J. Eukaryot. Microbiol., 54(5), 2007 pp. 418–426

DOI: 10.1111/j.1550-7408.2007.00281.x

Microheterogeneity and Coevolution: An Examination of rDNA Sequence Characteristics in *Neoparamoeba pemaquidensis* and Its Prokinetoplastid Endosymbiont

CHARLES G. B. CARAGUEL,[a,b] CHARLES J. O'KELLY,[c] PIERRE LEGENDRE,[d] SALVATORE FRASCA Jr.,[e] REBECCA J. GAST,[f] BÉATRICE M. DESPRÉS,[a] RICHARD J. CAWTHORN[a,b] and SPENCER J. GREENWOOD[a,b]

[a]*AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, C1A 4P3, Canada, and*

[b]*Department of Pathology & Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, C1A 4P3, Canada, and*

[c]*Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine 04575, USA, and*

[d]*Département de Sciences Biologiques, Université de Montréal, C.P. 6128 succursale Centre-ville, Montréal, QC, H3C 3J7, Canada, and*

[e]*Department of Pathobiology & Veterinary Science, University of Connecticut, Storrs, Connecticut 06269-3089, USA, and*

[f]*Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA*

ABSTRACT. *Neoparamoeba pemaquidensis*, the etiological agent of amoebic gill disease, has shown surprising sequence variability among different copies of the 18S ribosomal RNA gene within an isolate. This intra-genomic microheterogeneity was confirmed and extended to an analysis of the internal transcribed spacer (ITS) region. High levels of intra-genomic nucleotide diversity (Pi = 0.0201–0.0313) were found among sequenced ITS regions from individual host amoeba isolates. In contrast, the ITS region of its endosymbiont revealed significantly lower levels of intra-genomic nucleotide diversity (Pi = 0.0028–0.0056) compared with the host *N. pemaquidensis*. Phylogenetic and ParaFit coevolution analyses involving *N. pemaquidensis* isolates and their respective endosymbionts confirmed a significant coevolutionary relationship between the two protists. The observation of non-shared microheterogeneity and coevolution emphasizes the complexity of the interactions between *N. pemaquidensis* and its obligate endosymbiont.

Key Words. Internal transcribed spacer, intra-genomic variability, parasome, *Perkinsiella amoebae*-like organism, 18S ribosomal RNA gene.

THE amphizoic marine amoeba *Neoparamoeba pemaquidensis* (Page 1970) Page, 1987 is recognized as the etiological agent of amoebic gill disease (AGD) in sea-farmed salmonids (Kent, Sawyer, and Hedrick 1988; Munday et al. 1990, 1993; Roubal, Lester, and Foster 1989), and non-salmonid fish hosts (Dyková, Figueras, and Novoa 1995, 1999; Dyková et al. 1998; Fiala and Dyková 2003). In addition, there is evidence that *N. pemaquidensis* is the agent causing paramoebiasis in American lobster (Mullen et al. 2004, 2005), and wasting disease in green sea urchins (Jones 1985, as *Paramoeba invadens* Jones 1985; Mullen et al. 2005). *Neoparamoeba pemaquidensis* is, in part, identified by the possession of one or several membrane-bound inclusions (''paranuclear organelle'' or ''parasome'') localized near the amoeba's nucleus. Amoebae with parasomes were previously placed in a single genus, *Paramoeba* Schaudinn 1896, although they are dissimilar to each other in locomotive form (Chatton 1953) and in ultrastructure (Cann and Page 1982; Grell and Benwitz 1970; Page 1987; Perkins and Castagna 1971). Consequently, some parasome-containing amoebae were removed from *Paramoeba* and moved to other genera (Chatton 1953, *Janickina*; Page 1987, *Neoparamoeba*). *Neoparamoeba* species belong to a separate lineage of amoebae, recently recognized at the molecular level (Fiala and Dyková 2003; Peglar et al. 2003), but the relationships of the other parasome-containing amoebae to each other and to other Gymnamoebae have not yet been elucidated.

The structure and reproduction of the parasome have been examined many times (Chatton 1953; de Faria, da Cunha, and Pinto 1922; Grell 1961, 1968; Grell and Benwitz 1970; Hollande 1940; Janicki 1912, 1928; Kudo 1966; Minchin 1922; Page 1970; Perkins and Castagna 1971; Schaudinn 1896; Sprague, Beckett, and Sawyer 1969), but the exact origin and biological significance of this structure proved difficult to determine. Hollande (1980) investigated the ultrastructure of the inclusion within *Janickina pigmentifera* (Chatton 1953) and defined the median segment as dispersed DNA and concluded that the inclusion was a eukaryotic organism, a kinetoplastid flagellate endosymbiont that he called *Perkinsiella amoebae*. During a comprehensive re-analysis of the genus *Paramoeba*, Dyková, Figueras, and Peric (2000) renamed the endosymbiont of amoebae from the genera *Paramoeba* and *Neoparamoeba* as a *P. amoebae*-like organism (PLO). However, the genus *Perkinsiella* Kirkaldy 1903 was previously defined and used to designate the genus of three species of sugarcane planthopper from Australia (Kirkaldy 1903). To avoid any nomenclatural confusion, we suggest not using the PLO designation. A name change for the endosymbiont is further indicated by recent phylogenetic studies based on the 18S ribosomal RNA gene (Dyková et al. 2003; Moreira, Lopez-Garcia, and Vickerman 2004), which showed that the PLO is more closely related to the kinetoplastid, *Ichthyobodo necator*. Therefore, we propose that the eukaryotic endosymbiont be more correctly called *I. necator*-related organism (IRO).

Because of the difficulties in separating *Neoparamoeba* species morphologically and ultrastructurally, there has been an increasing use of molecular tools to study this genus (Dyková et al. 2005). To date, only the 18S rRNA gene has been used from the nuclear genomes of both the host amoebae and the IROs to establish species concepts and phylogenetic positions of the organisms (Dyková et al. 2003, 2005; Elliot, Wong, and Carson 2001; Fiala and Dyková 2003; Mullen et al. 2005; Peglar et al. 2003; Wong, Carson, and Elliott 2004). The 18S rRNA gene is relatively well conserved and has proven to be a good marker for species concepts, but its variability has been inadequate for strain identification. The closely associated internal transcribed spacer (ITS) region contains both variable and conserved domains that have been used to examine both intra-specific and inter-strain variation, as well as intra-genomic variability in various organisms (Hillis and Dixon 1991). However, successful use of these genes for taxonomic and phylogenetic studies is based on the assumption that the many copies present in the nuclear genomes are either

Corresponding Author: S. J. Greenwood, AVC Lobster Science Centre and Department of Pathology & Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3, Canada—Telephone number: 902 566 6002; FAX number: 902 566 0851; e-mail: sgreenwood@upei.ca

completely homogeneous in primary sequence or have relatively rare alternate alleles with only small divergence from the most frequent allele. This assumption may not be appropriate for *Neoparamoeba* nuclear genomes, since Dyková et al. (2005) found more nucleotide differences among copies of the 18S rRNA gene from a single *Neoparamoeba* isolate (microheterogeneity) than is typical for eukaryotes. If this level of microheterogeneity also exists in the ITS1 and ITS2 sequences, it may limit the utility of this region of DNA for strain identification and detection purposes.

In this study, we undertook an investigation of the intra-specific variability of the ITS region and estimated the level of sequence microheterogeneity of nuclear and IROs from six *N. pemaquidensis* isolates. Additionally, informative sites obtained from sequences allowed for parallel phylogenetic studies, which have led to a better understanding of the interactions between *N. pemaquidensis* and its endosymbiont.

MATERIALS AND METHODS

Amoeba isolates and cultures. Six isolates of *N. pemaquidensis* and one of *Neoparamoeba aestuarina* (Page 1970) Page, 1987 were obtained from private and public culture collections (Table 1). The two Culture Collection of Algae and Protozoa (CCAP) isolates were grown in MY75S agar medium at room temperature (19 °C–22 °C). The American Type Culture Collection (ATCC) isolates were cultured in ''ATCC medium 994'' agar medium at room temperature (19 °C–22 °C) bacterized with *Klebsiella pneumoniae*. Two urchin amoebae (UA1 and UA6) isolated from green sea urchins (*Strongylocentrotus droebachiensis*) in the Gulf of Maine were cultivated at 15 °C in L1 agar medium and fed with *Enterobacter aerogenes*. The cultures of *N. aestuarina* were maintained in liquid ''ATCC medium 994'' at room temperature (19 °C–22 °C).

Clonal culture procedure. The UA6 isolate of *N. pemaquidensis* was subcloned from a 10-day-old L1 agar plate culture by serial dilution to achieve clonal cultures derived from a single amoeba. Briefly, a swab of amoebae was suspended in a 10-µl drop of L1 liquid medium on a sterile transparent support. A serial dilution of the amoebae was performed using 5 µl of amoeba suspension added to an equal volume of L1 liquid medium under an inverted microscope and repeated until the number of amoebae present in the suspension was less than five. At this point, a single amoeba was transferred into a new 5-µl drop of L1 medium. Visualization under microscopy confirmed the presence of a single amoeba before transfer to a new L1 agar plate in a laminar-flow hood. Agar plates of clonal cultures were maintained at 15 °C for 2 wk before DNA extraction.

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 6,500 *g*. DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Ltd. Oakville, ON, Canada). DNA concentration was determined spectrophotometrically and quality was assessed by electrophoretic separation in a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide.

Amplification and sequencing of ITS region. The ITS region of *Neoparamoeba* spp. was amplified using universal eukaryote primers NLF 1624/20/SSU rDNA (5′-TTTGYACACACC GCCCGTCG-3′), positioned on the 3′-end of the 18S rRNA gene and NLR 204/21 (5′-ATATGCTTAARTTCAGCGGGT-3′), positioned on the 5′-end of the 28S rRNA gene (Van der Auwera, Chapelle, and De Wachter 1994). Approximately 10–50 ng of genomic DNA were amplified in a 50-µl reaction containing 5 pmol of each primer NLF 1624/20/SSU rDNA and NLR 204/21 in the presence of the following reagents (Fermentas International Inc., Burlington, ON, Canada): 200 µM of each dNTP (A, G, C and T), 1.5 mM $MgCl_2$, 10 × PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.8% [v/v] Nonidet P40), and 1.25 U of *Taq* DNA polymerase. Negative controls were included in each amplification experiment and consisted of the same reaction mixture, with molecular biology grade water (Sigma-Aldrich Ltd.) instead of template DNA. The amplification protocol was carried out in a MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories Inc., Toronto, ON, Canada) under the following conditions: an initial denaturation at 94 °C for 2.5 min, followed by 25 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °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 a specific ITS forward primer IRO-F-ITS (5′-GCGCACTACAATGACAA AGTG-3′) positioned on the 3′-end of the 18S rRNA gene and a universal eukaryote reverse primer ITS4 (5′-TCCTCCGCTTATT GATATGC-3′), positioned on the 5′-end of the 28S rRNA gene (Ristaino et al. 1998). Each 50-µl reaction included 100 ng of genomic DNA with the same concentration of reagents as described above. Thermocycling conditions were as follows: 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. Amplified ITS products were cloned directly into plasmid

Table **1.** *Neoparamoeba* spp. isolates and corresponding endosymbiont *Ichthyobodo necator* related organism information.

Isolate	Identification	Origin	Location
CCAP 1560/4	*N. pemaquidensis*	Environmental	Gwynedd, Wales
CCAP 1560/5	*N. pemaquidensis*	Environmental	Gwynedd, Wales
UA 1	*N. pemaquidensis*	*Strongylocentrotus droebachiensis*	Maine, USA
UA 6	*N. pemaquidensis*	*S. droebachiensis*	Maine, USA
ATCC 30735	*N. pemaquidensis*	Environmental	Virginia, USA
ATCC 50172	*N. pemaquidensis*	*Oncorhynchus kisutch* (AGD)	Washington, USA
ATCC 50806	*Neoparamoeba aestuarina*	Environmental	/
IRO-CCAP 1560/4	*I. necator* Related organism	*N. pemaquidensis* (CCAP 1560/4)	Gwynedd, Wales
IRO-CCAP 1560/5	*I. necator* Related organism	*N. pemaquidensis* (CCAP 1560/5)	Gwynedd, Wales
IRO-UA 1	*I. necator* Related organism	*N. pemaquidensis* (UA 1)	Maine, USA
IRO-UA 6	*I. necator* Related organism	*N. pemaquidensis* (UA 6)	Maine, USA
IRO-ATCC 30735	*I. necator* Related organism	*N. pemaquidensis* (ATCC 30735)	Virginia, USA
IRO-ATCC 50172	*I. necator* Related organism	*N. pemaquidensis* (ATCC 50172)	Washington, USA
IRO-ATCC 50806	*I. necator* Related organism	*N. aestuarina* (ATCC 50806)	/

AGD, amoebic gill disease; CCAP, Culture Collection of Algae and Protozoa; UA, urchin amoeba; ATCC, American Type Culture Collection; IRO, *I. necator* related organism.

pCR 2.1 using the TOPO TA Cloning® Kit (Invitrogen Canada Inc., Burlington, ON, Canada). Plasmids containing inserts were isolated and purified from recombinant *Escherichia coli* using the GenElute™ Plasmid Mini-Prep Kit (Sigma-Aldrich Ltd.). Plasmid inserts were sequenced using M13 forward and reverse primers on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems Inc., Foster City, CA) at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, Ontario, Canada).

ITS region analysis. The quality of all sequence data was verified by examining electropherograms and confirming that only single peaks were present. Each sequence was identified by NCBI-BLAST (National Center for Biotechnology Information—Basic Local Alignment Search Tool) (Altschul et al. 1997) based on the 18S rRNA gene 3′-extremity to confirm the origin of the amplicon. Nucleotide sequence analysis was refined on the ITS region by removing the vector extremities. Internal transcribed spacer region sequences were assembled by multiple alignment using ClustalW algorithm in BioEdit (Hall 1999), then the 18S rRNA 3′-end (183 nucleotides for *Neoparamoeba* spp. and 335 nucleotides for IROs) and 28S rRNA 5′-end (21 nucleotides for *Neoparamoeba* spp. and 35 nucleotides for IROs) were removed. Estimations of the nucleotide diversity (Pi) and respective standard deviations (SD) were conducted using the DnaSP software (Rozas and Rozas 1999), according to Nei (1987). A regular statistical *Z*-test ($\alpha = 0.05$) was used to compare Pi's from different sequence sets. DnaSP was used to estimate the number of net nucleotide substitutions per site between strains (Da) with the Jukes and Cantor (JC) correction. Graphical analyses of Pi values were computed with DnaSP using a sliding window approach (window length: 20 base pairs (bp), step size: 10 bp) on the total length of the obtained sequences. The Arlequin software (Schneider, Roessli, and Excoffier 2000) was used to perform a hierarchical analysis of molecular variance (AMOVA) (Excoffier, Smouse, and Quattro 1992), using the Kimura 2-parameter distance method and considering the inter-strain level (four defined strains: CCAP, UA, ATCC 30735 and ATCC 50172), the inter-isolate within strain level (two isolates, CCAP 1560/4&5, within strain CCAP and two isolates, UA1 and UA6, within strain UA), and the intra-isolate level.

Neighbor-joining (NJ), Kimura 2-parameter model with gaps and missing data handled by complete deletion and maximum parsimony (MP) phylogenies were constructed using MEGA-2 software (Molecular Evolutionary Genetics Analysis-2) (Kumar et al. 2001). Maximum likelihood (ML) phylogenies were constructed with PAUP*4 β program version 10 (Swofford 2003) using the GTR+G+I (*N. paramoeba*-ITS alignments) and HKY+G (IRO-ITS alignments) evolutionary model selected by the Akaike Information Criterion in ModelTest 3.7 (Posada and Crandall 1998). Statistical support for ML, MP, and NJ tree topologies were bootstrap-resampled 1,000 times (Felsenstein 1985). Bootstrap support values (%) of the ML, MP, and NJ analysis were superimposed on the 50% majority-rule consensus tree from the ML analysis.

A host:parasite coevolution test, using ParaFit (Legendre, Desdevises, and Bazin 2002), was conducted to test the null hypothesis (H_0) that each IRO associates randomly with a host. The alternative hypothesis was that the individual host:IRO associations are not random but fixed according to the genetic distances within the two groups of organisms. This method combined the information from three data matrices: matrix *A* (0-1 data) contained a description of the observed host:parasite/relationship links, matrix *B* contained principal coordinates (Gower 1966) with Lingoes correction (Legendre and Legendre 1998) representing the IRO genetic distances (Kimura 2-parameter), and matrix *C* contained principal coordinates representing the host genetic distances. A matrix $D = CA'B$ was computed, and a trace statistic was used to evaluate the hypothesis of coevolution through a test of significance incorporating 9,999 random permutations.

RESULTS

***Neoparamoeba pemaquidensis* ITS region nucleotide variability.** Eight clones of the ITS region were sequenced from PCR amplicons generated from each of six different *N. pemaquidensis* isolates, yielding a total of 48 ITS sequences accessible in GenBank (Table 2).

CCAP isolates. The total length of the eight CCAP 1560/4 sequences varied from 748–752 bp with an intra-isolate nucleotide diversity (Pi) of 0.0201. For the eight CCAP 1560/5 sequences, the total length was 746–751 bp and the Pi was 0.0288. The difference between the two intra-isolate nucleotide diversities was minimally significant ($P = 0.03$). Based on the alignment of the 16 sequences from the two CCAP isolates, no fixed nucleotide difference was observed and the estimation of the number of net nucleotide substitutions per site between the strains (Da) with the JC correction was -0.0003. Therefore, based on the ITS region, both the CCAP 1560/4 and CCAP 1560/5 isolates were considered to represent the same strain (renamed CCAP). The CCAP strain sequences had a Pi of 0.0238, not significantly different from the two CCAP isolates Pi's ($P = 0.25$ and 0.21, respectively).

UA isolates. The total length of the eight UA1 sequences varied from 734–740 bp with a Pi of 0.0296. For the eight UA6 sequences, the total length was 731–740 bp and the Pi was 0.0311. No significant difference was observed between the two intra-isolate nucleotide diversities ($P = 0.68$). Based on the alignment of the 16 sequences from the two UA isolates, no fixed nucleotide difference was observed (Da[JC] = -0.0001). Therefore, based on the ITS region, both the UA1 and UA6 isolates were considered to represent the same strain (renamed UA). The UA strain sequences had a Pi of 0.0294, not significantly different from the two UA isolates Pi's ($P = 0.94$ and 0.58, respectively). However, 53 fixed nucleotide differences have been observed between the sequences of the CCAP and UA strains (Da[JC] = 0.105). The CCAP and UA strains were considered different based on the ITS region.

ATCC isolates. The total length of the eight ATCC 30735 sequences varied from 734–739 bp with an estimated Pi of 0.0216. For the eight ATCC 50172 sequences, the total length was 770–785 bp and the Pi was 0.0313. Based on the alignment of the 16 sequences from the two ATCC isolates, 52 fixed nucleotide differences were observed. The Da(JC) was estimated at 0.095. The two isolates were considered different and represent separate strains. The ATCC 30735 isolate sequences also shared 48 fixed nucleotide differences when compared with the CCAP strain (Da[JC] = 0.086); and 31 when compared with the UA strain (Da[JC] = 0.057). The ATCC 50172 sequences had 24 fixed nucleotide differences when compared with the CCAP strain (Da[JC] = 0.044); and 54 when compared with the UA strain (Da[JC] = 0.105).

Based on the nucleotide divergence values, four distinct strains could be defined in this study: CCAP, UA, ATCC 30735, and ATCC 50172. High levels of microheterogeneity were present in the ITS1 and ITS2 regions compared with the low levels found within the ribosomal DNA genes (Fig. 1A).

AMOVA. The AMOVA confirmed that most of the variation came from inter-strain variability (76.6%), but also noteworthy was the intra-isolate variability (23.8%) (Table 3). The inter-isolate variability within strains was negative and not significantly different from zero. Negative variance components usually indicate an absence of genetic structure (Schneider et al. 2000).

Table **2.** Internal transcribed spacer sequences information for *Neoparamoeba pemaquidensis* isolates and respective endosymbiont *Ichthyobodo necator* related organisim.

Source	No. of Clones sequenced	ITS region total length (bp)	ITS region Pi (SD)	ITS 1 Pi (SD)	5.8S Pi (SD)	ITS 2 Pi (SD)	GenBank accession numbers
N. pemaquidensis							
CCAP 1560/4[1]	8	748–752	0.0201 (0.00240)^{A}	0.0369 (0.00431)^{A,B}	0.0100 (0.00306)^{A}	0.0129 (0.00212)^{A}	DQ167506-13
CCAP 1560/5[1]	8	746–751	0.0288 (0.00339)^{B,C}	0.0405 (0.00522)^{A,B}	0.0017 (0.00120)^{B,C}	0.0325 (0.00400)^{B}	DQ167514-21
CCAP[2]	16	746–752	0.0238 (0.00216)^{A,B}	0.0376 (0.00347)^{A,B}	0.0058 (0.00207)^{A,B}	0.0221 (0.00313)^{C}	
UA1[1]	8	734–740	0.0296 (0.00246)^{C}	0.0319 (0.00322)^{A}	0.0033 (0.00158)^{B,D}	0.0395 (0.00332)^{B}	DQ167522-29
UA6[1]	8	731–740	0.0311 (0.00273)^{C}	0.0438 (0.00442)^{B}	0.0017 (0.00120)^{B,C}	0.0353 (0.00392)^{B}	DQ167530-37
UA[2]	16	731–740	0.0294 (0.00145)^{C}	0.0358 (0.00294)^{A,B}	0.0025 (0.00115)^{B,D}	0.0369 (0.00217)^{B}	
ATCC 30735[1,2]	8	734–739	0.0216 (0.00229)^{A}	0.0339 (0.00395)^{A}	0 (0)^{C}	0.0226 (0.00253)^{C}	DQ167538-45
ATCC 50172[1,2]	8	770–785	0.0313 (0.00307)^{C}	0.0463 (0.00658)^{B}	0.0034 (0.00242)^{B,C,D}	0.0325 (0.00359)^{B}	DQ167546-53
I. necator related organism							
IRO-CCAP 1560/4[1]	4	357	0.0028 (0.00095)^{D}	0.0054 (0.00288)^{C}	0.0031 (0.00164)^{B,C}	0 (0)^{D}	DQ167481-84
IRO-CCAP 1560/5[1]	4	357	0.0042 (0.00153)^{D,E}	0.0054 (0.00288)^{C}	0.0062 (0.00209)^{A,D}	0 (0)^{D}	DQ167485-88
IRO-CCAP[2]	8	357	0.0035 (0.00108)^{D,E}	0.0054 (0.00253)^{C}	0.0046 (0.00165)^{A,B}	0 (0)^{D}	
IRO-UA1[1]	4	369–371	0.0054 (0.00287)^{D,E}	0.0146 (0.00077)^{D}	0.0031 (0.00164)^{B,C,D}	0 (0)^{D}	DQ167489-92
IRO-UA6[1]	4	370–371	0.0054 (0.00183)^{D,E}	0.0048 (0.00255)^{C}	0.0093 (0.00338)^{A,D}	0 (0)^{D}	DQ167493-96
IRO-UA[2]	8	369–371	0.0052 (0.00194)^{D,E}	0.0090 (0.00469)^{C,D}	0.0062 (0.00240)^{A,B}	0 (0)^{D}	
IRO-ATCC 30735[1,2]	4	377	0.0053 (0.00143)^{D,E}	0.0045 (0.00237)^{C}	0 (0)^{C}	0.0146 (0.00531)^{A,C,E}	DQ167497-500
IRO-ATCC 50172[1,2]	4	356	0.0056 (0.00100)^{E}	0.0055 (0.00291)^{C}	0.0062 (0.00207)^{A,D}	0.0048 (0.00257)^{D,E}	DQ167501-04

[1]Isolates.
[2]Strains.
$^{A–E}$Within a column, means without a common superscript are significantly different ($P < 0.05$).
CCAP, Culture Collection of Algae and Protozoa; UA, urchin amoeba; ATCC, American Type Culture Collection; IRO, *Ichthyobodo necator* related organism; ITS, internal transcribed spacer; Pi, nucleotide diversity; SD, standard deviation of the estimate.

The absence of inter-isolate variability supported the designation of CCAP 1560/4 and CCAP 1560/5 as CCAP strain, and UA1 and UA6 as UA strain.

IRO-ITS region nucleotide variability. Four clones of the ITS region were sequenced from PCR amplicons generated from IROs isolated from six different *N. pemaquidensis* isolates, yielding a total of 24 ITS sequences accessible in GenBank (Table 2).

IRO-CCAP. The total length of the IRO-CCAP1560/4 and IRO-CCAP 1560/5 sequences were 357 bp with an estimated Pi of 0.0028 and 0.0042, respectively. No significant difference was observed between the two intra-isolate nucleotide diversities ($P = 0.43$). Based on the alignment of the eight sequences from the two isolates, no fixed nucleotide difference was observed (Da[JC] = 0). Therefore, the isolates IRO-CCAP 1560/4 and IRO-CCAP 1560/5 were considered to represent the same strain (renamed IRO-CCAP). The IRO-CCAP strain sequences had a Pi of 0.0035, not significantly different from the two IRO-CCAP isolates Pi's ($P = 0.62$ and 0.70, respectively).

IRO-UA. The total length of the four IRO-UA1 sequences varied from 369–371 bp with an estimated Pi of 0.0054. For the four IRO-UA6 sequences, the total length was 370–371 bp and the Pi is 0.0054. No significant difference was observed between the two intra-isolate nucleotide diversities ($P = 1$). Based on the alignment of the eight sequences from the two IRO-UA isolates, no fixed nucleotide difference was observed (Da(JC) = − 0.0003). Therefore, based on the ITS region, the isolates IRO-UA1 and IRO-UA6 were considered to represent the same strain (renamed IRO-UA). The IRO-UA strain sequences had a Pi of 0.0052, not significantly different from the two IRO-CCAP isolates Pi's ($P = 0.95$ and 0.94, respectively). However, 22 fixed nucleotide differences were observed between the sequences of the IRO-CCAP and IRO-UA strains (Da(JC) = 0.067). The IRO-CCAP and IRO-UA strains were considered different based on the ITS region.

IRO-ATCC. The total length of the four ATCC 30735 sequences was 377 bp with an estimated Pi of 0.0053. The total length of the four ATCC 50172 sequences was 356 bp with a Pi of 0.0056. Based on the alignment of the eight sequences from the two IRO-ATCC isolates, 29 fixed nucleotide differences were observed. The Da(JC) was estimated at 0.087. The two isolates were considered different and represent separate strains. The IRO-ATCC 30735 isolate sequences also shared 21 fixed nucleotide differences when compared with the IRO-CCAP strain (Da(JC) = 0.067); and 33 when compared with the IRO-UA strain (Da(JC) = 0.102). The IRO-ATCC 50172 sequences had 18 fixed nucleotide differences when compared with the IRO-CCAP strain (Da(JC) = 0.051); and 32 when compared with the IRO-UA strain (Da(JC) = 0.096).

Based on these nucleotide divergence values, four distinct strains were defined in the present study: IRO-CCAP, IRO-UA, IRO-ATCC 30735, and IRO-ATCC 50172. Low or non-existent levels of microheterogeneity were found within the ITS sequence region (Fig. 1B).

AMOVA. The AMOVA revealed that most of the variation was explained by inter-strain variability (95.1%). We also noted low intra-isolate variability (5.0%) and negligible inter-isolate variability within strains (Table 3). The absence of inter-isolate variability confirmed the designation of the IRO-CCAP 1560/4 and IRO-CCAP 1560/5 isolates as IRO-CCAP strain, and IRO-UA1 and IRO-UA6 isolates as IRO-UA strain.

***Neoparamoeba aestuarina* ITS region.** Two clones of the ITS region were sequenced from the *N. aestuarina* isolate and the respective IRO. Pairwise sequences comparison revealed 11 nucleotide substitutions for the amoeba's ITS region and only one for the IRO's. A single clone of the ITS region was used as outgroup in the phylogenetic analyses. The ITS region sequence length was 737 bp for *N. aestuarina* and 366 bp for the endosymbiont (accessible in GenBank, Table 2).

***Neoparamoeba* spp. phylogenetic analysis.** The alignment of 828 nucleotides of ITS region sequences from 49 *Neoparamoeba* spp. was assessed by ML, MP and NJ analysis (Fig. 2A). The ML 50% majority-rule consensus tree rooted with a *N. aestuarina*

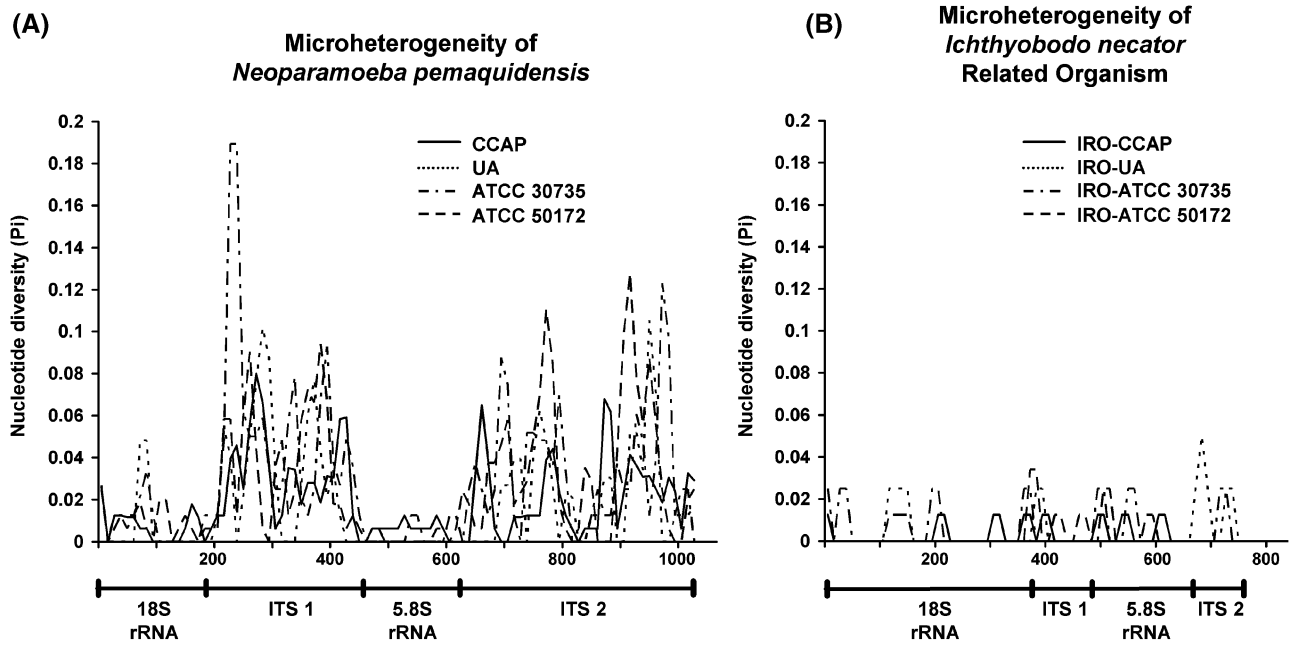

Fig. **1.** Microheterogeneity variation along the internal transcribed spacer region. (**A**) Four *Neoparamoeba pemaquidensis* strains (1032 nucleotide alignment). (**B**) Four *Ichthyobodo necator* related organism strains (753 nucleotide alignment). The *X*-axis represents the nucleotide position in the alignment. Sliding window approach: 20 nucleotide length and 10 nucleotide steps.

outgroup, represents the branching order among the four *N. pemaquidensis* strains (Fig. 2A). *N. pemaquidensis* sequences were separated into two distinct sister groups. Within these groups, all clones from a single strain were consistently grouped together and formed a cluster supported by high bootstrap values (92%–100%). The sister groups, UA and ATCC 30735 strains, were supported by moderate bootstrap values (65%–84%). The CCAP and ATCC 50172 strains formed a monophyletic group supported by high bootstrap values (98%–99%) (Fig. 2A). The general branching structure produced by all analyses did not indicate a phylogeographic pattern.

***Ichthyobodo necator*-related organism phylogenetic analysis.** The alignment of the 383 nucleotides from the 25 IRO ITS region sequences was assessed by ML, MP, and NJ analysis (Fig. 2B). The ML 50% majority-rule consensus tree rooted with an IRO-*N. aestuarina* outgroup, represents the branching order among the four *N. pemaquidensis* strains (Fig. 2B). All clones from a single strain were consistently grouped together and formed a cluster supported by high bootstrap values (94%–100%). The IRO-UA strain sequences produced a well-supported monophyletic group (bootstrap value of 99%–100%) (Fig. 2B). The IRO-CCAP and IRO-ATCC 50172 strain sequences clustered together to form a sister group supported by low to moderate bootstrap values of 55%–84%. The IRO-ATCC 30735 strain's association with the IRO-CCAP and IRO-ATCC 50172 sister group was supported by low bootstrap values (50%–65%) (Fig. 2B).

Host:parasite coevolution test. Genetic distances based on the Kimura 2-parameter model were computed from the six aligned ITS consensus sequences from *N. pemaquidensis* isolates and compared with the genetic distances computed from the six aligned IRO-ITS consensus sequences. The ParaFit test indicated that there was a global relationship between the host and parasite

Table **3.** Analyses of molecular variance (AMOVA) within the ITS sequence: (A) *Neoparamoeba pemaquidensis*; (B) *Ichthyobodo necator* related organism.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
(A) Neoparamoeba pemaquidensis ITS region				
Among strain	3	1551.81	43.75	76.6
Among isolates within a strain	2	23.62	− 0.22	− 0.4
Within isolate (microheterogeneity)	42	570.50	13.58	23.8
Total	47	2145.94	57.10	100
(B) Ichthyobodo necator related organism ITS region				
Among strain	3	334.92	19.16	95.1
Among isolates within a strain	2	1.87	− 0.02	− 0.1
Within isolate (microheterogeneity)	18	18.25	1.01	5.0
Total	23	355.04	20.15	100

The hierarchical structure of the analysis is the same for both organisms; 4 strains: CCAP, UA, ATCC 30735 and ATCC 50172; two isolates within the strain CCAP: CCAP 1560/4 and CCAP 1560/5; two isolates within the strain UA: UA1 and UA6.

ATCC, American type culture collection; CCAP, Culture Collection of Algae and Protozoa; ITS, internal transcribed spacer.

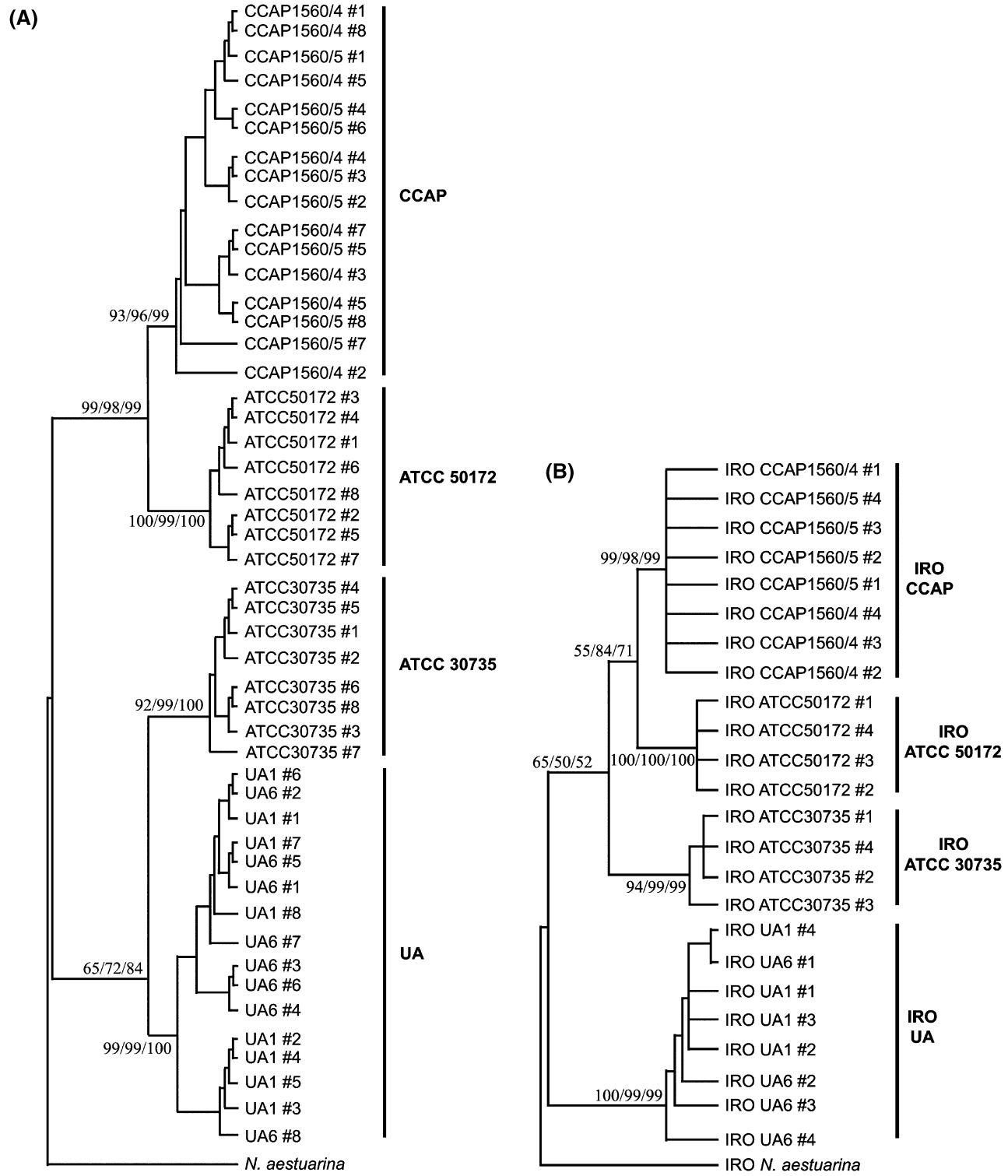

Fig. **2.** The 50% majority-rule consensus phylogenetic trees for *Neoparamoeba pemaquidensis* and *Ichthyobodo necator* related organism strains based on internal transcribed spacer sequences. (**A**) *Neoparamoeba pemaquidensis* based on the alignment of 828 nucleotides. (**B**) *Ichthyobodo necator* related organism based on the alignment of 383 nucleotides. Values at nodes represent the bootstrap percentages from 1,000 replicates for maximum likelihood, maximum parsimony and neighbor-joining, respectively. Each strain includes the cluster of all cloned sequences.

(endosymbiont) phylogenies, mediated by the table of host:parasite association links ($P < 0.001$). The test confirmed that the phylogenies were generally congruent. Additionally, the ParaFit test for individual host:parasite links indicated significant coevolution ($P < 0.02$) for all established associations except for the *N. pemaquidensis* ATCC 30735 strain and its respective

endosymbiont IRO-ATCC 30735 where the null hypothesis H_0 was not rejected ($P = 0.30$).

DISCUSSION

Evaluation of the *N. pemaquidensis* ITS region revealed quantitative intra-specific variability that permitted the definition of four strains among the six isolates studied. In two cases, we observed that phenotypically distinguishable clones of *N. pemaquidensis* (CCAP 1560/4 and CCAP 1560/5, UA1 and UA6) could not be separated at the molecular level, and thus we assigned the clones to the same strain. Genetic distances between strains (Da) revealed that the ITS region is an efficient subspecies marker for *N. pemaquidensis*. Nevertheless, the presence of high intra-isolate variability in *N. pemaquidensis* suggests that microheterogeneity may confound our ability to differentiate isolates.

Microheterogeneity. We observed significant levels of microheterogeneity in the nuclear rDNA of all six strains of *N. pemaquidensis*. Some microheterogeneity was found throughout the stretch of rDNA that we studied (1–3 differences from pairwise comparisons out of the 183 nucleotides sequenced of the 3′-end of the 18S rDNA), but most of it occurred in the ITS1 and ITS2 regions. Approximately 24% of the ITS region total intraspecific variation observed in *N. pemaquidensis* was explained by microheterogeneity (Table 3). Dyková et al. (2005) reported surprisingly high divergence levels among cloned 18S rDNA sequences (microheterogeneity) with 16–52 differences observed within an isolate from pairwise comparisons. In contrast, several previous studies did not report or describe any microheterogeneity for 18S rDNA sequences (Elliot et al. 2001; Fiala and Dyková 2003; Peglar et al. 2003; Wong et al. 2004). We believe that the levels of microheterogeneity that we and Dyková et al. (2005) have observed are the rule in *Neoparamoeba*.

In the current study, we demonstrated higher microheterogeneity than previously reported from any *Neoparamoeba* spp. or closely related organism. Low to non-existent levels of microheterogeneity have been found in studies from different amoebae: *Entamoeba* sp. (Som et al. 2000), *Naegleria* sp. (De Jonckheere 2004), and *Acanthamoeba* (Stothard et al. 1998). However, significant intra-specific polymorphism has been found in the marine alveolate protozoan *Perkinsus marinus* (Brown, Hudson, and Reece 2004). Brown et al. (2004) detailed intra-isolate variation in the ITS region (0.001–0.015) with the highest variation of 0.031 occurring in the ITS1 locus. The microheterogeneity found among the *N. pemaquidensis* isolates varied from 0.0201–0.0313 for the entire ITS region with the highest variation in ITS1 (0.032–0.046). Given the relatively small number of clones examined per isolate, perhaps we detected only a fraction of the ITS region heterogeneity present within the genome. Thus, actual levels of microheterogeneity are probably higher than the current estimates.

Several origins for the observed level of microheterogeneity are plausible. The production of sequence heterogeneity from a single *Neoparamoeba* isolate may be the result of PCR artefact (Tindall and Kunkel 1988; Pääbo, Irwin, and Wilson 1990). However, using the same PCR reagents, we obtained low or non-existent microheterogeneity within the IRO sequences (Table 3 and Fig. 1B). Therefore, biased PCR is not a likely reason to explain the observed microheterogeneity, whereas it could minimally result in overestimates. Alternatively, sequence heterogeneity could be accounted for if the *N. pemaquidensis* cultures were not clonal and therefore, contained several different isolates. We tested the nonclonality hypothesis by establishing new clonal cultures from the UA6 isolate. The UA6 *Neoparamoeba* and IRO nucleotide diversity levels (0.0307 and 0.0041, respectively) were not significantly different ($P = 0.92$ and 0.58, respectively) from the nucleotide diversities found in the initial UA6 clone sequences. We can therefore reject the hypothesis that a non-clonality effect could account for the observed microheterogeneity.

Our results suggest that the assumption of concerted evolution, as it is normally perceived to operate in eukaryotic cells (Dover 1982; Elder and Turner 1995), is not totally appropriate for *Neoparamoeba*. Multiple functional rDNA copies have been found within the apicomplexan parasite, *Plasmodium*. However, the microheterogeneity observed in *N. pemaquidensis* is different than the poly-allelic pattern described in the 18S rDNA gene copies in the *Plasmodium vivax* genome (Li et al. 1997). The variation observed between the four to eight dispersed genomic rDNA copies in *Plasmodium* is correlated with the stage-specific expression of the parasites in different hosts (mosquito and human) and cell types (erythrocytes and hepatocytes) (Li et al. 1997). For *N. pemaquidensis*, the amoeba trophozoite is the only known life stage and sexual reproduction has never been observed. Therefore, the likelihood that the microheterogeneity exhibited within the ITS regions of *N. pemaquidensis* correlates with life stages or the varying life history strategies of this amphizoic amoeba appears remote. Interestingly, sequence microheterogeneity does not introduce so much ''noise'' into the data that phylogenetic relationships among the strains are obscured, a result also obtained by Burreson, Reece, and Dungan (2005) for *Perkinsus*. This observation suggests that nucleotide sequence homogeneity among rDNA copies is still maintained within a strain by partial mechanisms of homogenization. The IROs that we examined showed far lower levels of rDNA microheterogeneity, levels more consistent with those observed in the great majority of eukaryotes. Whatever the extent of genomic integration that exists between the IRO and its host, it has not extended to the control of the mechanism of rDNA evolution. Therefore, the morphological (Martin 1987), antigenic (Villavedra et al. 2005), and now genetic plasticity present in *N. pemaquidensis* may be considered the result of a complex adaptation of the amoeba and its endosymbiont to a wide range of life styles and environments.

Coevolution. The topologies of the *N. pemaquidensis* and IRO phylograms differ. The dissymmetry between the trees is caused by the unresolved branching order between the UA cluster, the ATCC 30735 cluster and the cluster formed by the CCAP and ATCC 50175 strains. The lack of congruence between these phylogenetic analyses does not refute the hypothesis of coevolution. Nevertheless, the ParaFit test to estimate the robustness of the coevolution hypothesis between *N. pemaquidensis* and the IRO supported the hypothesis that the two protists followed coordinated evolution and shared specific relationships. Even if the individual ATCC 30735 association is not confirmed, the global coevolution pattern is established and corroborates the congruent phylogenies previously observed by Dyková et al. (2003) and Dyková and Lom (2004) from three IRO types and their *N. pemaquidensis* host. Additional studies should be done with *N. aestuarina* and *N. branchiphila* to verify if the observed pattern is ubiquitous within the genus *Neoparamoeba*.

This result is consistent with the little that we know of the biology of the *Neoparamoeba*/IRO association. To our knowledge, no cell of *Neoparamoeba* has ever been observed without an IRO, and the two protists have never been separated from each other experimentally, nor isolated nor cultured independently (Hollande 1980). Assuming that the two organisms could not be separated and are following coordinated evolution, our results establish that *N. pemaquidensis* and the IRO are intimately and obligately associated. This conclusion however may not extend to other parasome-containing amoebae. O'Kelly et al. (2001) noted that the entity known as *Korotnevella nivo* Smirnov, 1997 is indistinguishable at the ultrastructural level from *Paramoeba eilhardi* Schaudinn, 1896, except that the former lacks a parasome. Molecular sequence data should provide further insights into the relative

phylogenetic closeness of *K. nivo* and *P. eilhardi*. Until then, it is intriguing to consider that symbiont-free cells of a susceptible species are available for infection in nature. Endosymbiotic relationships suggest some level of dynamic cytonuclear association with different degrees of mutual exchange. Further genomic comparisons between *Neoparamoeba* and its endosymbiont should therefore reveal some level of reciprocal genomic transfers. Indeed, *Paramoeba* and *Neoparamoeba* may therefore represent two stages in the development of an obligate symbiotic relationship between two lineages of free-living heterotrophic protists, and may provide an excellent model in which to study genomic integration.

ACKNOWLEDGMENTS

We thank Dr. H. Strynh for critical review of the statistical analysis, and Dr. N. Donay for assistance and expertise with the *Neoparamoeba* cultures. We also thank Dr. Y. Wang and H. Andawa for discussions regarding the ML analysis. Funding for the Canadian Lobster Health Research Project delivered by the AVC Lobster Science Centre represents a consortium of private sector companies, fishermen's organizations, First Nations, provincial and federal government agencies, including the Atlantic Innovation Fund through the Atlantic Canada Opportunities Agency.

LITERATURE CITED

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**:3389–3402.

Brown, G. D., Hudson, K. L. & Reece, K. S. 2004. Multiple polymorphic sites at the ITS and ATAN loci in cultured isolates of *Perkinsus marinus*. *J. Eukaryot. Microbiol.*, **51**:312–320.

Burreson, E. M., Reece, K. S. & Dungan, C. F. 2005. Molecular, morphological, and experimental evidence support the synonymy of *Perkinsus chesapeaki* and *Perkinsus andrewsi*. *J. Eukaryot. Microbiol.*, **52**:258–270.

Cann, J. P. & Page, F. C. 1982. Fine structure of small free-living *Paramoeba* (Amoebida) and taxonomy of the genus. *J. Mar. Biol. Assn.*, **62**:25–43.

Chatton, E. 1953. Ordre des Amoebiens nus. *In*: Grassé, P. P. (ed.), Traité de Zoologie. Masson et Cie, Paris. p. 3–148.

De Faria, J. G., da Cunha, A. M. & Pinto, C. 1922. Estudos sobre Protozoarios do mar. *Mem. Inst. Oswaldo Cruz*, **15**:186–208.

De Jonckheere, J. F. 2004. Molecular definition and the ubiquity of species in the genus *Naegleria*. *Protist*, **155**:89–103.

Dover, G. 1982. Molecular drive: a cohesive mode of species evolution. *Nature*, **299**:111–117.

Dyková, I. & Lom, J. 2004. Advances in the knowledge of amphizoic amoeba infecting fish. *Folia Parasitol.*, **51**:81–97.

Dyková, I., Figueras, A. & Novoa, B. 1995. Amoebic gill infection of turbot, *Scophthalmus maximus*. *Folia Parasitol.*, **42**:91–96.

Dyková, I., Figueras, A. & Novoa, B. 1999. Epizoic amoebae from the gills of turbot *Scophthalmus maximus*. *Dis. Aquat. Org.*, **38**:33–38.

Dyková, I., Figueras, A. & Peric, Z. 2000. *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Dis. Aquat. Org.*, **43**:217–223.

Dyková, I., Fiala, I., Lom, J. & Lukeš, J. 2003. *Perkinsiella* amoebae-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. *Eur. J. Protistol.*, **39**:37–52.

Dyková, I., Figueras, A., Novoa, B. & Casal, J. F. 1998. *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Dis. Aquat. Org.*, **33**:137–141.

Dyková, I., Nowak, B. F., Crosbie, P. B., Fiala, I., Peckova, H., Adams, M. B., Machackova, B. & Dvorakova, H. 2005. *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *J. Fish. Dis.*, **28**:49–64.

Elder, J. F. & Turner, B. J. 1995. Concerted evolution of repetitive DNA-sequences in eukaryotes. *Quart. Rev. Biol.*, **70**:297–320.

Elliot, N. G., Wong, F. & Carson, J. 2001. Detection and abundance of *Paramoeba* species in the environment. Project Report, FRDC Project, Hobart, TAS 7001. 68 p. 98/209.

Excoffier, L., Smouse, P. E. & Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**:479–491.

Felsenstein, J. 1985. Confidence-limits on phylogenies – an approach using the Bootstrap. *Evolution*, **39**:783–791.

Fiala, I. & Dyková, I. 2003. Molecular characterisation of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus*. *Dis. Aquat. Org.*, **55**:11–16.

Gower, J. C. 1966. Some distant properties of latent root and vector methods used in multivariate analysis. *Biometrika*, **53**:325–338.

Grell, K. G. 1961. Uben den ''Nebenkörper'' von *Paramoeba eilhardi* Schaudinn. *Arch. Protistenkd.*, **105**:303–312.

Grell, K. G. 1968. Protozoologie. 2nd ed. Springer, Berlin.

Grell, K. G. & Benwitz, G. 1970. Ultrastrukturb Mariner Amoben I. *Paramoeba eilhardi* Schaudinn. *Arch. Protistenkd.*, **112**:119–137.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT/XP. *Nucleic Acids Symp. Ser.*, **41**:95–98.

Hillis, D. M. & Dixon, M. T. 1991. Ribosomal DNA – molecular evolution and phylogenetic inference. *Quart. Rev. Biol.*, **66**:410–453.

Hollande, A. 1940. Le ≪ Nebenkörper ≫ de certains *Cryptomonas* et la critique du cycle de *Paramoeba eilhardi* selon Schaudinn. *Bull. Soc. Zool.*, **65**:211–216.

Hollande, A. 1980. Identification du parasome (Nebenkern) de *Janickina pigmentifera* à un symbionte (*Perkinsiella amoebae* nov. gen. nov. sp.) apparenté aux flagellés Kinoplastidiés. *Protistologica*, **16**:613–625.

Janicki, C. 1912. Untersuchungen an Parasitischen Arten der Gattun *Paramoeba* Schaudinn (*P. pigmentifera* Grassi und *P. chaetognathi* Grassi). *Verh. Naturforsch. Ges. Basel*, **23**:1–16.

Janicki, C. 1928. Studien am Genus *Paramoeba* Schaudinn. *Z. Wiss. Zool.*, **131**:588–644.

Jones, G. M. 1985. *Paramoeba invadens* n. sp. (Amoebida, Paramoebidae), a pathogenic amoeba from the sea urchin, *Strongylocentrotus droebachiensis*, in eastern Canada. *J. Protozool.*, **32**:564–569.

Kent, M. L., Sawyer, T. & Hedrick, R. P. 1988. *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of Coho salmon *Oncorhynchus kisutch* reared in sea water. *Dis. Aquat. Org.*, **5**:163–169.

Kirkaldy, G. W. 1903. Miscellanea Rhynchotalia. *Entomologist*, **36**:179–181.

Kudo, R. R. 1966. Protozoology. 5th edn. Thomas, Springfield. p. 111.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**: 1244–1245.

Legendre, P. & Legendre, L. 1998. Numerical Ecology. 2nd English edn. Elsevier Science BV, Amsterdam. p. 870.

Legendre, P., Desdevises, Y. & Bazin, E. 2002. A statistical test for host-parasite coevolution. *Syst. Biol.*, **51**:217–234.

Li, J., Gutell, R. R., Damberger, S. H., Wirtz, R. A., Kissinger, J. C., Rogers, M. J., Sattabongkot, J. & McCutchan, T. F. 1997. Regulation and trafficking of three distinct 18S ribosomal RNAs during development of the malaria parasite. *J. Mol. Biol.*, **269**:203–213.

Martin, R. E. 1987. Adhesion, morphology, and locomotion of *Paramoeba pemaquidensis* Page (Amoebida, Paramoebidae): effects of substrate charge density and external cations. *J. Protozool.*, **34**:345–349.

Minchin, E. A. 1922. An Introduction to the Study of Protozoa. Arnold, London. p. 517.

Moreira, D., Lopez-Garcia, P. & Vickerman, K. 2004. An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. *Int. J. Syst. Evol. Microbiol.*, **54**:1861–1875.

Mullen, T. E., Nevis, K. R., O'Kelly, C. J., Gast, R. J. & Frasca, S. Jr. 2005. Nuclear small-subunit ribosomal RNA gene-based characterization, molecular phylogeny and PCR detection of the *Neoparamoeba* from Western Long Island Sound lobster. *J. Shellfish Res.*, **24**:719–732.

Mullen, T. E., Russel, S., Tucker, M. T., Maratea, J. L., Koerting, C., Hinckley, L., de Guyse, S., Frasca, S. Jr. & French, R. A. 2004. Paramoebiasis associated with mass mortality of American lobster (*Homarus americanus*) in Long Island Sound, USA. *J. Aquat. Anim. Health*, **16**:29–38.

Munday, B. L., Foster, C. K., Roubal, F. R. & Lester, R. J. G. 1990. Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout, *Salmo gairdneri*, in Tasmania. *In*: Perkins, F. O. & Cheng, T. C. (ed.), Pathology in Marine Science. Academic Press, London. p. 215–222.

Munday, B. L., Lange, K., Foster, C. K., Lester, R. J. G. & Handlinger, J. 1993. Amoebic gill disease of sea-caged salmonids in Tasmanian waters. *Tasman. Fish. Res.*, **28**:14–19.

Nei, M. 1987. Molecular Evolutionary Genetics. DNA Polymorphism Within and Between Populations. Columbia University Press, New York. p. 254–257.

O'Kelly, C. J., Peglar, M. T., Black, M. N. D., Sawyer, T. A. & Nerad, T. A. 2001. *Korotnevella hemistylolepis* n. sp. and *K. monacantholepis* n. sp., two new scale-covered mesohaline amoebae. *J. Eukaryot. Microbiol.*, **48**:655–662.

Pääbo, S., Irwin, D. M. & Wilson, A. C. 1990. DNA damage promotes jumping between templates during enzymatic amplification. *J. Biol. Chem.*, **265**:4718–4721.

Page, F. C. 1970. Two new species of *Paramoeba* from Maine. *J. Protozool.*, **17**:421–427.

Page, F. C. 1987. The classification of 'naked' amoeba (phylum Rhizopoda). *Arch. Protistenkd.*, **133**:199–217.

Peglar, M. T., Amaral Zettler, L. A., Anderson, O. R., Nerad, T. A., Gillevet, P. M., Mullen, T. E., Frasca, S. Jr., Silberman, J. D., O'Kelly, C. J. & Sogin, M. L. 2003. Two new small-subunit ribosomal DNA gene lineages within the subclass Gymnamoebia. *J. Eukaryot. Microbiol.*, **50**:224–232.

Perkins, F. O. & Castagna, M. 1971. Ultrastructure of the Nebenkörper or ''secondary nucleus'' of the parasitic amoeba *Paramoeba perniciosa* (Amoebida, Paramoebidae). *J. Invert. Pathol.*, **17**:186–193.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**:817–818.

Ristaino, J. B., Madritch, M., Trout, C. L. & Parra, G. 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Appl. Environ. Microbiol.*, **64**:948–954.

Roubal, F. R., Lester, R. J. G. & Foster, C. K. 1989. Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae, Paramoebidae) and the cyto-pathology of Paramebic Gill Disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *J. Fish. Dis.*, **12**:481–492.

Rozas, J. & Rozas, R. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**:174–175.

Schaudinn, F. 1896. Der Zeugungskreis von *Paramoeba eilhardi* nov. gen. nov. sp. *Sitzungsberichte der Koniglich Preussiscken Akademie der Wissensckaften zu Berlin*, **14**:31–41.

Schneider, S., Roessli, D. & Excoffier, L. 2000. Arlequin: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland. http://lgb.unige.ch/arlequin.

Som, I., Azam, A., Bhattacharya, A. & Bhattacharya, S. 2000. Inter- and intra-strain variation in the 5.8S ribosomal DNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii*, and *Entamoeba invadens*. *Int. J. Parasitol.*, **30**:723–728.

Smirnov, A. V. 1997. Two new species of marine amoebae: *Hartmannella lobifera* n. sp. and *Korotnevella nivo* n. sp. (Lobosea, Gymnamoebia). *Arch. Protistenkd.*, **147**:283–292.

Sprague, V., Beckett, R. L. & Sawyer, T. 1969. A new species of *Paramoeba* (Amoebida, Paramoebidae) parasitic in the crab *Callinectes sapidus*. *J. Invert. Pathol.*, **14**:167–174.

Stothard, D. R., Schroeder-Diedrich, J. M., Awwad, M. H., Gast, R. J., Ledee, D. R., Rodriguez-Zaragoza, S., Dean, C. L., Fuerst, P. A. & Byers, T. J. 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J. Eukaryot. Microbiol.*, **45**:45–54.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b 10. Sinauer Associates, Sunderland, MA.

Tindall, K. R. & Kunkel, T. A. 1988. Fidelity of DNA-synthesis by the *Thermus aquaticus* DNA-polymerase. *Biochemistry*, **27**:6008–6013.

Van der Auwera, G., Chapelle, S. & De Wachter, R. 1994. Structure of the large ribosomal subunit DNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. *FEBS Lett.*, **338**:133–136.

Villavedra, M., McCarthy, K., To, J., Morrison, R., Crosbie, P., Broady, K. & Raison, R. L. 2005. Changes in antigenic profile during culture of *Neoparamoeba* sp., causative agent of amoebic gill disease in Atlantic salmon. *Int. J. Parasitol.*, **35**:1417–1423.

Wong, F. Y., Carson, J. & Elliott, N. G. 2004. 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. *Dis. Aquat. Org.*, **60**: 65–76.

Received: 04/25/06, 09/21/06, 06/27/07; accepted: 06/29/07