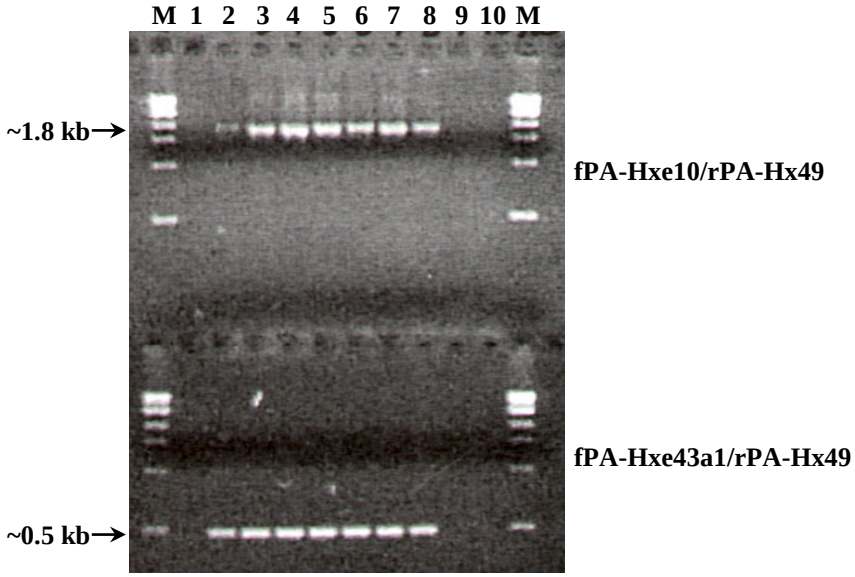

Figure 6.3. Optimization of $MgCl_2$ concentrations for PCR of *N. pemaquidensis* ATCC 50172 18S rDNA using primer sets fPA-Hxe10/rPA-Hx49 and fPA-Hxe43a1/rPA-Hx49. Lanes 1 to 8, separate reactions using 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM, and 4.0 mM of $MgCl_2$ respectively; lanes 9 to 10, reaction using 2.5 mM $MgCl_2$ on *Pseudoparamoeba pagei* CCAP 1566/1 DNA and negative control reaction without DNA template respectively; and lane M, New England Biolabs 1 kb DNA ladder. Arrows indicate positions of the expected specific PCR products.

6.2.1.2 PCR detection limits for *N. pemaquidensis* DNA

The minimum limits of PCR detection of DNA in free solution, extracted from monoxenic culture of PA 027, were determined for primer sets fPA-Hxe10/rPA-Hx49, fPA-Hx23b1/rPA-Hx49 and fPA-Hx43a1/rPA-Hx49. PCRs using these primer sets had minimum detection limits of 10 ng, 0.1 ng, and 0.01 ng respectively (Table 6.2). The average detection limit among the primer sets tested was 3.4 ng (3400 pg) of extracted cellular DNA. PCR detection using primer set fPA-Hx43a1/rPA-Hx49 was at least 10-fold more sensitive than reactions incorporating primers that produced larger amplification products.

Table 6.2. Respective minimum PCR detection limits of three primer sets for total DNA in free solution extracted from *N. pemaquidensis* PA 027 culture suspension.

PA 027 template DNA		Specific PCR amplification[a]		
(ng)	(pg)	fPA-Hxe10/ rPA-Hx49	fPA-Hx23b1/ rPA-Hx49	fPA-Hx43a1/ rPA-Hx49
100[b]	$1x10^5$	+	+	+
10	$1x10^4$	±	+	+
1	1000	–	+	+
0.1	100	–	±	+
0.01	10	–	–	±
0.001	1	–	–	–
0[c]	0	–	–	–

[a]PCR detection of expected amplification product (+, positive; ±, weak positive; –, negative).

[b]Positive control sample containing 100 ng template DNA.

[c]Negative control sample with no template DNA added.

6.2.2 Specificity of Diagnostic Primers

6.2.2.1 Cross-reaction with *Neoparamoeba aestuarina*

The battery of eight presumptive *N. pemaquidensis* specific primers (Table 6.1) was developed for PCR testing before the 18S rDNA sequence of *N. aestuarina* could be determined. However, both *Neoparamoeba* species were later confirmed by 18S rDNA analysis to be closely related and share a high level of 18S rDNA sequence similarity (Section 5.2.2.2). In fact, *N. aestuarina* possessed identical sequences to *N. pemaquidensis* at the respective annealing sites of seven out of the eight PCR primers. This apparent cross-specificity was confirmed when these primers were applied to PCR of *N. aestuarina* DNA (e.g. primer set fPA-Hx43a1/rPA-Hx49, Figure 7.1). PCRs produced amplification products of the expected molecular size that were indistinguishable between the two species.

6.2.2.2 *N. pemaquidensis* specific primer set

Only one of the presumptive *N. pemaquidensis* specific PCR primers was able to distinguish this species from *N. aestuarina*. Primer fPA-Hxe23b1 has seven non-complementary bases within the 24 bp sequence region corresponding to the primer annealing site on the *N. aestuarina* 18S rDNA (Figure 6.4). Primer fPA-Hxe23b1 was used with rPA-Hx49 to further develop a diagnostic PCR protocol that will specifically detect *N. pemaquidensis* without co-reacting with *N. aestuarina* (Section 7.0).

N. pemaquidensis	5'-GTGAGTGATGAGTAGACCTACTGG-3'
N. aestuarina	5'-GTGCGTGGTAGGAGGACCTATTGG-3'
primer fPA-Hx23b1	5'-GTGAGTGATGAGTAGACCTACTGG-3'
consensus	5'-*** *** * * ****** ***-3'

Figure 6.4. Respective 18S rDNA sequence regions of *N. pemaquidensis* and *N. aestuarina* corresponding to the annealing site of primer fPA-Hxe23b1. Asterisks denote consensus (homologous) base positions.

6.2.2.3 Specificity against other genera

None of the diagnostic PCR primers used (Table 6.1) were complementary to other potentially contaminating marine aquatic organisms when subjected to BLAST analyses, including other members of the protozoan subclass Gymnamoebia that were used for comparative sequence analyses in this study (Section 5.0).

The fPA-Hxe10/rPA-Hx49, fPA-Hxe23b1/rPA-Hx49 and fPA-Hx43a1/rPA-Hx49 primer sets were further tested for specificity using PCR. Each of these primer sets produced the expected amplification product following PCR of DNA from *N. pemaquidensis*, regardless of the source and country of origin of the isolate (Figure 6.5). In contrast, no amplification product was produced from DNA extracted from either the non-parasomal amoeba co-isolated from gills of AGD infected fish (*Paraflabellula hoguae*) or from *Pseudoparamoeba pagei* strain CCAP 1560/7. Specificity testing of the *N. pemaquidensis* species-specific primer set fPA–Hxe23b1/rPA-Hx49 is further described in Section 7.0.

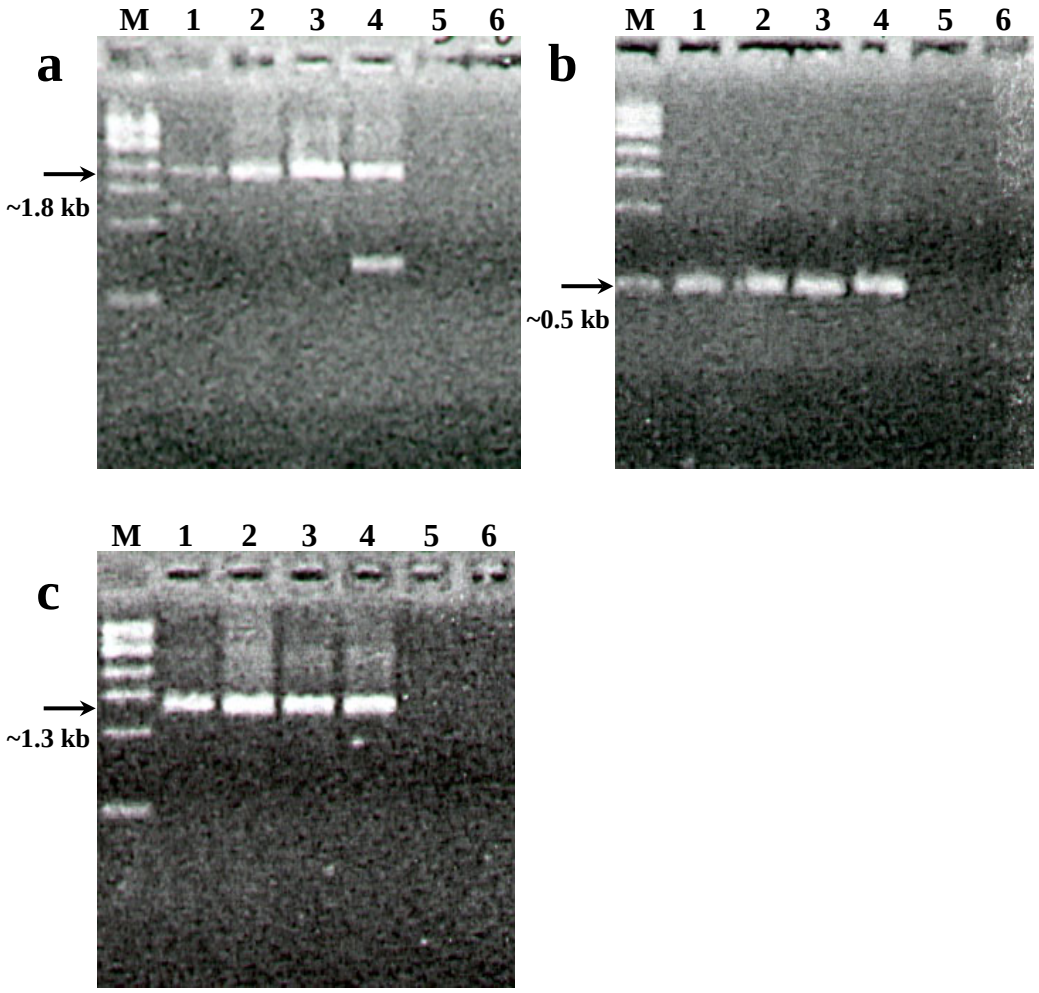

Figure 6.5. Specificity screening of PCR using (a) primer set fPA-Hxe10/rPA-Hx49, (b) primer set fPA-Hx43a1/rPAHx49 and (c) primer set fPA-Hxe23b1/rPA-Hx49 on a panel of *N. pemaquidensis* strains of different origins and selected non-*Neoparamoeba* amoeba species. Lanes 1 to 4, *N. pemaquidensis* AGD isolate from Tasmania (PA 027), AGD isolate from the USA (ATCC 50172), free-living isolate from the USA (ATCC 30735) and free-living isolate from Wales, UK (CCAP 1560/4) respectively; lane 5, *Pseudoparamoeba pagei* strain CCAP 1566/1; lane 6, amoeba gill isolate UP 1/6 from Tasmania (presumptive *Paraflabellula hoguae*); and lane M, New England Biolabs 1 kb DNA ladder. Arrows indicate the respective positions and approximate sizes of the expected specific PCR product of each primer set.

7. PCR ANALYSES OF ENVIRONMENTAL SAMPLES

7.1 Methods

7.1.1 DNA Extraction from Environmental Samples

Environmental samples selected for preliminary testing of the *N. pemaquidensis* specific PCR protocols were divided into three types of environmental matrices (seawater, cage biofouling and sediment) and fish gill samples. Samples were derived from the immediate environment associated with commercial Atlantic salmon sea-cages at three farm sites located at Hideaway Bay, North West Bay and Garden Island in south-east Tasmania. All cages sampled contained fish at the time of sampling. These samples were tested either crude or following enrichment in MYS culture medium. In addition, seawater collected from a South Australian Atlantic salmon farm and ethanol-fixed chinook salmon gill samples from New Zealand were analyzed by PCR. Sample processing varied for each sample type in order to maximize the extraction of representative cellular DNA from each particular matrix. In general, sample fractions were processed for DNA extraction according to the standard protocol described in Section 5.1.2.

7.1.1.1 Seawater samples

Seawater was sampled from either immediately outside or within sea-cages at two Tasmanian Atlantic salmon farm sites (Hideaway Bay and North West Bay) and one South Australian farm site. M. Helders-Douglas of the School of Aquaculture, University of Tasmania, collected the South Australian and Hideaway Bay samples. Where possible, seawater samples were processed immediately following receipt at the laboratory. Samples of 50 to 500 mL volumes were each passed through a 0.45 μm cellulose nitrate membrane filter (Whatman, Maidstone, England) with the aid of a low-pressure vacuum pump. The membrane, containing retained filter residue, was transferred to a sterile 10 mL capped tube with 1 to 2 mL sterile PBS (Appendix 3). Trapped material was loosened into solution by vigorous vortexing before discarding the membrane. The entire suspension was further concentrated by centrifugation at 10 000 x *g* for 10 min and the pellet resuspended in 600 μL lysis buffer (Appendix 3) and subjected to DNA extraction using the standard protocol (Section 5.1.2). Samples producing a weak (barely visible) pellet following centrifugation were resuspended in300 μL lysis buffer and half-volumes used for all subsequent extraction reagents.

Seawater samples with a total volume of 10 mL or less were concentrated by centrifugation at 10 000 x *g* for 10 min. Pellets were resuspended in 300 μL lysis buffer and subjected to DNA extraction.

7.1.1.2 Cage biofouling samples

Biofouling material found on the submersed netting of salmon sea-cages were divided into 11 different sub-fractions for PCR analyses. Biofouling sub-fractions typically consisted of identifiable macrofouling organisms commonly associated with salmon cage netting (Table 7.1). The majority of biofouling samples were collected by C. Tan of the School of Aquaculture, University of Tasmania as part of a biofouling field survey (Tan, 2000). Biofouling samples originated from sea cages located at the Hideaway Bay, North West Bay and Garden Island farm sites. Samples from a farm site were collected from the same sea cages where possible.

Each biofouling sample fraction was placed in a sterile microcentrifuge tube and thoroughly macerated in 400 μL lysis buffer (Appendix 3) and 100 μL 10%(w/v) CTAB solution (Ausubel *et al*., 1998) using a sterilized tube-pestle. Digestion was allowed to occur at 55°C overnight and the sample subjected to phenol/chloroform extraction and DNA isolation as described in Section 5.1.2.

Table 7.1. Biofouling sub-fractions/organisms associated with Atlantic salmon cage netting and analyzed by PCR in this study

Common name	Identified taxon[a]	Sample type
Red algae	*Porphyra* sp.	fraction
Biofilm on mussel shell	unidentified microfouling	scraping
Biofilm on net	unidentified microfouling	scraping
Bryozoan	*Scrupocellaria bertholetti*	unidentified fraction
Colonial ascidian	unidentified	unidentified fraction
Copepod	unidentified	multiple organisms
Crustaceans (mixed)	unidentified	multiple organisms
Hydroid	*Obelia australis*	whole organism
Mussel	*Mytilus edulis*	gill fraction
Amphipod	*Caprella* sp.	whole organism
Solitary ascidian	*Ciona intestinalis*	unidentified fraction

[a]Identification of taxa performed by C. Tan (pers. communication, 2000; Tan, 2000).

7.1.1.3 Marine sediment samples

The efficacy of DNA extraction and PCR from a complex sample matrix was evaluated on marine sediments. Sediment samples tested by PCR originated from the three Tasmanian salmon farm sites at Hideaway Bay, Garden Island and North West Bay. Marine sediments from Hideaway Bay and Garden Island were collected at respective operating salmon farms in May 2000 (M. Helders-Douglas, pers. communication, 2000). North West Bay sediment samples were collected at around the same time from an ex-farm site that had been vacant for the past nine months (C. Macleod, Marine Environment Program, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Taroona; pers. communication, 2000).

Total cellular DNA was extracted from marine sediment samples using a modification of the method of Ogram *et al*. (1995). All sediments tested had been stored frozen at minus 20°C since the time of sampling. Samples were allowed to thaw at room temperature for 10 to 20 min immediately prior to processing. Three sub-samples each containing 0.5 g of a sediment sample were transferred to separate tubes and mixed with 1.5 mL extraction buffer (Appendix 3). Cell lysis was effected by addition of 150 μL 10% (w/v) SDS and 10 μL proteinase K (20 mg/mL). Samples were mixed by vortexing and incubated at 65°C for 2 h with periodic gentle mixing. Lysis was continued by freezing at minus 80°C for 30 min followed by immediate thawing at 65°C for 15 min. Samples were centrifuged at 12 000 x *g* at 10°C for 15 min and the supernatants from the triplicate sub-samples pooled in a fresh tube.

The supernatant was treated with 120 μL 10% (w/v) CTAB solution (Section 5.1.2) and re-incubated at 65°C for at least 30 min. Phenol/chloroform extraction was performed as previously described and repeated until an aqueous fraction without visible particulate matter was obtained. DNA was precipitated overnight at minus 20°C with 0.6 volume isopropanol and pelleted by centrifugation at 17 000 x *g* for 30 min. The DNA pellet was washed twice with 70% (v/v) ethanol at 5 min each and dried in a vacuum centrifuge. DNA was resuspended in 100 μL distilled water and allowed dissolve overnight at 4°C. DNA extracts were used directly as template for PCR. Selected sediment DNA extracts diluted 0, 10, 20 and 30 times with distilled water were respectively seeded with 100 ng PA 027 DNA to test for the possibility of PCR inhibition in samples known to be positive for *N. pemaquidensis* 18S rDNA.

Crude sediment extracts were also further gel-purified to minimize interference by potential PCR inhibitors (Porteous and Armstrong, 1993). Approximately 15 μL of DNA extract was electrophoresised in a 1% (w/v) low melting point agarose gel (GibcoBRL) prepared in 1X TAE buffer (Appendix 3). Agarose plugs containing the genomic DNA fragment bands were excised from the gel using wide-bore pipette tips (ca. 2 mm diameter) and transferred to individual tubes. Plugs were melted at 65°C for 15 min, an equal volume of distilled water added to each sample and thoroughly vortexes. Gel-purified DNA samples were reheated to 65°C for 10 min immediately before use as template for PCR. PCR was also tested on selected gel-purified extracts seeded with 100 ng of positive control PA 027 DNA.

7.1.1.4 Fish gill samples

Intact gills.
Both fresh and 70% (v/v) ethanol preserved gill sections were tested for evidence of *N. pemaquidensis* using PCR analysis. Fresh gills were sampled from Tasmanian Atlantic salmon with apparent AGD-like gill lesions as diagnosed by gill inspection at the farm. Gills were immediately excised from freshly euthanised fish and subjected to DNA extraction. Total DNA was extracted from fresh gill samples following the method of Moran *et al*. (1999). Essentially, 20 to 25 mg of gill tissue (lamellae) were macerated in 500 μL solution of 10 mM Tris, 1 mM EDTA (pH 8.0), 1% (w/v) SDS, and 200 μg/mL proteinase K using a sterilized tube-pestle and incubated at 37°C for at least 4 h. The lysed suspension was subjected to phenol/chloroform extraction and DNA precipitated using isopropanol as previously described (Section 5.1.2).

Ethanol preserved gills from New Zealand chinook salmon were rehydrated by removing the alcohol fraction and drying the samples in a vacuum centrifuge for 5 min followed by resuspension and maceration in 500µL lysis buffer (Appendix 3). DNA extractions were performed using the standard protocol described in Section 5.1.2.

Gill scrapings.
Gill mucus scrapings were processed by diluting 100 µL in 500 µL lysis buffer (Appendix 3). Samples were subjected to DNA extraction according to the standard protocol.

7.1.1.5 Culture enriched environmental samples.

Culture enriched seawater, biofouling and gill scraping samples were also obtained from various collaborating laboratories for PCR testing. In these cases, crude samples had been spread or impregnated into MYS agar plates (Section 5.1.1.4) and incubated at 20°C for 7 to 10 d. Growth on plates was harvested and concentrated in sterile seawater as described in Section 5.1.1.4. Total cellular DNA was extracted from the suspensions following the protocol described in Section 5.1.2 and used directly as template for PCR.

7.1.2 Nested Two-Step PCR Detection of *N. pemaquidensis*

Preliminary PCR analysis of extracted DNA samples suggested that cells (and DNA) of *N. pemaquidensis* occurred at low concentrations where present in the various environmental matrices. A nested two-step PCR procedure was developed using *N. pemaquidensis* specific primers to maximize the chances of positive detection of the AGD pathogen from environmental samples.

7.1.2.1 Primary amplification step

Crude DNA extracted from environmental samples was subjected to an initial PCR amplification utilizing the *Neoparamoeba* specific forward primer fPA-Hxe23a1, with the universal 18S rDNA reverse primer Med.18Sr (Tables 5.2 and 6.1). Each 50 µL PCR reaction mix contained 5 µL 10X PCR Gold™ buffer, 5 µL of 25 mM $MgCl_2$ solution, 1 µL of 10 mM dNTP solution, 1.5 µL each of 10 µM primer solutions, 0.2 µL (1 U) of Amplitaq Gold™ DNA polymerase, 30.8 µL MilliQ water and 5 µL of DNA template. PCR cycling conditions included an initial denaturation at 95°C for 10 min followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, and 68°C for 2 min. A final 10 min extension step at 72°C was added following completion of the cycling steps. In all PCR preparations, 50 ng of cellular DNA isolated from *N. pemaquidensis* strain PA 027 was used as the positive control reaction and a reaction without added DNA served as the negative control. Each primary PCR amplified reaction was retained for use as template for the secondary PCR step.

7.1.2.2 Nested secondary amplification step

The secondary PCR step utilized specific primers that target sequences internal to the primary PCR product. The secondary primer set consisted of the *N. pemaquidensis* specific forward primer fPA-Hxe23b1 and the reverse primer rPA-Hx49 (Table 6.1). This primer set effectively amplifies 18S rDNA of *N. pemaquidensis* to the exclusion of DNA from all other species (Section 6.0). Each 50 μL secondary PCR reaction mix consisted of 5 μL 10X PCR Gold™ buffer, 4 μL of 25 mM $MgCl_2$ solution, 1 μL of 10 mM dNTP solution, 0.5 μL each of 10 μM primer solutions, 0.2 μL (1 U) of Amplitaq Gold™ DNA polymerase, 37.8 μl MilliQ water and 1 μl of the amplified primary PCR reaction as template. Due to some 3'-end base homology of primer fPA-Hxe23b1 with the *N. aestuarina* sequence, PCR was performed using high stringency conditions consisting of initial denaturation at 95°C for 10 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min; and final extension at 72°C for 10 min. A 10 μL aliquot of the secondary amplified reaction was subjected to agarose gel electrophoresis and amplification products visualized as described in Section 5.1.3.2.

7.1.2.3 Specificity screening of *N. pemaquidensis* specific nested PCR.

Specificity of the nested two-step PCR was tested by screening against cellular DNA extracts from target and non-target organisms (Table 5.1). Atlantic salmon DNA was also subjected to the *N. pemaquidensis* PCR to test for possible cross-reactivity, since the procedure was to be applied to samples from the salmon farm environment. Specificity of the secondary primer pair (fPA-Hxe23b1/rPA-Hx49) when used in the nested two-step PCR protocol was compared to a single step PCR amplification using these primers.

7.1.2.4 Determination of PCR sensitivity for cells in seawater.

The sensitivity of detection of the nested PCR procedure for whole cells of *N. pemaquidensis* suspended in seawater was determined using serial cellular dilutions of the Tasmanian strain PA 027. A stock suspension of 512 cells/100 μL was prepared in filter-sterilized seawater by M. Helders-Douglas (School of Aquaculture). Respective DNA extractions were performed on 500 μL cell suspensions starting at the stock concentration, followed by two-fold serial dilutions thereafter up to a concentration of 8 cells/100 μL. Each DNA extract was suspended in 50 μL distilled water from which a 5 μL aliquot was used as template for nested two-step PCR amplification as described above.

The efficacy of DNA extraction from each sample was considered an important limiting step affecting the sensitivity of *N. pemaquidensis* detection by PCR. Consequently, in addition to the DNA extraction procedure for seawater samples described in Section 7.1.1.1, DNA was also extracted using a commercial extraction kit based on spin-column separation of total cellular DNA (DNeasy™ Tissue Kit, Qiagen). The relative efficiencies of DNA extraction and downstream nested PCR detection of *N. pemaquidensis* 18S rDNA between the two methods were compared. Duplicate extracts were prepared for PCR at each cell concentration.

7.1.3 DNA Sequence Verification of PCR Products

The *N. pemaquidensis* specific 18SrDNA PCR products of arbitrarily selected positive environmental samples were subjected to DNA sequencing to verify that the respective amplicons were actually derived from that species. PCR products were purified and directly DNA sequenced as described in Section 5.1.3.3. 18S rDNA sequences were obtained using the *Neoparamoeba* primers fPA-Hx43a1, fPA-Hxe23b1 and rPA-Hx49 (Table 6.1), and the eukaryotic 18S rDNA primers Elw.892f, PA.1300f and Elw.1277r (Table 5.2). These sequencing primers anneal to positions internal to the PCR product. The DNA sequences obtained from PCR positive samples were aligned to 18S rDNA sequences of the various *N. pemaquidensis* and *N. aestuarina* strains (Section 5.0) and homologies assessed.

7.2 Results

7.2.1 Specificity of Nested PCR for *N. pemaquidensis*

The nested two-step PCR using two primer sets was found to be specific for all strains of *N. pemaquidensis* regardless of origin, while not cross-reacting with DNA from non-target organisms (Figure 7.1). The nested PCR did not cross-react with the closely related species *N. aestuarina*, nor with morphologically similar *Pseudoparamoeba pagei* and other unrelated amoeba species co-isolated from AGD-infected fish gills. The *N. pemaquidensis* specific PCR primers did not react with Atlantic salmon DNA, allowing PCR detection in fish tissue samples without interference from the host DNA. No variation in PCR specificity was observed between single step PCR amplification and secondary amplification in the nested two-step PCR procedure, using the primers fPA-Hxe23b1 and rPA-Hx49 (Figure7.1).

7.2.2 PCR Analyses of Environmental Samples

7.2.2.1 Seawater samples

The *N. pemaquidensis* nested PCR confidently detected cells in seawater to a concentration of 16 cells/100 μL (Figure 7.2a). In sensitivity tests performed in this study, this was equivalent to the positive detection of approximately 80 *N. pemaquidensis* cells in the 500 μL sample. PCR positives were also obtained for suspensions at a concentration of 8 cells/100 μL, although inconsistent results were obtained for replicate preparations at this concentration.

In addition to the standard enzymatic extraction method, DNA template for the primary PCR step was also extracted from equivalent dilutions of cell-seeded seawater using the QIAGEN Dneasy™ Tissue Extraction kit. However, results of nested two-step PCR using DNA prepared with this kit were inconsistent and lacked the sensitivity of detection from DNA samples prepared with the standard method (Figure 7.2b). PCR detection may have been compromised by a decrease in the quality or quantity of *N. pemaquidensis* DNA prepared by the DNeasy™ kit and highlights the DNA extraction step as an important limiting factor in the ability of PCR to confidently detect specific cells from environmental samples.

Crude seawater samples.
A total of 15 crude non-culture enriched seawater samples were processed for *N. pemaquidensis* PCR (Table 7.2). Hideaway Bay and South Australian samples had been screened for the AGD pathogen using an immunoblot detection method developed by M. Helders-Douglas (School of Aquaculture, pers. communication, 2000). The Hideaway Bay samples were immunoblot positive while South Australian samples were collectively blot negative. However, none of the immunoblot positive samples tested positive with *N. pemaquidensis* specific nested PCR.

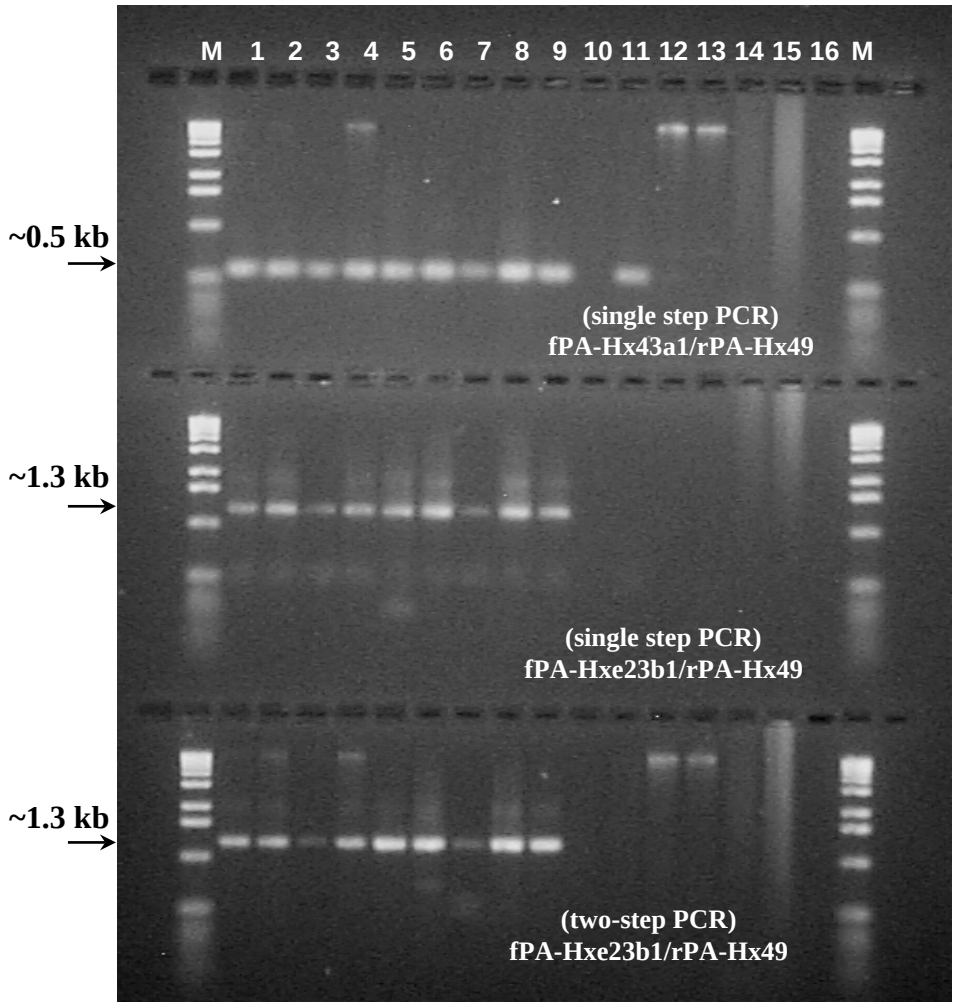

Figure 7.1. Specificity screening of *N. pemaquidensis* specific primers fPA-Hxe23b1 and rPA-Hx49 in single step PCR and as secondary primers in nested two-step PCR on cellular DNA extracts from selected target and non-target organisms. The relative specificity of single step PCR using different primer set fPA-Hxe43a1/rPA-Hx49 was included for comparison. Lanes 1 to 4, Tasmanian *N. pemaquidensis* isolates PA 027, PA 010, PA 011 and PA 111; lanes 5 to 8, *N. pemaquidensis* reference strains ATCC 30735, ATCC 50172, CCAP 1560/4 and CCAP 1560/5; lane 9, Irish *N. pemaquidensis* strain AVG 8194; lane 10, *Pseudoparamoeba pagei* strain CCAP 1566/1; lane 11, *N. aestuarina* strain CCAP 1560/7; lanes 12 and 13, presumptive *Paraflabellula hoguae* gill isolates UP 1/6 and UP 4a/2; lanes 14 and 15, Atlantic salmon genomic DNA; lane 16, negative control; and lane M, GibcoBRL DNA marker. Arrows indicate the positions of the expected specific PCR products. Note the cross-reactivity of the fPA-Hxe43a1/rPA-Hx49 primer set for *N. aestuarina* CCAP 1560/7 (lane 11) absent with the *N. pemaquidensis* specific primer set fPA-Hxe23b1/rPA-Hx49.

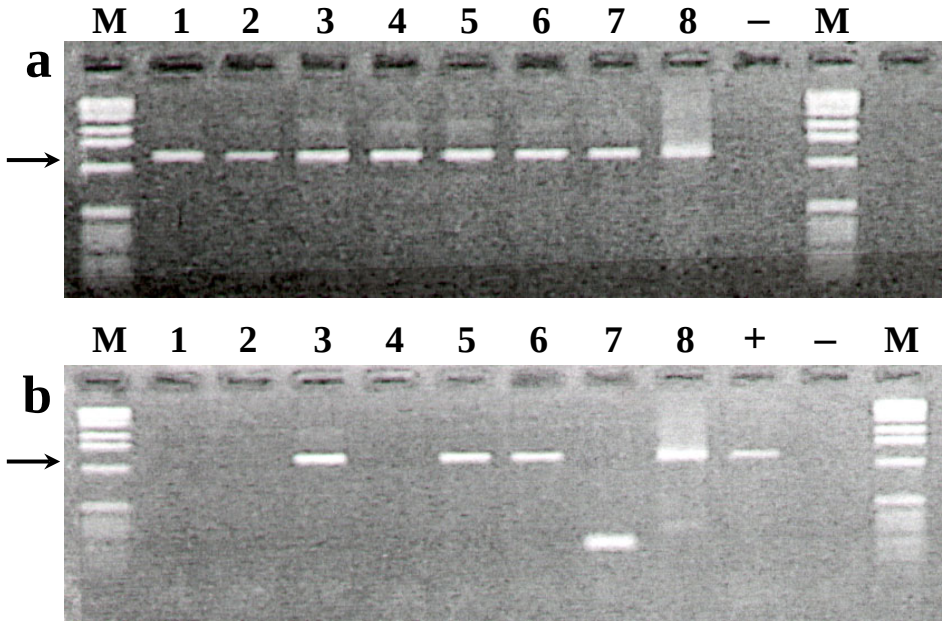

Figure 7.2. Detection sensitivity of *N. pemaquidensis* specific nested two-step PCR on filtered seawater seeded with cells of *N. pemaquidensis* strain PA 027. Template DNA was prepared using either (a) standard enzymatic method with phenol/chloroform extraction or (b) the Qiagen DNeasy Tissue DNA extraction kit. DNA was respectively extracted from serially diluted cell suspensions at 512 cells/100 mL (lane 1), 256 cells/100 mL (lane 2), 128 cells/100 mL (lane 3), 64 cells/100 mL (lane 4), 32 cells/100 mL (lane 5), 16 cells/100 mL (lane 6) and 8 cells/100 mL (lane 7). Lane 8, nested PCR with positive control PA 027 DNA; lane "+", single-step PCR with positive control PA 027 DNA; "–", negative control with no template DNA; and M, GibcoBRL DNA marker. Note inconsistency of PCR sensitivity in samples prepared with the Qiagen kit (b). Arrows indicate the expected positions of the ca.1.3 kb specific PCR product of the fPA-Hxe23b1/rPA-Hx49 secondary primer set.

Only one crude water sample, collected in April 2000 from the Hideaway Bay farm site was PCR positive for *N. pemaquidensis* (Table7.2). The positive seawater sample produced an expected amplification product of approximately 1.3 kb using the *N. pemaquidensis*-specific primer set fPA-Hxe23b1/rPA-Hx49 in the secondary PCR reaction.

Table 7.2. *N. pemaquidensis* specific nested PCR of non-culture enriched seawater samples from Atlantic salmon farm sites

Date[a]	Sample #	Sample processing	Origin	Immuno-blot[b]	Nested PCR[c]
04/00	1	centrifuge	Hideaway Bay, Tas.	nt	–
	2	centrifuge	Hideaway Bay, Tas.	nt	+
06/00	4	centrifuge	Hideaway Bay, Tas.	+	–
	11	centrifuge	Hideaway Bay, Tas.	+	–
	13	centrifuge	Hideaway Bay, Tas.	+	–
	15	centrifuge	Hideaway Bay, Tas.	+	–
08/00	1	filter	North West Bay, Tas.	nt	–
	2	filter	North West Bay, Tas.	nt	–
	3	filter	North West Bay, Tas.	nt	–
	4	filter	North West Bay, Tas.	nt	–
07/00	1	filter	South Australia	–	–
	3	filter	South Australia	–	–
	4	filter	South Australia	–	–
	5	filter	South Australia	–	–
	6	filter	South Australia	–	–

[a]Date (month/year) in which sample was collected.

[b]Immunoblot results (+, blot positive; –, blot negative; nt, not tested). Immunoblot analyses performed on centrifuge concentrated sample fractions by M. Helders-Douglas (School of Aquaculture).

[c]PCR results (+, PCR positive;–, PCR negative).

Enriched seawater samples.

N. pemaquidensis PCR was also tested on 11 culture enriched seawater samples collected from the Hideaway Bay site. These were miscellaneous samples processed by Tan (2000) to investigate the distribution of *N. pemaquidensis* in the salmon farm environment using IFAT detection. Four (36%) of the enriched samples were PCR positive for *N. pemaquidensis* (Table 7.3). One of these positive samples was also IFAT positive, while two others were weakly fluorescent. A fourth PCR positive sample was not tested by IFAT. Two of the seawater samples collected in April 2000 were also tested by PCR prior to culture enrichment. *N. pemaquidensis* was detected by PCR in both crude and enriched fractions of sample #2, but was not detected in either fraction in sample #1 (Tables 7.2 and 7.3).

Table 7.3. *N. pemaquidensis* specific nested PCR of culture enriched seawater samples originating from the farm site at Hideaway Bay, Tasmania

Date[a]	Sample #	Sample processing	IFAT[b]	Nested PCR[c]
04/00	1	centrifuge	–	–
	2	centrifuge	+	+
	3	centrifuge	–	–
	4	centrifuge	–	–
08/00	5	centrifuge	–	–
	6	centrifuge	±	–
	7	centrifuge	±	+
	8	centrifuge	±	+
	9	centrifuge	nt	+
	10	centrifuge	nt	–
	11	centrifuge	nt	–

[a]Date (month/year) in which sample was collected.

[b]IFAT results (+, positive detection; –, negative detection; ±, low level detection; nt, not tested). IFATs performed on centrifuge concentrated, MYS culture enriched sample fractions by C. Tan (School of Aquaculture).

[c]PCR results (+, PCR positive;–, PCR negative).

7.2.2.2 Cage biofouling samples

Crude biofouling samples.
All crude non-cultured biofouling samples tested by *N. pemaquidensis* PCR were collected in a single April 2000 sampling trip from the Hideaway Bay site. Eleven samples each comprising a different biofouling sub-fraction (Table 7.1) were analyzed by PCR. Only one crude biofouling sample (9% of total) consisting of the bryozoan *Scrupocellaria bertholetti* was positive for *N. pemaquidensis* when tested by nested two-step PCR. All other crude biofouling samples were PCR negative.

Enriched biofouling samples.
Nine culture enriched biofouling samples collected from Hideaway Bay in March and April 2000 were tested by *N. pemaquidensis* PCR (Table 7.4). These samples had also been tested by IFAT (Tan, 2000), allowing comparison between PCR and a non-DNA based detection method. Analysis of the enriched samples also resulted in a PCR positive bryozoan *Scrupocellaria bertholetti* sample (11% of total samples). This confirmed the positive PCR detection of *N. pemaquidensis* from the crude sub-sample prepared from this particular macro-organism.

Fifteen culture enriched net smears (biofilm) sampled from bag-cage systems in August 2000 from the North West Bay site were also tested by PCR. Eight of these samples (53%) were positive for *N. pemaquidensis*.

Table 7.4. *N. pemaquidensis* specific nested PCR of culture enriched cage micro-biofouling samples from the Hideaway Bay farm site, Tasmania

Date[a]	Sample sub-fraction	Taxon	IFAT[b]	Nested PCR[c]
03/00	Biofilm smear 1	unidentified	±	–
	Biofilm smear 2	unidentified	±	–
04/00	Solitary ascidian	*Ciona intestinalis*	+	–
	Colonial ascidian	unidentified	–	–
	Bryozoan	*Scrupocellaria bertholetti*	+	+
	Hydroid	*Obelia australis*	–	–
	Mussel gill	*Mytilus edulis*	–	–
	Biofilm smear 1	unidentified	–	–
	Biofilm smear 2	unidentified	–	–

[a]Date in which sample was collected.

[b]IFAT results (+, positive detection; –, negative detection; ±, low level detection). IFATs performed on culture enriched sample fractions by C. Tan (School of Aquaculture).

[c]PCR results (+, PCR positive;–, PCR negative).

Antifouling paint trial samples.

Culture enriched biofouling samples collected from salmon cages at the Garden Island farm site were also screened by PCR (Figure 7.3). These samples were collected to investigate the effects of antifouling paint treatments on the distribution of *N. pemaquidensis* on sea-cage netting (Tan, 2000). Samples comprised cultured net-biofilm smears collected from five separate cages over two sampling trips (weeks 2 and 8) spanning 6 weeks of the antifouling treatment study. Netting from these cages had been treated with Netclear® (Wattyl Paints) antifouling paint (cage N), the copper dioxide-based Hempanet® (Hempel Paints) antifouling paint (cages H1 and H2), or left as untreated controls (cages C1 and C2) (Tan, 2000). In each sampling trip, replicate cultures were obtained from each cage by randomly transferring up to five net smears onto separate MYS culture plates (Tan, 2000). PCR was used to screen 25 culture enriched biofilm samples consisting of five samples from each cage in Trial Week 2; and 15 biofilm samples consisting of three samples from each cage in Trial Week 8 (Figure 7.3). *N. pemaquidensis* was detected by PCR in net biofilm sampled from all three treated cages and from the untreated control cage C2 in both weeks 2 and 8 of the antifouling treatment study. In contrast, *N. pemaquidensis* was not detected by PCR in any of the control cage C1 samples examined. Sub-samples of the same enriched biofilm samples used for PCR were also examined by IFAT (Tan, 2000). Good correlation of *N. pemaquidensis* positive samples were obtained for Netclear®, Hempanet®, and control treatments using both PCR and IFAT in the week 2 samples, but lower numbers of PCR positives were recorded in week 8 (Table 7.5).

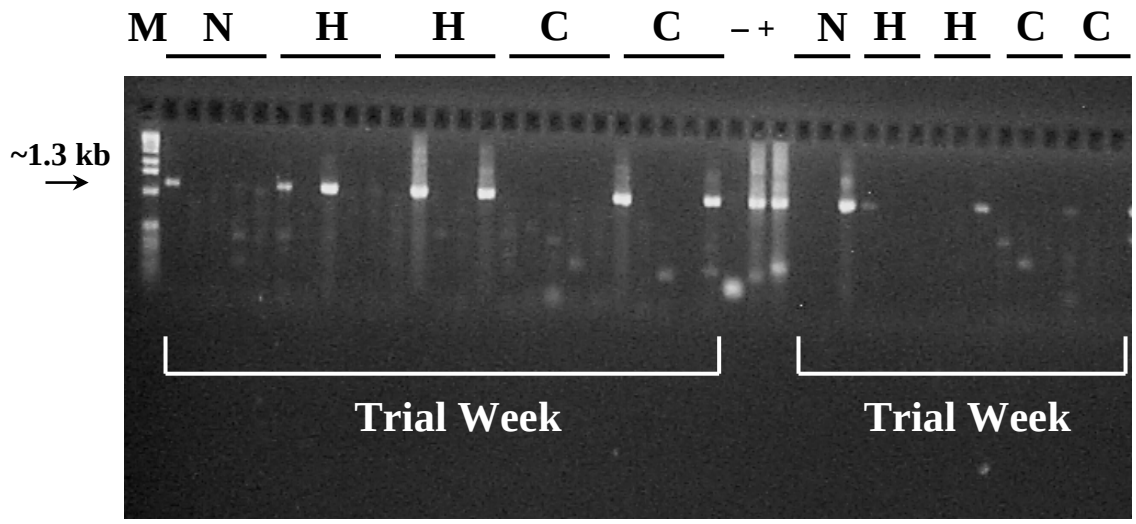

Figure 7.3. *N. pemaquidensis* specific nested PCR of enriched biofilm samples from five Atlantic salmon cages undergoing a 10-week antifouling paint treatment trial at the Garden Island farm site, Tasmania. Biofilm smear samples were collected from treated netting at the same cages during week 2 (five samples per cage) and week 8 (three samples per cage) of the trial. Lane M, GibcoBRL DNA marker; lanes N, samples from Netclear® treated cage; lanes H1 and H2, samples from two separate Hempanet® treated cages; lanes C1 and C2, samples from two separate untreated control cages; lane "–", negative control; and lanes "+", positive control *N. pemaquidensis* PA 027 DNA. The arrow indicates the position of the expected specific PCR products from positive samples.

Table 7.5. Relative percentages of *N. pemaquidensis* positive enriched net biofilm samples as detected by IFAT and PCR for each treatment in weeks 2 and 8 of the antifouling paint trial

Trial period	Antifouling treatment	% positive samples	
		IFAT[a]	Nested PCR
Week 2	Netclear®	40%	40%
	Hempenet®	40%	50%
	control	20%	20%
Week 8	Netclear®	58%	33%
	Hempenet®	59%	33%
	control	10%	17%

[a]Percentage (%) positive values for IFAT taken from Tan(2000).

7.2.2.3 Sediment samples.

Relatively good quality cellular DNA was obtained from all marine sediment samples using the described extraction method (Figure 7.4). All crude DNA extracts derived from sediment samples were negative when tested by *N. pemaquidensis* specific nested PCR. However, dilutions of sediment DNA extract seeded with PA 027 DNA were also PCR negative, suggesting the presence of PCR inhibitors. In contrast, the same sediment extracts that had been gel-purified to minimize the presence of potential PCR inhibitors prior to seeding with PA 027 DNA resulted in positive PCR amplification. All sediment extracts were re-analyzed by PCR following gel-purification but remained negative for *N. pemaquidensis* 18S rDNA (Table 7.6).

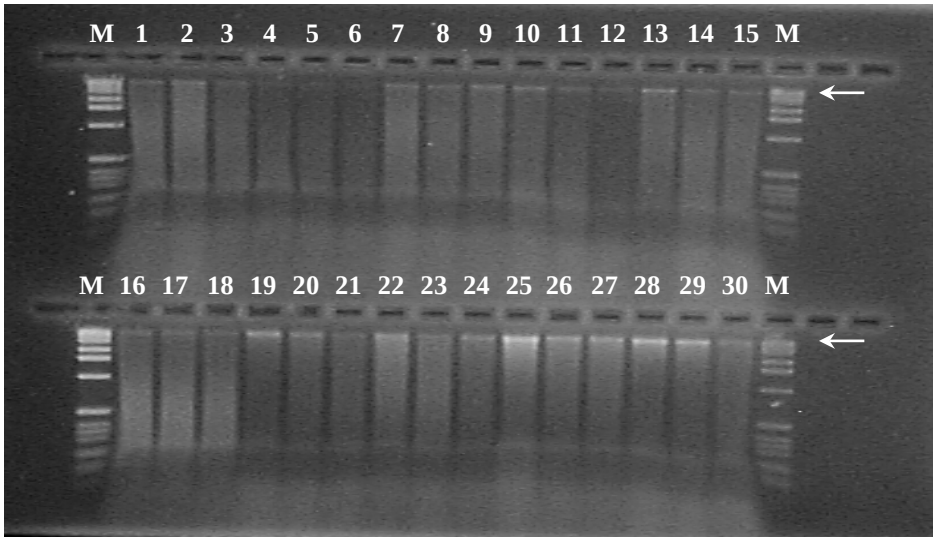

Figure 7.4. Total crude cellular DNA extracts from marine sediment samples collected from three Tasmanian Atlantic salmon farm sites. Lane M, GibcoBRL DNA marker; lanes 1 to 18, sediment samples from the North West Bay farm site; lanes 19 to 21, sediment samples from the Garden Island farm site; and lanes 22 to 30, sediment samples from the Hideaway Bay farm site. Arrows indicate the positions of the high molecular weight DNA bands. Agarose plug samples were excised at these cellular DNA bands for gel-purification and *N. pemaquidensis* specific PCR.

Table 7.6. *N. pemaquidensis* specific nested PCR of gel-purified marine sediment samples associated with Atlantic salmon farming sites in Tasmania

Date[a]	Origin	Sample	Position	Nested PCR
05/00	Garden Island	Transect #1 sub-sample 1	non-cage[b]	–[e]
	Garden Island	Transect #1 sub-sample 2	non-cage	–
	Garden Island	Transect #1 sub-sample 3	non-cage	–
05/00	Hideaway Bay	Transect #1 sub-sample 1	non-cage	–
	Hideaway Bay	Transect #1 sub-sample 2	non-cage	–
	Hideaway Bay	Transect #1 sub-sample 3	non-cage	–
	Hideaway Bay	Transect #2 sub-sample 1	cage[c]	–
	Hideaway Bay	Transect #2 sub-sample 2	cage	–
	Hideaway Bay	Transect #2 sub-sample 3	cage	–
	Hideaway Bay	Transect #3 sub-sample 1	cage	–
	Hideaway Bay	Transect #3 sub-sample 2	cage	–
	Hideaway Bay	Transect #3 sub-sample 3	cage	–
05/00	North West Bay	Transect #1 sub-sample 1	non-cage[d]	–
	North West Bay	Transect #1 sub-sample 2	non-cage	–
	North West Bay	Transect #1 sub-sample 3	non-cage	–
	North West Bay	Transect #1 sub-sample 1	cage	–
	North West Bay	Transect #1 sub-sample 2	cage	–
	North West Bay	Transect #1 sub-sample 3	cage	–
	North West Bay	Transect #2 sub-sample 1	non-cage	–
	North West Bay	Transect #2 sub-sample 2	non-cage	–
	North West Bay	Transect #2 sub-sample 3	non-cage	–
	North West Bay	Transect #2 sub-sample 1	cage	–
	North West Bay	Transect #2 sub-sample 2	cage	–
	North West Bay	Transect #2 sub-sample 3	cage	–
	North West Bay	Transect #3 sub-sample 1	non-cage	–
	North West Bay	Transect #3 sub-sample 2	non-cage	–
	North West Bay	Transect #3 sub-sample 3	non-cage	–
	North West Bay	Transect #3 sub-sample 1	cage	–
	North West Bay	Transect #3 sub-sample 2	cage	–
	North West Bay	Transect #3 sub-sample 3	cage	–

[a]Date (month/year) in which sample was collected.

[b]Sediment collected at farm lease site, but not in immediate vicinity of salmon cage.

[c]Sediment collected at farm lease site under cage.

[d]Sediments from North West Bay with the same transect reference number were collected either immediately beside (0 m) an ex-cage site (cage samples); or distant (150 m) from that site (non-cage samples).

[e]PCR negative.

7.2.2.4 Tasmanian Atlantic salmon gill samples.

Miscellaneous samples were obtained from both apparently healthy and infected gills of Tasmanian Atlantic salmon for testing by *N. pemaquidensis* specific PCR. These samples comprised intact gill tissue and mucus scrapings from 23 Atlantic salmon collected from the Hideaway Bay and North West Bay sites, and two fish from Port Esperance (Tables 7.7 and 7.8). Only two of the 25 (8%) gill samples, both from the North West Bay farm site, had evidence of *N. pemaquidensis* contamination when tested by PCR (Table 7.8). North West Bay gill samples were also sub-sampled for testing by immunological detection methods including IFAT and immunoblot (Table 7.8).

Table 7.7. *N. pemaquidensis* specific PCR of Atlantic salmon gill samples originating from Tasmanian farm sites

Date[a]	Sample #	Gill patches[b]	Sample	Origin	Nested PCR[c]
04/00	1	–	Intact gill	Port Esperance	–
	2	–	Intact gill	Port Esperance	–
04/00	1	+	Intact gill	Hideaway Bay	–
	2	+	Intact gill	Hideaway Bay	–
	3	+	Intact gill	Hideaway Bay	–
08/00	1	+	Mucus	Hideaway Bay	–
	2	+	Mucus	Hideaway Bay	–
	3	+	Mucus	Hideaway Bay	–
	4	+	Mucus	Hideaway Bay	–
	5	+	Mucus	Hideaway Bay	–

[a]Date(month/year) in which sample was collected.

[b]Presence (+) or absence (–) of AGD-like gill patches/lesions as determined by farm management.

[c]PCR result (–, PCR negative).

Table 7.8. Comparison of IFAT, immunoblot, and PCR results for detection of *N. pemaquidensis* in Atlantic salmon gill samples originating from the North West Bay farm site, Tasmania

Date[a]	Sample #	Gill patches[b]	Sample	IFAT[c]	Immuno-blot[d]	Nested PCR[e]
08/00	1	+	Intact gill	–	+	–
	5	+	Intact gill	–	–	–
	6	+	Intact gill	–	–	–
	7	+	Intact gill	–	–	–
	8	+	Intact gill	–	+	–
	9	+	Intact gill	±	±	–
	10	+	Intact gill	±	+	±
	11	+	Intact gill	–	–	–
	12	+	Intact gill	+	+	+
	13	+	Intact gill	±	–	–
	14	+	Intact gill	–	+	–
	15	+	Intact gill	–	+	–
	16	+	Intact gill	–	±	–
	17	+	Intact gill	–	–	–
	18	+	Intact gill	–	+	–

[a]Date (month/year) in which sample was collected.

[b]Presence (+) or absence (–) of AGD-like gill patches/lesions as determined by farm management.

[c]IFAT diagnosis performed at FHU, DPIWE (+, positive detection; –, negative detection; ±, low level detection).

[d]Immunoblot diagnosis performed by M. Helders-Douglas of the School of Aquaculture (+, positive blot; –, negative blot; ±, weak positive).

[e]PCR result (+, PCR positive; –, PCR negative; ±, weak PCR positive).

7.2.2.5 New Zealand chinook salmon gill samples.

Four ethanol fixed culture suspensions of *Paramoeba* sp. received from the Wallaceville Research Centre, Ministry of Agriculture and Forestry (MAF), New Zealand were examined by *N. pemaquidensis* specific PCR. These cultures were originally obtained from AGD infected chinook salmon sea-farmed in Marlborough Sounds, New Zealand. Initial identification of *Paramoeba* sp. as the gill parasite had been made by histological methods. Two of these culture-derived suspensions were confirmed to consist of *N. pemaquidensis* following positive amplification by nested PCR.

In addition, 19 ethanol preserved chinook salmon gill samples collected in March 2000 from Marlborough Sounds were also tested by PCR. These samples were obtained from 14 fish from two farm sites. None of the fish sampled showed any evidence of AGD-like infection by gill inspection. All of these samples were PCR negative for *N. pemaquidensis* (Table 7.9).

Table 7.9. *N. pemaquidensis* specific PCR of ethanol-fixed chinook salmon gill samples originating from sea farms in Marlborough Sounds, New Zealand[a]

Date[b]	Fish	Gill patches[c]	Sample #	Sample	Nested PCR[d]
03/00	1	–	5	Intact gill	–
		–	6	Intact gill	–
		–	7	Mucus	–
	2	–	8	Intact gill	–
		–	9	Mucus	–
	3	–	10	Mucus	–
	4	–	11	Mucus	–
	5	–	12	Mucus	–
	6	–	13	Mucus	–
	7	–	14	Mucus	–
	8	–	15	Mucus	–
	9	–	16	Mucus	–
	10	–	17	Mucus	–
		–	18	Mucus	–
	11	–	19	Mucus	–
		–	20	Mucus	–
	12	–	21	Mucus	–
	13	–	22	Mucus	–
	14	–	23	Mucus	–

[a]Details of New Zealand samples were provided by C. Anderson of the Wallaceville Research Centre, National Centre for Disease Investigation, MAF, New Zealand.

[b]Date (month/year) in which sample was collected.

[c]Presence (+) or absence (–) of AGD-like patches/lesions on sampled gill.

[d]PCR result (+, PCR positive;–, PCR negative; w, weak PCR positive).

7.2.3 Sequence Analysis of PCR Positive Samples

DNA sequences were obtained from the PCR products amplified from two different *N. pemaquidensis* positive biofilm samples from the North West Bay farm site in Tasmania. These biofilm samples originated from bag-cages that were utilized by this farm. The sequences obtained from cage biofilm were homologous to the 18S rDNA sequence of *N. pemaquidensis*, with 99.0% sequence similarity to Tasmanian strain PA 027 when up to 1212 bases were aligned and compared. Sequences from both samples were identical. This confirmed that the PCR method was specifically detecting DNA from *N. pemaquidensis* in the samples, although the existence of strain differences could not be determined since the entire 18S rDNA was not analyzed.

DNA sequences were also determined for two PCR positive chinook salmon gill cultures from Marlborough Sounds, New Zealand. Both of these sequences were identical and were homologous to *N. pemaquidensis* 18S rDNA, with approximately 98.4% sequence similarity over 925 bases. Base differences between sequences obtained from the New Zealand gill cultures and strain PA 027 were expected since cells were isolated from different countries and salmonid species. As for above, the extent of genetic divergence that may exist between these isolates cannot be deduced since sequences from the entire 18S rDNA were not compared.

8. DISCUSSION

8.1 AGD associated Paramoeba are *Neoparamoeba pemaquidensis*

The genus *Neoparamoeba* contains two species *Neoparamoeba pemaquidensis* and *N. aestuarina*, both formerly classified as members of the genus *Paramoeba* (Page, 1987; Cann and Page, 1982). This new genus has recently been accepted and used by a major international culture collections (ATCC) as a separate classification distinct from the genus *Paramoeba*, as proposed by Page (1987). The genus nomenclature "*Neoparamoeba*" is used in this study when referring to the parasomal marine amoebae previously known as *Paramoeba pemaquidensis* and *P. aestuarina*.

The implicated parasitic agent of AGD in Tasmanian sea-farmed Atlantic salmon had been identified as *Paramoeba* sp. since 1987 (Foster and Percival, 1988). *Paramoeba* sp. has also been reported to cause outbreaks of AGD in other salmonid species farmed in other countries (Findlay and Munday, 1998; Palmer, 1997). The Tasmanian parasite was found by morphological, ultrastructural and pathological studies to most closely resemble *N. pemaquidensis*, a paramoeba species that causes AGD-like gill disease in sea-farmed coho salmon *Oncorhynchus kisutch* in the USA (Roubal *et al*., 1989; Munday *et al*., 1990; Kent *et al*., 1988). Our 18S rDNA analyses have confirmed that *Paramoeba* sp. is homologous and identical to the species *Neoparamoeba pemaquidensis*.

Comparison of the entire 18S rDNA showed that *Paramoeba* sp. isolates from infected gills of Atlantic salmon farmed in Tasmania and Ireland had greater than 98% sequence similarity with the parasitic *N. pemaquidensis* strain isolated from infected coho salmon in the USA. The AGD isolates also had the same high level of sequence similarity (at least 98%) with free-living isolates of *N. pemaquidensis* originating from coastal waters in the USA and UK. This level of similarity is consistent with a single homologous 18S rDNA sequence type within the same species (Gast *et al*., 1996). The AGD paramoeba isolates originating from different countries possessed greater sequence homogeneity than different strains within a single *Acanthamoeba* species (Gast *et al*., 1996), but had slightly greater intra-specific sequence variation than that reported for *Hartmannella vermiformis* (Weekers *et al*., 1994). These species represent other members of the non-testate, lobose, non-spore forming amoeboid subclass Gymnamoebia to which the genus *Neoparamoeba* belongs.

Four isolates of *N. pemaquidensis* from AGD outbreaks in Tasmania shared identical 18S rDNA sequences. Although the specific isolation details of the four isolates are uncertain, the isolates had been separately obtained in November 1993 and April 1994 from farmed Atlantic salmon held by Saltas at Dover. Our sequence data supports other existing identification criteria that suggest AGD outbreaks affecting Tasmanian Atlantic salmon farms are caused by the same organism. *N. pemaquidensis* is recognized as a relatively common, mostly free-living marine amoeba (Page, 1973). Sequence identification of isolates from Tasmania, the UK and USA suggests that free-living and parasitic isolates are homologous, and supports previous findings that the species is widely distributed. 18S rDNA sequences were not able to separate fish

gill-associated isolates from free-living seawater isolates. Further molecular characterization using a greater number of isolates would be needed to determine whether actual strain or clonal differentiation exists between parasitic and free-living isolates.

8.2 *N. pemaquidensis* closely related to *N. aestuarina*

The two species *N. pemaquidensis* and *N. aestuarina* lack cell surface microscales and together with other ultrastructural differences, are taxonomically differentiated from the genus *Paramoeba* represented by the type species *Paramoeba eilhardi* (Page, 1987). 18S rDNA sequences support the distinction of two separate but closely related *Neoparamoeba* species. A diverse group of *N. pemaquidensis* isolates was found to be monophyletic and fall into a single sequence type that was easily differentiated from the sequence of the *N. aestuarina* reference strain CCAP1560/7. The *N. aestuarina* strain had at least 4.3% sequence dissimilarity with isolates of *N. pemaquidensis*. This amount of sequence variation is close to the approximately 5% dissimilarity levels used to distinguish different 18S rDNA sequence types of the genus *Acanthamoeba* (Stothard *et al*., 1998).

The ability to differentiate between *N. pemaquidensis* and *N. aestuarina* is an important consideration for future ecological and environmental studies of the AGD pathogen. Recent AGD studies suggest that confusion exists as to the actual distinction between the two species (Dyková *et al*., 2000). Dyková *et al.* (2000) could not distinguish *N. aestuarina* strain CCAP 1560/7 from various paramoeba isolates from gill disease of non-salmonid fish, sea bass and turbot (Dyková *et al*. 1998), based on morphological and ultrastructural comparisons of amoeba cells. However, that study did not examine any *N. pemaquidensis* (the type species of the genus) reference strains or salmonid AGD isolates, despite suggesting that both *Neoparamoeba* species may be agents of aetiologically similar AGD in fish. Our present study confirmed that paramoebae associated with AGD in sea-farmed salmonids collectively belonged to the species *N. pemaquidensis* and not its sister species. However, whether AGD reported in non-salmonid marine fishes are also caused by *N. pemaquidensis*, or are in fact due to *N. aestuarina* or another species is unknown. Prior to the report by Dyková *et al*. (2000) *N. aestuarina* had only been reported as free-living trophozoites and had not been associated with disease in fish.

8.3 Phylogenetic Relationships of *N. pemaquidensis*

N. pemaquidensis was not analogous to any protistan taxa that presently exist in the eukaryotic 18S rDNA sequence database. The sequence data obtained from our study represents important new information for the characterization and identification of this organism. The respective relationships obtained among the selection of diverse taxa examined in this study are consistent with the accepted phylogenies representing the major eukaryotic and protistan lineages (Sogin and Silberman, 1997). Amoeboid protozoans have been found to be evolutionarily diverse and separate into an unknown number of independent lineages (Sogin and Silberman, 1997). Previous phylogenetic studies have found that some members of the amoeboid subclass Gymnamoebia, including the genera *Acanthamoeba* and *Hartmannella*, may be

broadly related and occupy one of these lineages (Weekers *et al*., 1994; Sims *et al*., 1999). Our findings support the relationship between those genera and also placed the additional genera of *Neoparamoeba* and *Paraflabellula* into that broad phylogenetic cluster. Although another Gymnamoebia, *Vannella anglica*, was found to be excluded from this cluster (Sims *et al*., 1999), results suggest that many of the non-testate, lobose and non-sporulating amoebae, including the paramoebae, may be derived from a common evolutionary lineage.

8.4 Specific PCR detection of *N. pemaquidensis*

A specific detection method for *N. pemaquidensis* is required for epidemiological and environmental studies, since the AGD-causing organism is closely related to *N. aestuarina* and possibly other as yet uncharacterized Gymnamoebia. Currently available methods for detection of the AGD parasite are mainly based on immunodetection. However, antisera on which the immunodetection methods are based (IFAT and immunoblot) are known to cross-react with *N. aestuarina* and *Pseudoparamoeba pagei* (Douglas-Helders *et al*., 2001) but not with a range of other amoebae found associated with the gills of fish with AGD (Howard and Carson 1993). This cross-specificity has not been found to be important for detection of *N. pemaquidensis* in gill samples or on culture-purified isolates, but represents a significant limitation to detection in environmental samples where high numbers of cross-reacting organisms may be present. Both *Neoparamoeba* species are known to be widespread free-living organisms found in similar marine environments and samples may be cross-contaminated (Cann and Page, 1982). 18S rDNA sequence analyses have enabled us to develop a PCR detection method with high specificity for *N. pemaquidensis* based on the lack of cross-reactivity when tested against a selection of non-target species.

The 18S rDNA gene usually contains unique sequence segments (also known as expansion segments) that are highly variable between phylogenetically divergent or non-related organisms (Stothard *et al*., 1998; Sims *et al*., 1999). These sequence segments are situated in non-conserved regions of the gene and are often responsible for the variations in nucleotide length observed between 18S rDNAs of different organisms. Design of the eight presumptive specific PCR primers was based on annealing sites located within the 18S rDNA expansion segments of *N. pemaquidensis*. However, sequence analysis of the near complete *N. aestuarina* 18S rDNA revealed that it shared all equivalent nucleotide expansion segments identified in the *N. pemaquidensis* gene. These segments were absent or highly different in the 18S rDNA of all other taxa examined. Consequently, seven of the primers had annealing sites shared by both *Neoparamoeba* species. These PCR primers may be genus specific for *Neoparamoeba* as they did not cross-react with other taxa used for PCR testing. One of our PCR primer set comprising a species specific forward primer and a genus specific reverse primer, specifically PCR amplified *N. pemaquidensis* DNA without co-amplification of *N. aestuarina*. None of the genus or species specific PCR primers cross-reacted with other taxonomically related members of the Gymnamoebia. The PCR test was able to distinguish the non-parasomal amoeba species found colonising gills of AGD-infected Atlantic salmon in Tasmania, subsequently identified by 18S rDNA sequence as *Paraflabellula hoguae*. This species is a component of a complex fauna known to colonise the gills of AGD-

infected fish including *Acanthamoeba, Platyamoeba, Linguloamoeba, Vanella, Flabellula, Heteroamoeba* and *Vexillifera* (Howard and Carson, 1992).

A nested two-step PCR for the detection of *N. pemaquidensis* 18S rDNA was developed in this study. The PCR test relies on a semi-specific primary amplification step using a *Neoparamoeba* genus specific primer coupled with an 18S rDNA universal primer. This is followed by secondary amplification using an internally nested *N. pemaquidensis* specific primer set. A semi-specific primary PCR primer set was chosen instead of a totally universal 18S rDNA primer pair to minimize possible masking of target DNA by an excessive accumulation of non-target amplification products expected from certain environmental samples, in particular biofouling and macro-organism samples. Instead, the primary amplification step was designed to maximize the concentration of target DNA template for the secondary PCR step. Second round PCR using the species specific internal primers essentially serves to confirm that *Neoparamoeba* 18S rDNA was amplified in the first round, as well as increasing the sensitivity of detection for the target species by the cumulative PCR cycling. Specificity of the *N. pemaquidensis* primer set in the nested two-step PCR was shown to be retained when tested against a panel of DNA from target and non-target organisms.

8.5 Nested Two-Step PCR of Environmental Samples

The nested *N. pemaquidensis* PCR assay had a detection threshold of approximately 40 paramoeba cells in a filter-sterilized seawater sample that was subjected to DNA extraction and PCR. Determination of PCR sensitivity in laboratory samples only provides an estimate of the detection limit in actual seawater samples, since the presence of possible inhibitors of both DNA extraction and PCR amplification steps are minimized by filtration. The actual PCR detection limit for *N. pemaquidensis* genomic DNA in suspension could not be determined in this study since purified paramoeba cells were not obtained, although single step PCR was able to detect *N. pemaquidensis* DNA from approximately 10 pg of cellular DNA prepared from a monoxenic culture. However, this concentration is not really indicative of the amount of paramoeba DNA present in the sample as the majority of extracted DNA probably originated from the cultured bacterium.

Important factors that affect the sensitivity of PCR detection in environmental samples are the efficiency of DNA extraction from often low numbers of the target organism in a complex sample matrix, and the possible presence of PCR inhibitory substances in the DNA preparation. DNA prepared using two different methods of extraction, including the use of a commercial DNA extraction kit, produced different PCR results with the same seeded seawater samples. Enzymatic cellular lysis followed by phenol/chloroform extraction produced the most consistent results in this study. However, this method is both time consuming and requires the use of hazardous solvents. Choice of the most appropriate DNA extraction strategy would depend on the environmental sample and inherent matrix type examined.

Nested PCR was used to identify *N. pemaquidensis* in seawater, biofouling and sediment samples collected from Atlantic salmon farms in Australia. Crude and culture enriched samples of seawater and biofouling were tested, with enriched

samples producing a greater proportion of PCR positives. This was expected since selective culture enrichment of the samples serves to increase the numbers of detectable *N. pemaquidensis* cells. The process of culture enrichment and processing of cultures for PCR also minimizes the presence and potential impact of inhibitors that may have been present in the original sample. An added advantage of PCR testing culture enriched samples is that positive detection is more likely to reflect the presence of viable *N. pemaquidensis* cells.

N. pemaquidensis was detected in seawater and biofouling samples from Tasmanian salmon farms using PCR in this study and non-DNA methods in other studies (Tan, 2000), which suggests that the AGD parasite may occur as a free-living organism ubiquitous to the surrounding environment. Whether the organism is also present in the wider coastal environment remote from fish farming activities or is restricted to the immediate environment surrounding the sea-cages is unknown, and subject to further ecological study. PCR positive cage biofouling fractions included a common bryozoan species and scrapings of net biofilm. The association of *N. pemaquidensis* with cage biofouling was not restricted to particular fouling fractions and positive samples have included a mix of macro and microfouling species (Tan, 2000).

In general, only a small proportion of the seawater and biofouling samples tested were found to be PCR positive. This may reflect an actual sparse distribution of *N. pemaquidensis* in these environmental samples. However, samples obtained for PCR testing were miscellaneous samples collected primarily for other studies and as such, were not optimized for DNA-based analysis. Filter concentration of larger sample volumes of seawater may have improved the efficiency of PCR detection in crude samples. A more targeted approach to biofouling sampling concentrating on larger number of samples of fewer sub-fractions would also be more useful. Samples tested in the present study were mainly used for optimization of PCR detection protocols for a range of environmental samples relevant to salmon farms affected by AGD.

The requirement of *N. pemaquidensis* cells to be attached to substratum for population growth (Martin, 1985) suggests that cells may be present in marine sediment, at least at the surface layer. However, none of 30 sediment samples analyzed showed any evidence of *N. pemaquidensis* when tested by species specific nested PCR. This result was not entirely unexpected with the samples obtained from the North West Bay site as the samples consisted of sediment cores. Although sub-samples were taken from the ends of the cored sample, it is unknown whether the samples tested represented near-surface sediments. Sub-surface sediments are likely to be anaerobic and unsuitable for accumulation of paramoeba cells. Even surface or near-surface sediments situated immediately below operating sea-cages would represent relatively anaerobic and possibly unsuitable conditions. Sea-cage surfaces and associated biofouling are likely to provide more favourable substrates for *N. pemaquidensis*.

DNA extracts obtained from various sediments are usually unsuitable templates for PCR amplification due to the presence of PCR inhibitors such as humic acids and phenolics (Porteous and Armstrong, 1993). This was found to be the case with crude DNA extracts prepared from marine sediments tested in this study. However, simple gel-purification of the crude extracts effectively separated contaminating inhibitors

from the DNA and allowed positive PCR amplification of *N. pemaquidensis* DNA seeded samples.

8.6 Nested Two-Step PCR of Fish Gills

The nested PCR assay was generally unsuccessful in detecting *N. pemaquidensis* in fish gill samples (intact filaments and mucus scrapings) examined in this study. However, it should be noted that none of the gill samples tested by PCR were confirmed to be infected by the AGD parasite, despite the on-site identification of AGD-like mucoid gill patches in many of the sampled fish by farm management. This was highlighted by the North West Bay samples which were also tested by the non-DNA based IFAT smear test and by immunoblot. Despite the identification of mucoid patches on all of the sampled fish, only 27% (4 of 15) returned positive IFAT results of which only one was a definite positive. PCR analysis returned 13% positive samples. In contrast, immunoblotting detected a higher proportion (60%) of positive samples. There may be several possible reasons for the discrepancy between PCR and immunoblot detection. For instance, either the DNA extraction or the PCR step may have been inhibited by excessive mucus or blood in the gill samples. Blood is a recognised inhibitor of PCR (Wilson, 1997). Conversely, the immunological methods have less specificity for *N. pemaquidensis* and may be cross-reacting with other amoebae or cellular debris leading to false positives. However, previous testing had found no evidence of cross-reactions of *Paramoeba* sp. antisera with other amoebae associated with gills of AGD infected fish (Howard and Carson, 1993). The higher proportion of blot positives over IFAT positives maybe due to the greater sensitivity of the immunoblot technique. However, the overall low correlation between the presence of mucoid patches and *N. pemaquidensis* positive samples suggests that mucoid patches on these fish were due to aetiology unrelated to AGD. Fish from this farm were reported to be suffering from "clubbed gill" syndrome at the time of sampling. These low correlations between few observations of gill patches of AGD diagnosis have been previously recognised (Clark and Nowak, 1999).

PCR testing of fixed gill tissue from apparently healthy chinook salmon farmed in New Zealand detected no evidence of *N. pemaquidensis*. However, PCR analysis of ethanol preserved culture suspensions originating from AGD infected New Zealand chinook salmon positively identified the isolates as *N. pemaquidensis*. These cultures have also previously been positively identified using IFAT. The PCR results confirm that paramoebae implicated in AGD in three different salmonid species farmed in four different countries, namely the USA (coho salmon), New Zealand (Chinook), Ireland and Australia (Atlantic salmon), belonged to the same species *N. pemaquidensis*.

8.7 Comparison and Co-Validation of PCR with Non-DNA Based Methods

Certain samples that were subjected to *N. pemaquidensis* PCR analysis have also been sub-sampled for testing using currently available non-DNA identification tests. These included seawater and biofouling samples, and the fish gill samples mentioned above. Overall, PCR results obtained from analyses of crude samples correlated poorly with results obtained by immunological methods, IFAT and immunoblot. Immunological

methods usually produced a greater proportion of positive samples. As mentioned above, this apparent discrepancy is probably due to a combination of poor DNA extraction due to low concentrations of *N. pemaquidensis* in the complex sample matrices, PCR inhibition and possible cross-reactivity by the non-DNA methods. In contrast, there was reasonable correlation between the DNA and non-DNA methods when applied to culture enriched samples. This was not unexpected since the inherent properties of the original samples such as the presence of inhibitors, become diluted. Culture enriched samples also produce an increased concentration of the target organism.

PCR analysis of enriched biofilm samples from an antifouling paint trial study correlated well with results obtained from IFAT analysis (Tan, 2000). Essentially, both DNA and non-DNA detection methods showed that cage netting that had been treated with antifouling paints had increased prevalence of *N. pemaquidensis* compared with control untreated netting.

8.8 Direct Validation of Positive PCR by DNA Sequencing

DNA sequencing of the nested PCR product from positive biofilm samples collected in North West Bay produced sequences with high similarity to the Tasmanian *N. pemaquidensis* strain PA 027. However, some base differences were observed suggesting that different strains may exist within the Tasmanian Atlantic salmon farms and surrounding marine environment.

PCR positive New Zealand culture suspensions were also confirmed to be *N. pemaquidensis* by direct sequencing of the nested PCR product. The New Zealand sequences showed a similar level of sequence variation from Tasmanian strain PA 027 as strains originating from the USA and UK.

9. BENEFITS

The sea-cage farmers of Atlantic salmon are the ultimate beneficiaries of the species identification of the paramoeba associated with AGD, the DNA-based identification protocol for *N. pemaquidensis*, and optimised DNA extraction protocols for environmental samples.

Researchers and diagnostic laboratories are direct beneficiaries of the project outputs by having confirmed identification of the organism, and an alternative and more specific identification technique than the current immunoassay techniques available.

The protocols developed will allow a comprehensive and systematic environmental survey to be conducted for the presence of *N. pemaquidensis*. A greater understanding of its life cycle and epidemiology of AGD will lead to improved management strategies, and so reduce the current financial impact of this disease on the industry. AGD is currently estimated to cost the Tasmanian Atlantic salmon industry 10% of its GVP. Improved management of AGD will have flow-on production benefits by reducing stress levels in the salmon.

The optimisation of DNA extraction protocols for environmental samples provides generic technologies for a range of industries. The technology may be used for the detection of pathogens and other organisms in marine samples using existing PCR primers or developing new DNA probes as required.

10. FURTHER DEVELOPMENT

Environmental surveys
Despite numerous past and present projects investigating various aspects of AGD, knowledge about the assumed causative agent, *Neoparamoeba pemaquidensis*, within the local environment remains limited. A better understanding of its interactions within the environment and the epidemiology of AGD are crucial to the further development of management strategies for AGD.

The outputs of this project (species-specific PCR test and DNA extraction protocols) should be put to use for the benefit of the industry by undertaking a systematic environmental survey to investigate the distribution of *N. pemaquidensis* in association with salmon farm and non-farm (control) sites.

To date, several projects have involved short-term sampling of seawater, fish, net biofouling organisms and biofilms to detect for non-salmon sources of paramoeba in the farm environment. However, due to the sporadic nature of these studies, little information has been gained on the possible effects of changing environmental parameters on the distribution of paramoeba at and around farm sites.

Previous studies were also limited by the quality of the data which was based on detection methods available (eg. laboratory culture of paramoeba cells and IFAT), which were primarily developed for the diagnosis of AGD infected fish. The PCR technique developed provides a more specific detection method of AGD-associated paramoeba cells from environmental samples as it relies on the unique DNA signature of the target organism.

The life cycle of AGD-paramoeba is unknown and certain stages of the organism may not be able to be cultured or be detected by immunological methods. However, DNA remains constant regardless of life cycle stage and remains detectable by PCR amplification.

Enhancing PCR procedure
An issue concerning DNA-based detection methodologies is the interpretation of the results. PCR based methods are potentially highly sensitive and generally detect DNA, which is not necessarily an indication of intact viable (living) cells. A positive PCR signal does not always mean that viable cells are present in a sample. There is a risk that a positive PCR may be the result of amplification of detrital DNA (cell-free DNA in the environment) or DNA from non-viable or degraded paramoeba cells.

Samples taken from farm sites may contain high levels of dead paramoeba cells and cellular debris. These may be sloughed off into the surrounding environment during normal farm operations such as freshwater bathing, or during heavy weather conditions. Samples (e.g. seawater) obtained following these events and analysed by PCR may give “false” positive signals, which do not reflect the occurrence of viable paramoeba cells in the environment.

The sensitivity and specificity of the PCR procedure can be improved with the addition of an enrichment culture step to the sampling protocol; as undertaken with some environmental samples in Chapter 7. This additional step after sample collection would ensure only viable cells in a sample are detected as only live cells grown from these would be obtained from the enrichment plates.

Alternatively, or in conjunction with enrichment, the modification of the PCR procedure to a reverse transcription (RT)-PCR technique would allow identification of viable over non-viable paramoeba cells. The RT-PCR method would target the RNA rather than the rDNA.

RNA is a single strand of nucleic acids generated (in a process known as transcription) from respective complementary double stranded DNA templates. A RNA sequence in effect reflects the sequence of its complementary DNA. Because RNA is necessary for protein synthesis, transcription and so presence of RNA is an indication of actively growing cells. In addition, single stranded RNA is extremely susceptible to degradation by enzymes, and does not remain intact for long outside of living cells. Hence, the source of RNA detected in a sample would originate from intact viable cells.

A major proportion of total RNA extracted is composed of structural rRNA, such as that for the small subunit 18S gene used for the PCR technique developed in the current project. A RT-PCR technique could therefore be developed from the current protocol.

Value-adding to output

The diagnostic assay procedure developed in this project provides the most species-specific test available for identifying *N. pemaquidensis* in the environment. The value of the procedure could be enhanced to assist other areas of research by the development of fluorescently labelled nucleic acid (DNA/RNA) hybridisation probes for the specific *in-situ* detection (FISH) of whole paramoeba cells.

Host-pathogen interaction studies of *N. pemaquidensis* would benefit from the availability of an *in-situ* DNA hybridisation technique. The existing paramoeba ribosomal DNA (rDNA) PCR primers can be modified into rRNA probes. The host-paramoeba interaction studies are important for understanding some of the mechanisms of AGD infections and aid in the identification of possible virulence or attachment factors. Such studies may provide useful information for a more targeted approach to alternative treatments and/or vaccine development.

Labelled nucleic acid probes have advantages over immunological (labelled antibody) probes due to their smaller molecular size and greater permeability into tissues during hybridisation. This allows labelling of the target cells with minimal disruption of any inherent pathogen and host cell associations.

Labelled nucleic acid probes would also allow direct detection of whole paramoeba cells in certain environmental samples, such as filter-concentrated seawater and biological matrices (eg. cage biofilms).

11. PLANNED OUTCOME

The planned outcome of this project was for industry to have a greater ability to implement management strategies to limit the impact of AGD on salmon production. The major outputs of this project provide significant knowledge and analytical tools towards this outcome. Firstly, they provide a greater knowledge of the organism responsible for the disease by means of categorical identification of the species responsible. Secondly, they provide a very specific tool for identifying the organism in environmental samples, and thirdly they provide optimised DNA extraction protocols for examining complex environmental samples.

12. CONCLUSIONS

Objective 1. *Obtain a library of paramoeba isolates and a range of amoebae from infected Atlantic salmon and the environment, sequence regions of the small-subunit ribosomal DNA to identify DNA sequences specific to* Paramoeba *species.*

- Paramoeba and other amoebae isolates from locally infected Atlantic salmon were obtained. In addition isolates of known Paramoeba species were obtained from two international culture collections.

- DNA sequence data for the small-subunit ribosomal 18S gene was obtained for all isolates except the *P. eilhardii* reference culture.

- From the DNA sequence data, the assumed causative agent of amoebic gill disease on Tasmanian Atlantic salmon was positively identified as *Neoparamoeba pemaquidensis* Page 1970 (Sarcomastigophora: Paramoebidae).

- *N. pemaquidensis* is a widely distributed marine species existing both as a free-living marine organism and associated with AGD in various teleosts. Isolates from Australia, Ireland, Wales, and the USA shared 98 to 99% sequence similarity over 2104 base pairs of the 18S rDNA gene.

- Species specific DNA sequences within the 18S rDNA gene were identified for *N. pemaquidensis*.

Objective 2. *Using DNA sequences specific to* Paramoeba *species, develop and test a diagnostic assay procedure based on PCR (polymerase chain reaction) technology.*

- A DNA-based method, using PCR amplification was developed for identifying *N. pemaquidensis* in environmental samples.

- DNA extraction protocols for complex environmental samples were partly optimised, tested and co-validated with a non-DNA based test. Further optimisation may improve the sensitivity of the PCR technique, e.g. analysis of enrichment cultures, or the use of various reagents (e.g. BSA, Chelex resin) in the PCR reaction to minimise the effects of inhibitors.

- Contamination and slow growth of key reference cultures delayed progress in the development and validation of the species-specific PCR test.

Objective 3. *Using the diagnostic assay developed, identify the major sources and reservoirs of* Paramoeba *in the environment, in and adjacent to fish farms.*

- Seawater and biological samples associated with Atlantic salmon farms have been tested for the presence of for *N. pemaquidensis*.

- The presence of paramoeba has been confirmed in seawater collected from the vicinity of sea-cages, and in biological material collected from cage netting.

- Only a small number of environmental samples tested were found to be PCR positive. This may reflect an actual sparse distribution of *N. pemaquidensis* in the environment. However, samples examined were miscellaneous collections primarily for other studies and so were not optimised for either DNA-based analysis or systematic surveying of the environment.

- The identification of major sources and reservoirs of *N. pemaquidensis* requires a more detailed (sample sizes and spatial coverage) and extensive (spatial and temporal coverage) sampling program than could be accomplished in the time allocated within this project.

In summary, this project has:

1. For the first time unequivocally speciated the pathogen *Neoparamoeba pemaquidensis* Page 1970 (Sarcomastigophora: Paramoebidae) as the causative agent for AGD in Tasmanian Atlantic salmon.

2. Developed, optimised and tested a PCR-based species-specific test for *N. pemaquidensis* associated with AGD.

3. Optimised DNA extraction protocols for complex environmental samples.

4. Detected and co-validated by non-DNA based identification, the presence of *N. pemaquidensis* in environmental samples associated with sea-cage farming of Atlantic salmon.

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Toni Wagner, Fish Health Unit, Tasmanian Department of Primary Industries, Water and Environment
Dina Zilberg, School of Aquaculture, University of Tasmania

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APPENDIX 1: Intellectual Property

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

1. DNA sequences for the 18S rDNA gene of amoeba species associated with the gills of Atlantics salmon (sequences deposited in GenBank DNA sequence database under accession numbers AF371967 to AF371973).

2. *Neoparamoeba pemaquidensis* specific PCR amplification primers.

3. Optimisation of DNA extraction protocols from complex environmental samples.

4. Copyright in this report

APPENDIX 2: Staff

Staff engaged on the project:

Principal Investigator

Dr Nicholas Elliott	CSIRO Marine Research

Co-Principal Investigator

Dr Jeremy Carson	Fish Health Unit, Tasmanian Department of Primary Industries, Water and Environment

Research Assistant

Mr Frank Wong	CSIRO Marine Research

APPENDIX 3: Formulae of buffers and reagents

All solutions were prepared in distilled water.

Gel loading buffer (Ausubel *et al*., 1998):
- 2% (w/v) Ficoll 400
- 10 mM EDTA, pH 8.0
- 0.1% (w/v) SDS
- 0.025% (w/v) bromphenol blue

Extraction buffer (Ogram *et al*.,1995):

0.2 M	sodium phosphate
0.1 M	EDTA, pH 8.0

Lysis buffer (Ausubel *et al*., 1998):

10 mM	Tris.Cl, pH 8.0
1 mM	ethylenediamine tetraacetic acid (EDTA), pH 8.0
0.5% (w/v)	sodium dodecyl sulphate (SDS)
0.1 mg/mL	proteinase K

Phosphate buffered saline (PBS; Ausubel *et al*., 1998):

137 mM	NaCl
2.7 mM	KCl
4.3 mM	$Na_2HPO_4.7H_2O$
1.4 mM	KH_2PO_4
(pH 7.4)	

TAE electrophoresis buffer (1X; Sambrook *et al*., 1989):
- 40 mM Tris base
- 40 mM glacial acetic acid
- 1 mM EDTA, pH 8.0

TBE electrophoresis buffer (10X stock; Ausubel *et al*., 1998):
- 890 mM Tries base
- 890 mM boric acid
- 20 mM EDTA, pH 8.0

(diluted to 1X working concentration as required).

APPENDIX 4: Presentations made in relation to project

International conference presentations (first author presenting)

Wong, F., Elliott, N. and Carson, J. 2000. Application of SSU rRNA sequence analysis to the identification and detection of the agent of amoebic gill disease in sea-caged Atlantic salmon (*Salmo salar*). Genetics in Aquaculture VII (International Association for Genetics in Aquaculture Symposium), Townsville, Australia 15-22 July 2000

Nowak, B., Carson, J., Wong, F. and Elliott, N. 2000. Amoebae: fish parasitologists challenge. VIII European Multicolloquium of Parasitology, Poznan Poland 10-14 September 2000

Wong, F.Y., Carson, J. and Elliott, N. G. 2000. Molecular identification of paramoeba implicated in amoebic gill disease of sea-caged Atlantic salmon (*Salmo salar*) in Tasmania, Australia. Joint Meeting of the New Zealand Society for Parasitology and Australian Society for Parasitology. Wellington, NZ. September 2000.

Elliott, N. G., Wong, F., Powell, M.D., Nowak, B., Carson, J. 2001. Amoebic gill disease – What, where and why. The Cultivation of Salmon II, Bergen, Norway. May 2001.

In addition, presentations were made at the annual AGD workshops.

Publication of the research in the peer-reviewed international scientific literature is in progress at the time of publication.