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Characterization of *Neoparamoeba pemaquidensis* strains: PCR-RFLP of the internal transcribed spacer region from the amoeba and endosymbiont

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ABSTRACT: *Neoparamoeba pemaquidensis* continues to be an ongoing problem for commercial finfish aquaculture and has also sporadically been associated with mass mortalities of commercially relevant marine invertebrates. Despite the ubiquity and importance of this amphizoic amoeba, our understanding of the biology as it applies to host range, pathogenicity, tissue tropism, and geographic distribution is severely lacking. This may stem from the inability of current diagnostic tests based on morphology, immunology, and molecular biology to differentiate strains at the subspecies level. In the present study, we developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method based on the internal transcribed spacer (ITS) region that can accurately differentiate amoeba strains of *N. pemaquidensis*. The investigation focused on the complications of the amoeba ITS microheterogeneity in the development of a subspecies marker and the use of the endosymbiont, *Ichthyobodo necator* related organism (IRO), ITS region as an alternative marker. The combination of host amoeba and endosymbiont ITS PCR-RFLP analyses was successfully used to correctly identify and characterize an *N. pemaquidensis* isolate from an outbreak of amoebic gill disease in Atlantic salmon *Salmo salar* from the west coast of North America (Washington State, USA).

KEY WORDS: Internal transcribed spacer · Strain marker · PCR-RFLP · Microheterogeneity · *Ichthyobodo necator* related organism · Parasome

INTRODUCTION

Neoparamoeba pemaquidensis Page, 1987 is a ubiquitous marine amoeba present in free-living and parasitic forms. As a pathogen, *N. pemaquidensis* is recognized worldwide as the causative agent of amoebic gill disease (AGD) in sea-farmed salmonids (Munday et al. 2001) and non-salmonid fish hosts (Dyková et al. 1995, 1998, 1999, 2000, Fiala & Dyková 2003). Disease outbreaks in marine invertebrates have also been attributed to *N. pemaquidensis* in both American lobster *Homarus americanus* (Mullen et al. 2004, 2005) and green sea urchins *Strongylocentrotus droebachiensis* (as *Paramoeba invadens* Jones, 1985; Mullen et al. 2005).

The genus *Neoparamoeba* is identified, in part, by the presence of one or more membrane-bound inclusions ('paranuclear organelle' or 'parasome') near the nucleus of the amoeba. Recent molecular evidence places members of the genus *Neoparamoeba* in a tight sublineage within the Gymnamoebia (Fiala & Dyková 2003, Peglar et al. 2003). Molecular phylogenetic analysis has also revealed that the parasome is an endosymbiont closely related to the prokinetoplastid *Ichthyobodo necator* (Dyková et al. 2003, Moreira et al. 2004). This molecular association forced reconsideration of the

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proper name of this endosymbiont, and the more appropriate name of *Ichthyobodo necator* related organism (IRO) has been proposed (Caraguel et al. in press).

Several diagnostic methods have been developed for the identification of *Neoparamoeba pemaquidensis*. Isolation in culture of amoebae and histopathological identification represent primary techniques based on morphological features of the amoeba; however, both methods are inconsistent and lack specificity (Munday et al. 1993, Dyková & Novoa 2001, Dyková et al. 2005). More specific and sensitive immunological methods incorporating polyclonal antibodies have been designed for screening biological material, including an indirect fluorescent antibody test (IFAT; Howard & Carson 1993) and an immuno-dot blot test (Douglas-Helders et al. 2001). All current immunological techniques, however, have limitations in the specific identification of *N. pemaquidensis*, with reported cross-reactivity of the polyclonal antisera with *N. aestuarina* and *Pseudoparamoeba pagei* (Douglas-Helders et al. 2001).

Because of the difficulties in characterizing *Neoparamoeba* species, there has been increased use of molecular tools for identification and phylogenetic studies. The 18S ribosomal RNA (rRNA) gene was appropriately the first genetic marker used for molecular characterization of the genus *Neoparamoeba* (Elliot et al. 2001, Fiala & Dyková 2003, Peglar et al. 2003, Wong et al. 2004, Dyková et al. 2005, Mullen et al. 2005). As a useful specific marker for *Neoparamoeba* species (Dyková et al. 2005), the 18S rRNA sequences have high levels of similarity (98.1 to 99%) among sequences from different isolates of *N. pemaquidensis* (Wong et al. 2004). Two nested PCR methods for the detection of *N. pemaquidensis* based on the 18S rRNA gene have been developed to detect and identify the pathogen. The first is specific for *N. pemaquidensis* and is relatively efficient when used after culture-enrichment of environmental and host-derived samples (Elliot et al. 2001, Wong et al. 2004). The second nested PCR generates a 165 base pair product from Paramoebidae/Vexilliferidae amoebae but does not amplify templates from *Pseudoparamoeba pagei* or *Korotnevella hemistylolepis* and more importantly does not amplify templates from *N. pemaquidensis* strain ATCC 50172 (Mullen et al. 2005). These PCR protocols clearly illustrate useful features as well as some of the current limitations of molecular characterizations. As with all initial studies, the benefits and applicability will be assessed as more sequence data become available; until then, they should only be used with caution in large-scale diagnostic applications. Moreover, both methods are unable to discriminate isolates at the subspecies level due to the low degree of intraspecific variability of the 18S rRNA marker.

The ability to further characterize pathogens at a subspecies or strain level using genetic markers with sufficient variability has revealed previously unrecognized differences in parasite biology and has provided a clearer picture of host-pathogen interactions. In both *Giardia duodenalis* and *Cryptosporidium parvum*, genotyping and subtyping of isolates from different hosts have led to the discovery of new species, as well as the distinction of both host-adapted and cosmopolitan species (Caccio et al. 2005). Thus, the characterization of *Neoparamoeba pemaquidensis* at the subspecies level may provide new information and also allow us to reconsider our current understanding of the geographic distribution, host range, and tissue tropism of this amphizoic amoeba. Firstly, *N. pemaquidensis* has been isolated from a variety of marine coastal environments from around the world (Fiala & Dyková 2003) as well as from a diverse assemblage of marine organisms (Cann & Page 1982). However, it is unknown whether *N. pemaquidensis* detected in tissue or recovered from finfish have the potential to infect invertebrates in geographically nearby locales. Secondly, *N. pemaquidensis* have been characterized as 'pathogenic' when isolated from infected fish (e.g. ATCC 50172) and as 'environmental' when found in the marine ecosystem (e.g. CCAP 1560/5). Although pathogenic strains can lose virulence during *in vitro* culture (Morrison et al. 2005), it is currently unknown whether environmental strains can become pathogenic. Thirdly, *N. pemaquidensis* is an external parasite during finfish gill infections (Adams et al. 2004) but an internal parasite in lobsters and sea urchins (Mullen et al. 2004, 2005). Therefore, it has yet to be determined if particular strains of *N. pemaquidensis* have specific host or tissue tropisms. Clearly, subspecies identification of *N. pemaquidensis* by using a broadly applicable molecular marker could have direct applications in disease monitoring, surveillance, and epidemiological studies during outbreaks.

The internal transcribed spacer (ITS) region, located between the 18S and 28S rRNA genes, was targeted to explore the level of intra-specific and intra-genomic variability (Brown et al. 2004, Ruggiero & Procaccini 2004, Beszteri et al. 2005). The *Neoparamoeba pemaquidensis* ITS region showed both qualitative and quantitative inter-strain variability that was mainly localized to the more variable regions of the ITS 1 and ITS 2 (Caraguel et al. in press). A detailed investigation of intra-strain variability revealed the existence of very high levels of microheterogeneity in these ITS regions. The existence of this intragenomic variability potentially precluded the use of the *N. pemaquidensis* ITS region as a good genetic marker. Phylogenetic analysis of the *N. pemaquidensis* ITS region revealed that microheterogeneity did not

obscure the monophyletic nature of the sequences derived from individual isolates, which led to the further consideration of this region as a subspecies marker. Analysis of the endosymbiont (IRO) ITS region revealed both qualitative and quantitative inter-strain variability among the IRO isolates and showed low to non-existent levels of microheterogeneity that likewise did not obscure the phylogenetic monophyly of the IRO-ITS sequence data from individual isolates (Caraguel et al. in press). Since the IRO is intimately associated and coevolves with the amoeba host, the endosymbiont ITS region may provide an alternative target for *N. pemaquidensis* diagnostic test development (Caraguel et al. in press).

The purpose of the present study was to develop a molecular method based on restriction fragment length polymorphism (RFLP) of the *Neoparamoeba pemaquidensis* ITS region that would accurately identify amoeba strains. The investigation focused on the complications of the amoeba ITS microheterogeneity in the development of a subspecies marker and the use of the IRO ITS region as a complementary or alternative marker. The amoeba and IRO PCR-RFLP analyses were used to assess an outbreak of AGD in Atlantic salmon *Salmo salar* from the west coast of North America (Washington, USA).

MATERIALS AND METHODS

Amoeba isolates. Initially, all 6 isolates of *Neoparamoeba pemaquidensis* that were available from public culture collections at the American Type Culture Collection (ATCC), and the Culture Collection of Algae and Protozoa (CCAP) were acquired and analyzed. From these 6 isolates, 4 strains were defined within *N. pemaquidensis* by Caraguel et al. (in press) based on ITS sequence similarity (Table 1). The CCAP 1560/4 isolate, representing the strain CCAP, was grown in MY75S agar medium at room temperature (19 to 22°C). The isolate ATCC 30735, representing the strain ATCC 30735, and the isolate ATCC 50172, representing the strain ATCC 50172, were cultured in ATCC medium 994 agar medium at room temperature (19 to 22°C) bacterized with *Klebsiella pneumoniae*. Urchin amoeba UA6, representing the strain UA, was cultivated at 15°C in L1 agar medium and fed with *Enterobacter aerogenes. N. aestuarina* Page, 1987 was cultured in liquid ATCC medium 994 at room temperature (19 to 22°C).

Amoebae were isolated from an AGD outbreak in Atlantic salmon that occurred in late fall 2004 (Washington, USA). Several protists were extracted from gills by the method of Zilberg et al. (2001). Two morphologically distinct amoebae were isolated, and

Table 1. *Neoparamoeba* spp. reference isolates and respective endosymbiont *Ichthyobodo necator* related organisms (IRO). AGD: amoebic gill disease; CCAP: Culture Collection of Algae and Protozoa; UA: urchin amoeba; ATCC: American Type Culture Collection. Strains are according to the *Neoparamoeba pemaquidensis* strain definitions by Caraguel et al. (in press). Acc. no.: GenBank accession numbers. n.a.: not applicable

Reference isolate	Identification	Strain	Origin	Location	Acc. no.
CCAP 1560/4	*Neoparamoeba pemaquidensis*	CCAP	Environmental	Gwynedd, UK	DQ167506-13
UA 6	*Neoparamoeba pemaquidensis*	UA	*Strongylocentrotus droebachiensis*	Maine, USA	DQ167530-37
ATCC 30735	*Neoparamoeba pemaquidensis*	ATCC 30735	Environmental	Virginia, USA	DQ167538-45
ATCC 50172	*Neoparamoeba pemaquidensis*	ATCC 50172	*Oncorhynchus kisutch* (AGD)	Washington, USA	DQ167546-53
ATCC 50806	*Neoparamoeba aestuarina*	n.a.	Environmental	Unknown	DQ167554
IRO-CCAP 1560/4	*Ichthyobodo necator* related organism	IRO-CCAP	*N. pemaquidensis* (CCAP 1560/4)	Gwynedd, UK	DQ167481-84
IRO-UA 6	*Ichthyobodo necator* related organism	IRO-UA	*N. pemaquidensis* (UA 6)	Maine, USA	DQ167493-96
IRO-ATCC 30735	*Ichthyobodo necator* related organism	IRO-ATCC 30735	*N. pemaquidensis* (ATCC 30735)	Virginia, USA	DQ167497-500
IRO-ATCC 50172	*Ichthyobodo necator* related organism	IRO-ATCC 50172	*N. pemaquidensis* (ATCC 50172)	Washington, USA	DQ167501-504
IRO-ATCC 50806	*Ichthyobodo necator* related organism	n.a.	*N. aestuarina* (ATCC 50806)	Unknown	DQ167505

clonal cultures were established in solid ATCC medium 994 bacterized with *Klebsiella pneumoniae*. The first isolate, AVCLSC-001, was flattened and irregularly fan-shaped, and an IRO could be easily observed with light microscopy. The second amoeba, AVCLSC-002, was also flattened and irregular-shaped; however, unlike AVCLSC-001, AVCLSC-002 displayed numerous long and dark uroidal filaments and a denser intracellular compartment, and no IRO was observed.

Genomic DNA extraction. Amoebae were detached from the agar using 2 ml of sterile seawater spread directly on plates; cell suspensions were collected by centrifugation for 5 min at 6500 × *g* at room temperature. DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). DNA concentration was determined spectrophotometrically; quality was assessed by electrophoretic separation in a 0.8% agarose gel containing 0.5 µg ml^{-1} ethidium bromide.

Amplification of ITS regions. The ITS region of *Neoparamoeba pemaquidensis* was amplified using the specific forward *Neoparamoeba* spp. primer fNp-Hx49 (5′-GGGTAGAGCGAGTTTGTTGTG-3′), positioned on the 3′ end of the 18S rDNA gene (reverse complement of the primer rNp-Hx49 of Wong et al. 2004) and the universal reverse primer NLR 204/21 (5′-ATATGCTTAARTTCAGCGGGT-3′), positioned on the 5′ end of the 28S rDNA gene (Van der Auwera et al. 1994). Approximately 10 to 50 ng of genomic DNA were amplified in a 25 µl reaction containing 2.5 pmol of each primer fNp-Hx49 rDNA and NLR 204/21 in the presence of the following reagents contained in a pure TAq Ready-To-Go PCR Bead (Amersham Biosciences): 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9), 50 mM KCl, and 2.5 U of *Taq* DNA polymerase. The amplification protocol was carried out in an MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories) under the following conditions: an initial denaturation at 94°C for 2.5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min. Final extension was at 72°C for 10 min.

The ITS region of IROs was amplified using the specific ITS forward primer IRO-F-ITS (5′-GCGCACTA CAATGACAAAGTG-3′) positioned on the 3′ end of the 18S rDNA gene, and the universal eukaryote reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), positioned on the 5′ end of the 28S rDNA gene (Ristaino et al. 1998). Each 25 µl reaction included 50 ng of genomic DNA with the same concentration of reagents as described above. Thermocycling conditions were the same as above. Amplicon size and quality were assessed for both reactions by electrophoretic separation in a 1.0% agarose gel containing 0.5 µg ml^{-1} ethidium bromide.

Sequence analyses and restriction mapping. Restriction mapping was initially pursued by analyzing *Neoparamoeba* and IRO ITS region sequences available in GenBank (accession numbers in Table 1; Caraguel et al. in press). For each isolate, a consensus sequence was constructed and analyzed by restriction mapping using BioEdit software (Hall 1999). The restriction endonuclease *Ase*I was selected to discriminate *Neoparamoeba* ITS region PCR products, and 2 restriction endonucleases, *Ale*I and *Ngo*MIV, were chosen to separate IRO ITS region amplicons. The cleavage patterns of ITS sequences were predicted by *in silico* simulation using 'NEBcutter V2.0' software (http://tools.neb.com/NEBcutter2/index.php).

RFLP. Five µl of the 25 µl *Neoparamoeba* reaction mixture were digested with 5 U of the restriction enzyme *Ase*I (New England Biolabs) as directed by the manufacturer in a final volume of 20 µl at 37°C for either 1 or 3 h. Similarly, 8 µl of the 25 µl IRO reaction mixture were digested with 5 U of *Ngo*MIV and 2 U of *Ale*I (New England Biolabs) as directed by the manufacturer in a final volume of 20 µl at 37°C for either 1 or 3 h. Following incubation, the digestion products were separated by electrophoresis on a 2.0% agarose gel containing 0.5 µg ml^{-1} ethidium bromide.

Species confirmation. The identities of the unknown amoebal isolates were confirmed by partial sequencing of the 18S ribosomal RNA gene. The 18S rRNA gene was amplified using universal eukaryote primers Medlin A (5′-AACCTGGTTGATCCTGCCA GT-3′) and Medlin B (5′-TGATCCTTCTGCAGGT TCACCT-3′) and (Medlin et al. 1988). Approximately 10 to 50 ng of genomic DNA were amplified using PCR beads as previously described, under the following conditions: an initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. Final extension was at 72°C for 10 min. If the ITS sequence was successfully amplified first, then only the ITS region was sequenced. Amplified small subunit (SSU) and ITS products were cloned directly into plasmid pCR 2.1 using the TOPO TA Cloning® Kit (Invitrogen). Plasmids containing inserts were isolated and purified from recombinant *E. coli* using the GenElute™ Plasmid Mini-Prep Kit (Sigma-Aldrich). Plasmid inserts were sequenced on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems) at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, Ontario, Canada). Sequences from each amoeba were identified by a Basic Local Alignment Search Tool (BLAST) sequence similarity search (Altschul et al. 1997).

RESULTS

Neoparamoeba PCR-RFLP

The paired primers fNp-Hx49 rDNA and NLR 204/21 yielded an approximately 850 to 900 bp PCR product from the 4 *Neoparamoeba pemaquidensis* reference isolates CCAP 1560/4, UA 6, ATCC 30735, ATCC 50172 (Fig. 1A). Detection of a similarly sized band that was lighter in staining intensity from the *N. aestuarina* isolate confirmed the expected cross-specificity of the forward primer fNp-Hx49 rDNA previously described by Wong et al. (2004). The outbreak isolate AVCLSC-001 was successfully amplified with an approximately 900 bp band, whereas the outbreak-associated isolate AVCLSC-002 was not (Fig. 1A).

The restriction patterns of the PCR products generated by *Ase*I for the different incubation times are presented in Fig. 1B, and the number and calculated size of the restriction fragments are shown in Table 2. After 1 h of incubation, distinct cleavage patterns were observed from each of the *Neoparamoeba pemaquidensis* reference isolates and from the *N. aestuarina* isolate. By comparison to expected bands (Table 2), residual undigested or partially digested bands for all isolates except ATCC 30735 could be identified. After 3 h of incubation, distinct cleavage patterns were observed from each of the *N. pemaquidensis* reference isolates and from the *N. aestuarina* isolate. Residual undigested or partially digested bands were notable for most of the isolates. The cleavage pattern of the amplified isolate AVCLSC-001 matched the pattern of ATCC 50172, including the undigested band, and the AVCLSC-001 isolate was characterized as an isolate of the *N. pemaquidensis* ATCC 50172 strain.

IRO PCR-RFLP

The paired primers IRO-F-ITS and ITS4 successfully yielded an approximately 750 bp PCR product from each IRO isolate from the 4 *Neoparamoeba pemaquidensis* reference isolates (CCAP 1560/4, UA6, ATCC 30735, ATCC 50172; Fig. 2A). The detection of a similarly sized band for IRO-*N. aestuarina* extended the amplification by the IRO universal forward primer IRO-F-ITS to other members of the genus *Neoparamoeba*. Template from the test sample AVCLSC-001 successfully amplified, yielding an approximately 750 bp band, whereas that of the AVCLSC-002 isolate did not amplify (Fig. 2A).

The restriction patterns of the PCR products generated by *Ale*I and *Ngo*MIV for the different incubation times are presented in Fig. 2B; the number and calculated size of the restriction fragments are shown in

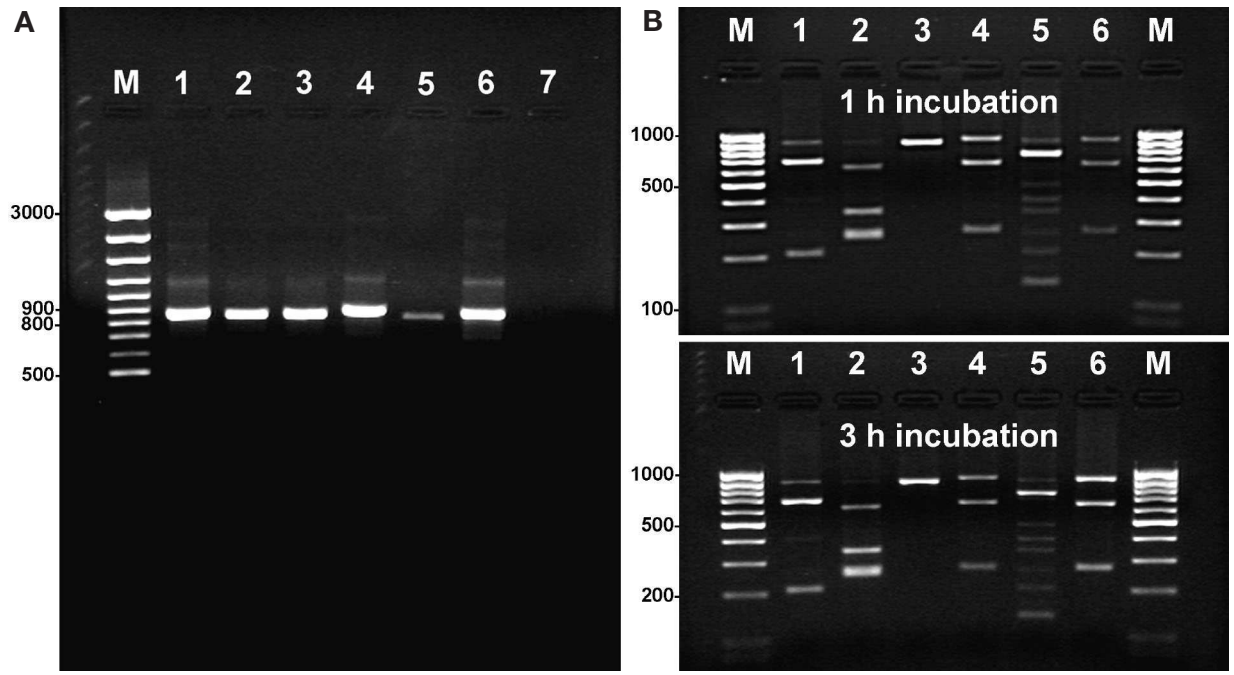

Fig. 1. *Neoparamoeba* spp. Agarose gel electrophoresis of (A) internal transcribed spacer (ITS) amplicons, and (B) restriction pattern of ITS amplicons digested with *Ase*I after 1 and 3 h of incubation. Source of templates for *Neoparamoeba* spp. PCR: Lane 1, CCAP 1560/4; 2, UA 6; 3, ATCC 30735; 4, ATCC 50172; 5, ATCC 50806; 6, AVCLSC-001; 7, AVCLSC-002. Lane M: DNA ladder. Gel features: (A) 1.0% agarose, GeneRuler™ 100 bp Ladder Plus, 80 V, 1 h; (B) 2.0% agarose, GeneRuler™ 100 bp Ladder, 80 V, 50 min

Table 2. After 1 h of incubation, a distinct cleavage pattern was observed from each of the IRO-*Neoparamoeba pemaquidensis* reference isolates and from the IRO-*N. aestuarina* isolate. By comparison with the expected bands (Table 2), residual undigested bands for IRO-ATCC 50172 and IRO-ATCC 50806 isolates were evident, as were 2 partially undigested bands for IRO-CCAP 1560/4. After 3 h of incubation, a distinct cleavage pattern was observed from each of the IRO-*N. pemaquidensis* reference isolates and from the IRO-

Table 2. *Neoparamoeba* spp. and respective endosymbiont *Ichthyobodo necator* related organism (IRO) restriction fragment length polymorphism (RFLP) patterns. Parallel comparison of number and size of restriction fragments obtained after NEBcutter 2.0 simulation (expected), or after 1 and 3 h incubation of PCR products. *Neoparamoeba* spp. amplicons were digested with *Ase*I and IRO amplicons with *Ale*I and *Ngo*MIV. n.a.: not applicable

Isolate	Expected		1 h incubation		3 h incubation	
	No. of fragments	Fragment sizes (bp)	No. of fragments	Approx. fragment sizes (bp)	No. of fragments	Approx. fragment sizes (bp)
CCAP 1560/4	2	665/210	5	870/650/400[a]/250[a]/210	3	870[a]/650/210
UA 6	3	340/265/255	4	860[a]/600[a]/350/250[b]	3	600[a]/350/250[b]
ATCC 30735	1	860	1	860	1	860
ATCC 50172	2	630/270	3	900/650/280	3	900/650/280
ATCC 50806	2	730/130	8	860/680[a]/500[a]/410[a]/ 380[a]/270[a]/210[a]/140	8	860/680[a]/500[a]/410[a]/ 380[a]/270[a]/210[a]/140
AVCLSC-001	Tested	n.a.	3	900/650/280	3	900/650/280
IRO-CCAP 1560/4	3	395/230/100	5	650[a]/500[a]/400/240/120	3	400/240/120
IRO-UA 6	1	740	1	750	1	750
IRO-ATCC 30735	2	400/350	2	400/360	2	400/360
IRO-ATCC 50172	2	390/235	3	725/500/250	3	725[a]/500/250
IRO-ATCC 50806	2	370/365	2	730/370[b]	1	370[b]
IRO-AVCLSC-001	Tested	n.a.	3	725/500/250	3	725[a]/500/250

[a]Light band; [b]double bands

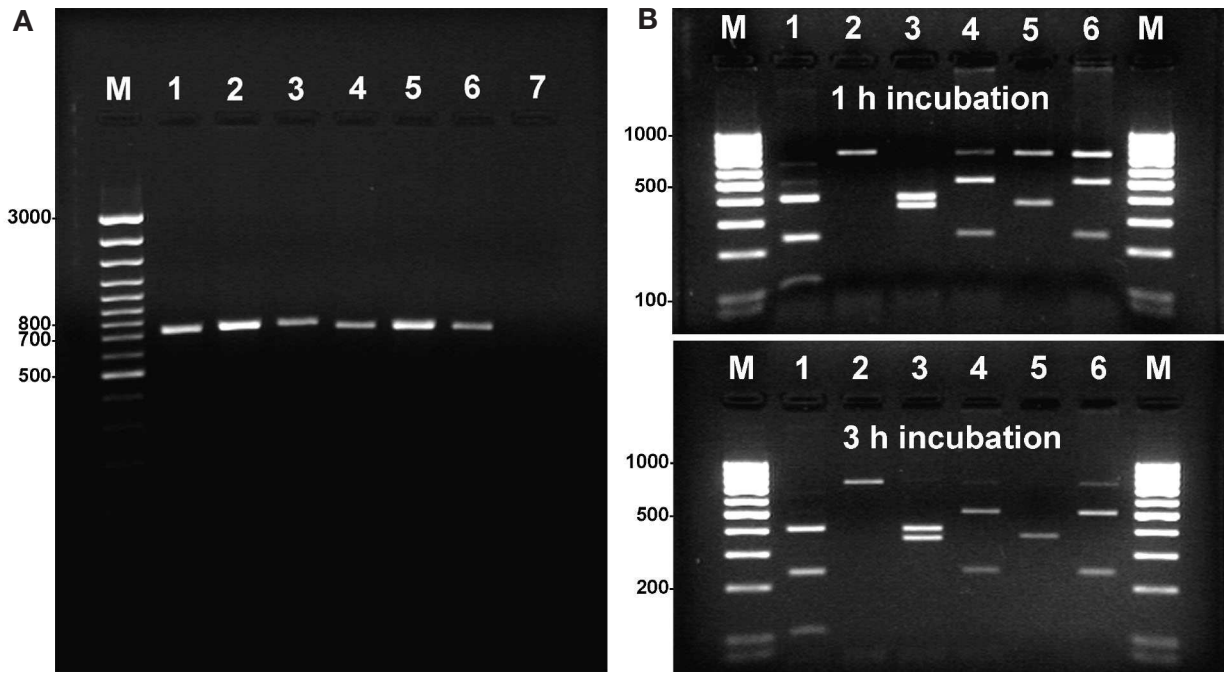

Fig. 2. *Ichthyobodo necator* related organism (IRO). Agarose gel electrophoresis of (A) ITS amplicons, and (B) restriction pattern of amplicons digested with *Ale*I and *Ngo*MIV after 1 and 3 h of incubation. Source of templates for IRO PCR: Lane 1, CCAP 1560/4; 2, UA 6; 3, ATCC 30735; 4, ATCC 50172; 5, ATCC 50806; 6, AVCLSC-001; 7, AVCLSC-002. Lane M: DNA ladder. Gel features: (A) 1.0% agarose, GeneRuler™ 100 bp Ladder Plus, 80 V, 1 h; (B) 2.0% agarose, GeneRuler™ 100 bp Ladder, 80 V, 45 min

N. aestuarina isolate; a single undigested band was identified from the IRO-ATCC 50172 isolate. The cleavage pattern of the episode isolate IRO-AVCLSC 001 was identical to the pattern of IRO-ATCC 50172, including the undigested band.

Species confirmation

After positive ITS region amplification, the AVCLSC-001 isolate was confirmed to be *Neoparamoeba pemaquidensis* and more specifically was most similar to ATCC 50172 using the ITS region sequences. The AVCLSC-001 amoeba ITS region sequence (GenBank accession number DQ660492) had 96 to 97.1% similarity with GenBank sequences for ATCC 50172 (DQ167546 to DQ167553) and only 83 to 89% similarity for all other *N. pemaquidensis* ITS sequences. Similarly, the AVCLSC-001 IRO ITS region sequence (DQ660493) had 99.6 to 99.9% similarity with GenBank sequences for IRO-ATCC 50172 (DQ167501 to DQ167504) and only 92.4 to 97.1% similarity for all other *N. pemaquidensis* IRO ITS sequences. However, the ITS region of the second isolate, AVCLSC-002, was not amplified. Consequently, the 18S rRNA gene was partially sequenced (700 bp of the 5′ end), and the isolate was identified as the leptomyxid amoeba *Paraflabellula hoguae* Sawyer, 1975 (99.4% similarity with GenBank sequences AF293899 and AY277797).

DISCUSSION

Despite the ubiquity of *Neoparamoeba pemaquidensis* and its important role as a pathogen in commercial finfish aquaculture, there are several questions still outstanding regarding our current understanding of the biology of this amphizoic amoeba, from the variation in host range, to the modes of pathogenicity and suspected tissue tropism, to its worldwide geographical distribution. This lack of knowledge may be further compounded because currently available methods used to identify *N. pemaquidensis* (Elliot et al. 2001, Munday et al. 2001, Wong et al. 2004, Mullen et al. 2005) lack the ability to differentiate isolates at the subspecies level. In this study, 2 complementary PCR-RFLP tests, based on the ITS regions from both *N. pemaquidensis* and its endosymbiont IRO, were developed and evaluated to determine their usefulness as methods to discriminate among different isolates.

Initial sequencing of the ITS regions from both the amoeba and endosymbiont showed sufficient inter-strain variability to allow for further consideration in the development of a discriminative tool. However, the *Neoparamoeba pemaquidensis* ITS region contained significant intra-genomic variability (and consequently intra-isolate and intra-strain variability) that was earlier recognized as microheterogeneity (Caraguel et al. in press). This microheterogeneity within the ITS region introduced the dilemma of a potential 'moving target' for the marker (i.e. the possibility that a discriminative restriction enzyme site may be gained or lost in a number of copies within the genome and therefore may obscure the real value of the ITS region as a useful marker). The reduced to absent microheterogeneity within the IRO-ITS and the intimate association of the endosymbiont and its amoeba host makes the IRO-ITS a useful alternative target for discriminating between *N. pemaquidensis* isolates (Caraguel et al. in press).

The ITS region PCR-RFLP was successfully used to separate the 4 distinct strains of *Neoparamoeba pemaquidensis* using either the amoeba or the IRO derived markers. Faint extra bands, however, were present in all sample lanes following restriction enzyme digestions of 1 or 3 h. These extra bands did not interfere with the comparison of the restriction patterns or with the interpretation of the results. Initially, these extra bands were thought to be the result of partial enzyme digestions from unsatisfactory restriction conditions (amplicon amount, endonuclease quantity and quality, buffer, incubation time). However, the survival of residual bands after optimizing the conditions revealed that microheterogeneity could account for appearing or disappearing restriction sites, dependent on the nature of the amplified ITS region copy. Therefore, based on the random proportion of amplified biased copies, microheterogeneity may explain the appearance of some unexpected light bands.

The effectiveness of the ITS region PCR-RFLP for differentiating strains of *Neoparamoeba pemaquidensis* was evaluated using amoebae isolated from Atlantic salmon during an AGD outbreak in Washington in the fall of 2004. DNA from the amoeba isolate AVCLSC-001 was successfully amplified using both *Neoparamoeba* and IRO region ITS primer sets and subsequently confirmed as belonging to the same strain as *N. pemaquidensis* (ATCC 50172). The second amoeba, AVCLSC-002, failed to produce a PCR product with either the *Neoparamoeba* or IRO region ITS primer sets. DNA from AVCLSC-002 was later amplified with 18S rRNA gene primers and subsequently identified by partial sequencing as *Paraflabellula hoguae*. The lack of amplified product from AVCLSC-002 with either ITS primer set supported the specificity of the primers for *Neoparamoeba* spp. and the associated IRO endosymbiont. *Paraflabellula hoguae* has previously been isolated together with *N. pemaquidensis* from diseased fish gills (Elliot et al. 2001, Wong et al. 2004). Given the recent evidence for AGD being associated with a scuticociliate in the absence of

any *Neoparamoeba* spp. in Irish salmonid aquaculture (Bermingham and Mulcahy 2004), it may be interesting to further investigate the potential role of this non-IRO carrying amoeba during AGD.

Interestingly, 20 yr after *Neoparamoeba pemaquidensis* ATCC 50172 (originally deposited as *Paramoeba pemaquidensis* in 1987) was isolated from the infected gills of coho salmon *Oncorhynchus kisutch* reared in seawater net-pens in Puget Sound (Washington, USA; Kent et al. 1988), the same strain is still present and able to cause AGD in sea-cage raised Atlantic salmon. This represents an expansion of the known host range of this isolate to include both coho and Atlantic salmon. During this period, the ITS regions from both the amoeba and the endosymbiont were stable and may therefore represent excellent geographical markers for the *N. pemaquidensis* isolate, and clearly illustrates the critical importance that sub-species markers may have in disease monitoring, surveillance, and epidemiological studies.

To develop a diagnostic tool that can detect all *Neoparamoeba* species, specific primers must be designed and tested for the ITS region. In the present study, the specific forward primer fNp-Hx49 rDNA successfully amplified both *N. pemaquidensis* and also *N. aestuarina* that contained 1 imperfect match in 21 nucleotides (Wong et al. 2004). Unfortunately, this would not be the case for the recently described *N. branchiphila* that has been isolated from salmon gills and is associated with AGD (Dyková et al. 2005). This primer theoretically would not anneal to the *N. branchiphila* 18S rRNA gene, as the same region contains 7 non-matching nucleotides (Dyková et al. 2005). We therefore recommend that as new isolates become readily available in public culture collections they must first be amplified (using either the above or universal primers) and subsequently sequenced to select the most appropriate and inclusive primer sites and the best discriminative restriction endonuclease(s) according to the pool of isolates evaluated.

The discriminative power of using complementary molecular markers from both the host and endosymbiont will only be truly evaluated as the collection of readily available and new isolates of *Neoparamoeba* increases. However, if the level of microheterogeneity found within *Neoparamoeba* cannot be resolved through practical troubleshooting, as shown in the present study, then the IRO ITS should be considered as a useful internal control and an acceptable alternative target. Nevertheless, the presence of microheterogeneity within *Neoparamoeba* spp. could lower the analytical specificity of the PCR. The design of specific primers or probes to detect a particular *N. pemaquidensis* strain must be selected and screened with caution, as they could result in false positive amplicons if the primers anneal non-specifically on regions affected by microheterogeneity (ITS 1 and ITS 2; Caraguel et al. in press).

In the present study, the required quantity of genomic DNA was 5 to 10 times higher for IRO amplification than for the amoeba host. Consequently, we suggest using both markers in series or in parallel, by first using the *Neoparamoeba* ITS region to maximize the analytical sensitivity of the detection and then using the IRO ITS region to maximize the analytical specificity of the characterization. Moreover, in this study, extraction and amplification protocols were established and standardized using pure amoeba cultures. At least 2 factors affect the sensitivity of amoeba detection in crude samples, i.e. the efficiency of DNA extraction from low numbers of amoebae in a complex sample matrix, and the possible presence of PCR inhibitors in the preparation (Elliot et al. 2001, Wong et al. 2004). The use of the methods developed in this study with isolated amoeba cultures will require further refinement and optimization for incorporation into diagnostic studies from amoeba-infected tissues and environmental samples to ensure analytical sensitivity.

The *Neoparamoeba* ITS inter-strain variability in conjunction with the complementary IRO ITS represents very attractive alternative features that could be used to develop more specific *in situ* hybridization detection. Incorporating these subspecies markers into infection experiments may help answer some of the unresolved questions surrounding the biology of *Neoparamoeba*.

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