DEVELOPMENT OF MOLECULAR DIAGNOSTICS FOR THE CHARACTERIZATION OF *NEOPARAMOEBA PEMAQUIDENSIS* PAGE, 1987

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Charles G. B. Caraguel

Charlottetown, P. E. I.

August, 2006

Your file Votre référence
ISBN: 978-0-494-22824-1
Our file Notre référence
ISBN: 978-0-494-22824-1

Chair of the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Charlottetown, P. E. I.

Canada, C1A 4P3

SIGNATURE PAGE

iii

REMOVED

SIGNATURE PAGE

iv

REMOVED

ABSTRACT

Neoparamoeba pemaquidensis Page, 1987 is an ongoing pathogen for commercial finfish aquaculture and has also sporadically been associated with mass mortality outbreaks of invertebrates. Despite the ubiquity and importance of this amphizoic amoeba, our understanding of its biology as applied to host range, pathogenicity, tissue tropism and geographic distribution, is severely lacking. This confusion may stem from the inability of current diagnostic tests based on morphology, immunology and molecular biology to differentiate strains at the subspecies level. This study focused on the identification of a subspecies marker able to characterize *Neoparamoeba pemaquidensis* strains. The inter-strain and intra-strain variability of the amoeba Internal Transcribed Spacer (ITS) region was estimated. This hypervariable region showed discriminative inter-strain variability among individual amoeba isolates. However, high levels of intra-genomic microheterogeneity were found among sequenced ITS regions. Further investigations on the ITS region of the *Neoparamoeba* eukaryotic endosymbiont, renamed *Ichthyobodo necator* Related Organism, revealed pertinent inter-strain variability and significantly lower levels of microheterogeneity. Phylogenetic and ParaFit coevolution analyses involving *Neoparamoeba pemaquidensis* isolates and their respective endosymbionts confirmed a significant coevolutionary relationship between the two protists. The combination of non-shared microheterogeneity and coevolution, presents the endosymbiont marker as a complementary or alternative target to differentiate *Neoparamoeba* strains. Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) diagnostic tests based on both ITS regions were developed. The investigations centred on the complications of the amoeba ITS microheterogeneity in the development of a subspecies marker and the use of the endosymbiont ITS region as an internal marker. Both amoeba and endosymbiont ITS PCR-RFLP analyses were successfully used to detect and characterize a *N. pemaquidensis* isolate from an episode of Amoebic Gill Disease in Atlantic salmon, *Salmo salar,* from the west coast of North America (Washington State, USA).

ACKNOWLEDGEMENTS

I gratefully acknowledge my supervisors Dr. Spencer Greenwood and Dr. Rick Cawthorn. First, Dr. Greenwood for his exceptional support and guidance throughout the program. I was proud to study under the direction of someone who combined strong teaching skills and high levels of research science. I was his first graduate student and I hope that many will follow. Also, Dr. Cawthorn for believing in my person and offering me the opportunity to work on this project. Furthermore, I would like to extend my gratitude to the others members of my supervisory committee, Dr. Basil Ikede, Dr. Rob Hurta, Dr. Jeff Lewis and Dr. Franck Berthe.

The technical support of Béatrice Després, Rachael Summerfield, Fraser Clark and Dr. Henrik Strynh was greatly appreciated. Also, thank you to the AVC Lobster Science Centre team, Dr. Jean Lavallée, Dr. Andrea Battison, Dr. Ian Keith, Lori Edwards, Barry Stahlbaum, Natasha Doyle, Jerry Amirault and Lorraine Gibson.

Thanks to all the people that I met in the lat two years:

- all the graduate students from the AVC (too many to be cited)
- many faculties and staffs from the different departments and services (too many to be cited)
- all my sport teammates from rugby, soccer and kite surfing (too many to be cited)
- all my French speaker friends that are now my Canadian family: Charles-André, Hélène, Robert et autres expatriés (trop nombreux pour être cités)

I am thankful to my family which support and encourage me in my professional carrier and still accept me even if know I speak few word of English. A special thank goes to Martine who help and support me since I arrived in Canada: Merci ma tante!

I deeply thank Nathanaelle for everything in this adventure, she was and she is still the perfect partner...You are my "IRO"!

The last but not the least, thanks to Jacob (the fake cat) for nothing related with this thesis...

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
AVCLSC	Atlantic Veterinary College Lobster Science Centre
Acc. No.	GenBank accession number
bp(s)	Base pair(s)
CCAP	Culture Collection of Algae and Protozoa
CTDEP	Connecticut Department of Environmental Protection
Da	Genetic distance between strains: number of net nucleotide substitutions per site between strains
df	Degree of freedom
DO	Dissolved oxygen
ELIS	Eastern Long Island Sound
IFAT	Indirect Immunofluorescent Antibody Test
IRO	*Ichthyobodo necator* Related Organism
ITS	Internal Transcribed Spacer
h	hour
JC	Jukes & Cantor correction
LIS	Long Island Sound
LC_{50}	Lethal Concentration 50 %
LT_{50}	Lethal Time 50 %
PCBs	Polychlorobiphenols
PCR	Polymerase Chain Reaction
Pi	Nucleotide diversity: number of nucleotide substitutions per site between sequences
rRNA	ribosomal ribonucleic acid
SSU	small subunit
t	metric tons
UA	Urchin Amoeba
USA	United States of America
WLIS	Western Long Island Sound

WNV	West Nile Virus
18S rRNA	Gene coding for the small subunit of the ribosome in Eukaryotes

Chapter I: GENERAL INTRODUCTION

The American lobster, *Homarus americanus* Edwards 1837, fishery provides significant sustainable income for local fishing communities from the eastern provinces of Canada (Sackton 2004). Of the 40,000 t lobster annual landings in Canada, half are processed and the balance are maintained in captivity to be sold as live product (Gardner Pinfold 2006). Live lobsters are stored in specialized holding facilities for periods from a few days to several months. Therefore, live product is available to the market all year around.

Confinement conditions vary considerably as there is no industry standard. However, all lobster holding facilities attempt to maintain product quality and saleability. An efficient storage method is to replicate, as close as possible, the lobster's natural overwintering conditions which induce a state of torpor (i.e. reduced metabolism at low water temperatures, 1.5 to 2 °C) (Lavallée 1999). Nonetheless, handling and high density storage produce adverse stress and immunosuppression of lobsters that result in losses. Anecdotally, significant mortality and weight loss that occur during holding (10-15 %) are referred to, in industry terms, as "shrinkage", and a major portion of the shrinkage is related to diseases. Historically, the three major diseases with economic impact are gaffkemia (bacterial disease caused by *Aerococcus viridans* var. *homari*) (see Stewart et al. 1969), bumper car disease (caused by the ciliate *Anophryoides haemophila*) (see Cawthorn 1997), and shell disease (cuticular lesions caused by various bacteria) (Smolowitz et al. 2005). The threat to any holding facility is the introduction of a pathogen to a previously stressed, at risk population. Therefore, addition of new

lobsters for long term storage must be critically evaluated in order to avoid major shrinkage associated with disease outbreaks.

The lobster industry in Canada and the USA are reciprocally interconnected. Canadian processors import up to 70 % of the annual landings from the USA to supplement the supply of raw material available from local fisheries (Gardner Pinfold 2006). Alternatively, up to 80 % of processed and live lobsters from Canada are exported to the USA (Gardner Pinfold 2006). A major concern, in both countries, is the introduction of live animals that are potential carriers of pathogens. Consequently, the Canadian industry is concerned with the origin and health status of imported lobsters. The recent collapse (1999) of the lobster industry in Long Island Sound generated significant concern among Canadian lobstermen that such catastrophes may occur in Canadian waters.

1.1 Long Island Sound Lobster Die Off

Long Island Sound (LIS), and particularly western LIS, was the site of a major mass mortality outbreak in American lobsters, *Homarus americanus* Edwards 1837, during fall 1999. The western LIS lobster industry declined up to 99 % from previous landings (CTDEP 2000). Collective research investigations identified atypical environmental and human stressors which, combined with the presence of the parasitic amoeba, *Neoparamoeba pemaquidensis*, resulted in significant lobster mortality. Simultaneously, in the eastern part of LIS, fishermen observed an increase in the prevalence of lobsters with shell disease lesions (Smolowitz et al. 2005). Since shell disease did not appear to be directly related to the die off, reports from eastern LIS

raised more concerns about the health status of the LIS lobster population and its sustainability (Pearce & Balcom 2005).

1.1.1 American lobster mass mortality

The chronology of the mortality events varied depending on locale and weather conditions. The observation of lethargic, moribund, and dead lobsters in traps started in late August and early September 1999 (CTDEP 2000, unpublished transcripts of First LIS Lobster Health Symposium 2000). With the exception of limp or lethargic lobsters, the difference between healthy and sick lobsters was difficult to assess by lobstermen. Reports also described mortalities in other marine invertebrates including blue crabs (*Callinectes sapidus* Rahtbun, 1896), rock crabs (*Cancer irroratus* Say, 1817), spider crabs (*Libinia emarginata* Leach, 1815), sea urchins (*Strongylocentrotus droebachiensis* Müller, 1776) and starfish (*Echinaster sepositus* Retzius, 1805). Lobster mortalities continued during the fall until lobstermen finally stopped fishing when the activity was no longer profitable. Analysis of the die-off revealed that mortality did not affect all harbours simultaneously. In the summer 1999, lobster boats from Greenwich, Connecticut, consistently trapped lobsters for 6 weeks (Pearce & Balcom 2005). Unfortunately, after a major rainstorm (50 mm rain), lobsters were no longer caught (Pearce & Balcom 2005). Later in the season, when lobsters returned, catches were one-tenth what they were prior to the storm and some dead lobsters were found in traps (Pearce & Balcom 2005). In New York, regular landings were reported until tropical storm Floyd occurred on September 16th. On September 20th, landings declined by at least 75 %, and dead lobsters were found in traps (Pearce & Balcom 2005).

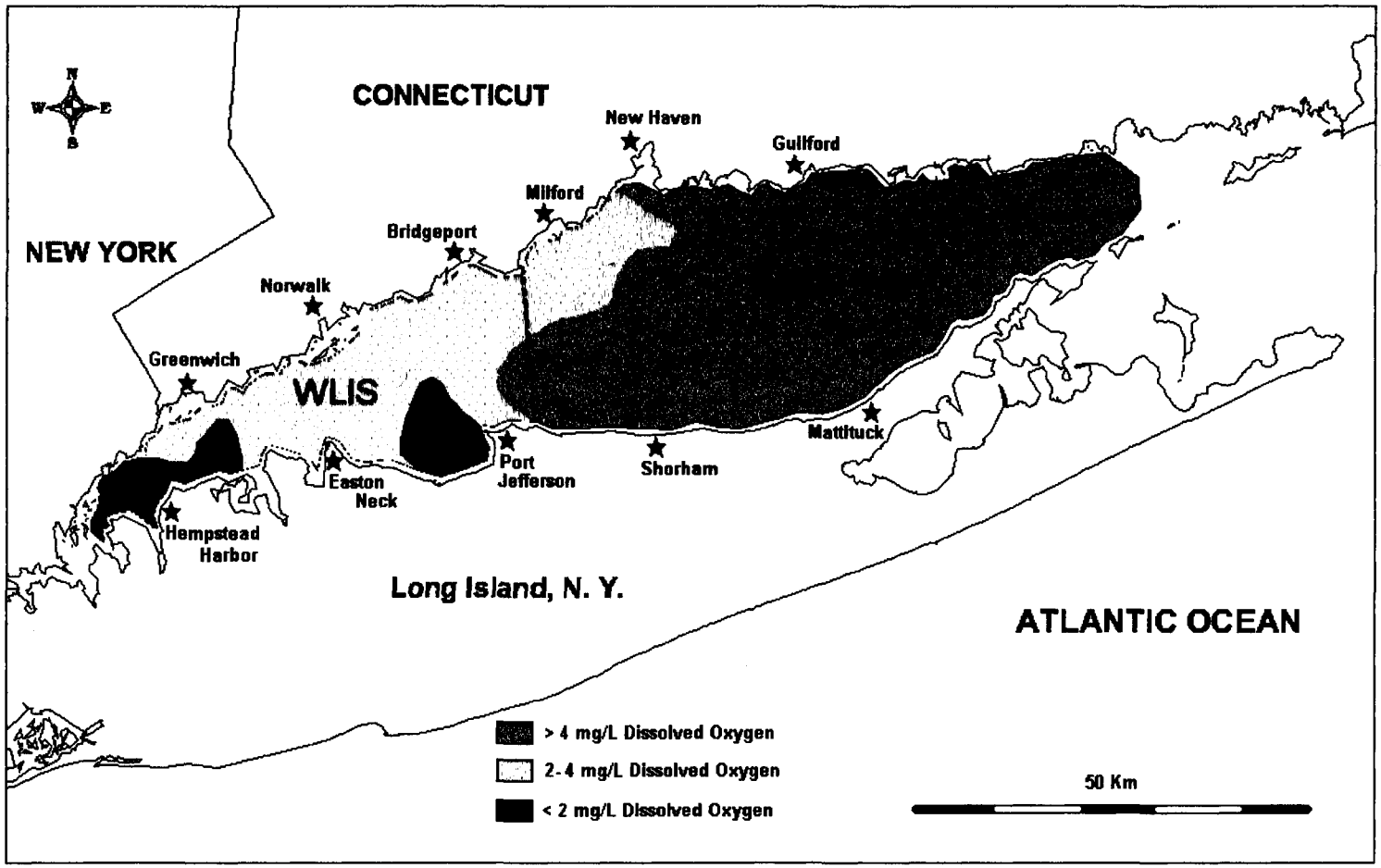


Figure 1.0. Long Island Sound map. Major commercial fishing harbours from Connecticut and New York states. The Sound is arbitrarily split into Western Long Island Sound (WLIS) and Eastern Long Island Sound (ELIS). The colour panels represent areas where the concentration of dissolved oxygen was determined between the August 2-5, 1999 (adapted from CTDEP Long Island Sound monitoring website).

Catches continued to remain low while the proportion of dead lobsters increased. In mid October 1999, lobster processors indicated that a considerable proportion of dead lobsters arrived at their plants and that high mortalities continued (Pearce & Balcom 2005).

At the end of the 1999 fishing season, dead lobsters were estimated at approximately 11 million which resulted in a 90-99 % reduction in landings in western LIS and the failure of the lobster fishery (Howell et al. 2005). Conditions in the western sound fisheries continued to deteriorate in the following four years (2000-2003). Referring to an industry panellist remark, Pearce & Balcom (2005) commented that warm waters and the additional adverse environmental factors might have increased lobster stress and susceptibility in LIS, which can occur in poorly maintained live holding tanks.

1.1.2 Environmental factors

The physiology of marine invertebrates is strongly affected by water temperature. As poikilotherms, growth, reproduction, metabolic rate and survival of lobsters are directly influenced by temperature (Cobb 1976, Aiken & Waddy 1986, Mercaldo-Allen & Kuropat 1994). Lobsters have a broad thermal range, as low as -1 °C or as high as 30.5 °C (Harding 1992). Optimal temperatures are 5 °C to 20 °C (Aiken & Waddy 1986), which can define areas of high population densities (Stewart 1972). At Ram Island, Connecticut, Stewart (1972) reported that bottom temperatures of 18.9 °C inhibited the movement of the lobsters. Respiration rate and stress are significantly

higher in lobsters at temperatures above the threshold of 20.5 °C (Chang 2004, Powers et al. 2004, Dove et al. 2005).

American lobsters occur south to North Carolina (Squires 1990); however, the latitude of LIS represents the warmest limit for commercial exploitation of lobsters (Stewart 1972, Lawton & Lavalli 1995). Typically, during the winter, bottom water temperatures decline to 0 °C to 1 °C in western LIS. However, in 1999, the water temperature never went below 3 °C (Pearce & Balcom 2005). Later in the summer, the temperature gradient between surface and bottom waters was 5 °C and water temperature continued to increase (CTDEP 1999, Wilson & Swanson 2005). On August 29, 1999, the association of strong winds from hurricane Dennis and displacement of a cold water front generated an up-welling pattern in LIS. This phenomenon produced vertical mixing of the water column combined with an increase of bottom temperature by several degrees, to > 22 °C (Wilson et al. 2004, Wilson & Swanson 2005). Persistence of the mixing pattern limited restratification on September 16, 1999 when tropical storm Floyd passed through the region. Comparing two monitoring stations, one 30 m deep in western LIS and the other 70 m deep in eastern LIS, CTDEP observed that bottom temperatures at the western station were above 20 °C for a total of 83 "stress degree days" (Pearce & Balcom 2005). A "stress degree day" is defined as the number of days per year when the bottom water temperature exceeded the 20 °C upper tolerance threshold for lobsters multiplied by the number of degree above 20 °C (Pearce & Balcom 2005). Therefore, one day at 23 °C is equal to three "stress degree days". In contrast to the large number of stress degree days in WLIS, the ELIS experienced only two "stress degree days" in 1999 when bottom temperatures never exceeded 18 °C or 19

°C (Miller 2004, Pearce & Balcom 2005). During the summer, LIS lobsters typically migrate to deeper, colder water when shallow water becomes too warm (Stewart 1972). During summer 1999, lobster movements from shallow waters to high concentration areas in deep waters were observed by lobstermen in central and western LIS (Pearce & Balcom 2005). Finally, long-term monitoring data confirmed strong correlation between mortality in the commercial catch and the mean summer bottom temperature over eight years encompassing the die-off (1996-2003) (Howell et al. 2005). Although bottom water temperature was identified as a significant contributing factor, additional factors were considered important in contributing to the mass mortality of lobsters.

Whereas high water temperature (24 °C) had no lethal effect on lobsters when dissolved oxygen (DO) levels were high (> 5 mg/L), low DO levels (< 2.5 mg/L) at the same temperature killed 50 % of the lobsters (LT_{50}) in 5 days (Draxler et al. 2005). Oxygen saturation level is negatively correlated with water temperature and depth. DO data were collected to assess severity and extent of hypoxic conditions that western LIS experienced in late summer (Pearce & Balcom 2005). Hypoxic conditions were observed from July 2 August 21, 1999 (CTDEP Long Island Sound Water Quality Monitoring Program website). There were strong correlations between elevated temperature and low DO level of bottom waters in a west-to-east gradient across the Sound (Figure 1.0). Lobsters were highly concentrated in areas where DO was > 2 mg/L, and rare or absent in hypoxic areas (DO $\leq$ 2 mg/L) (Pearce & Balcom 2005). Overall the western LIS lobster population, during summer 1999, moved from warm, hypoxic shallow waters to cooler, oxygenated deep waters. The consequent increase in density and interaction among lobsters may have contributed to increased stress, related

to territoriality, dominance behaviour and limited food resources (Karnofsky & Price 1989).

Proliferation of anaerobic microbial flora in the sediment-water interface induced increased organic decomposition associated with the release of sulfide and ammonia (Cuomo et al. 2005). These conditions were exacerbated by warm bottom water temperatures. Experimental trials demonstrated that exposure of lobster to the sulfide and ammonia levels reported in LIS (5.5 μM and 17 μM respectively) combined with low DO (< 2.5 mg/L) decreased the LT_{50} to 3.3 days (Draxler et al. 2005) and increased lobster susceptibility to the pathogen, *Aerococcus viridans*, at summer temperatures (Robohm et al. 2005). Exposure to hypoxia in conjunction with increased levels of sulfide and ammonia might stress LIS lobsters and increase their susceptibility to toxins or pathogenic agents.

1.1.3 Human factors: West Nile Virus and consequences of pesticide load

An epidemic of West Nile Virus (WNV) was reported for the first time in New York and Connecticut during the summer 1999. Seven people died following complications due to the mosquito-borne virus (Pearce & Balcom 2005). To limit propagation of the virus, a program to eradicate adult and larval mosquito populations was in place from early August to mid October 1999, with maximum application of pesticides occurring during the two last weeks of September (Miller et al. 2005, Wilson et al. 2005). Different methods of application and different pesticides were used in the control programs. Pyrethroids (resmethrin and sumithrin) and methoprene were used in New York and Connecticut; malathion was also applied in New York. However, these

pesticides may adversely impact local ecosystems, especially crustaceans that are closely related to insects (Pisani et al. 2004).

The Connecticut Department of Environmental Protection (CTDEP) tested LIS water samples at the University of Connecticut for various of compounds including pesticides, herbicides, PCBs, semi-volatile and volatile organic compounds, heavy metals and cyanide. Although all tests were negative, some compounds may have been present in lower concentrations than the parts per billion detection limits of the analytical equipment (CTDEP 2000). Two separate modeling simulations were performed to estimate levels of individual pesticides in the water column (Miller et al. 2005, Wilson et al. 2005). Both studies considered "Phase I" (which assumed all of the pesticide applied reached the water column and never decayed) (Miller et al. 2005) and "Phase II" scenarios (which assumed all of the pesticide applied reached the water column and then underwent decay) (Miller et al. 2005, Wilson et al. 2005). Even in the "worst case" scenarios, concentrations of the three pesticides did not reach the lethal concentration for either larval or adult lobsters (Miller et al. 2005, Zulkosky et al. 2005, Wilson et al. 2005).

Experimentally the pesticides malathion, resmethrin and methoprene (sumithrin was not considered) were immunotoxic to lobsters at low exposure levels (De Guise et al. 2004, De Guise et al. 2005, Walker et al. 2005, Zulkosky et al. 2005). Major sub-lethal effects were described on all life stages of lobsters, with resmethrin being most toxic, followed by malathion and methoprene (Zulkosky et al. 2005). Although pesticide application levels and residues could not explain the mortality event, low level exposure

of some lobsters in near-coastal waters could have stressed lobsters and weakened their immune systems.

1.1.4 Pathology and discovery of a causative agent

Necropsies were performed on dead and dying lobsters from LIS, and tissue samples were collected for histopathological, ultrastructural, microbiological, and toxicologic analyses (Mullen et al. 2004). Bacterial cultures of hepatopancreas and hemolymph revealed some potential pathogens, but not in any significant number or consistent pattern to be identified as a primary cause. There were no detectable amounts of pesticides such as malathion, methoprene, and resmethrin in hepatopancreas and muscle tissues. Gross lesions consisted of variable red discolouration of skeletal muscle and hemolymph, hyper pigmentation of hemocoelomic viscera, excessive coelomic hemolymph, and nodular hypertrophy of segmental ganglia of the ventral nerve cords. Microscopically, there was mild to moderate, multifocal, hemocytic infiltrates with intralesional protozoa in optic and antennal nerves, supra-esophogeal ganglia and segmental ganglia of the ventral nerve cord. The amoeboid protozoan was morphologically characterized as round to elongate (10-15 μm diameter) with a round, well-defined nucleus and a small Feulgen-positive organelle adjacent to the nucleus. Electron microscopy confirmed the presence of the singular Feulgen-stained organelle, as a parasome, and provided the ultrastructural evidence that the parasite was a member of the group *Paramoeba* Schaudinn, 1896. No protozoa or other microbial agents were detected in healthy control lobsters (Mullen et al. 2004).

Further investigations identified the parasome-containing amoeba as *Neoparamoeba pemaquidensis* Page, 1987 based on the 98 % similarity of consensus sequences constructed from 18S rRNA gene fragments (Mullen et al. 2005). Disease was successfully reproduced experimentally by direct contact between five healthy lobsters from Maine and ten limp lobsters from western LIS. After three weeks post exposure, paramoebiasis was confirmed by histopathology in all ten LIS limp lobsters and in all five Maine lobsters (Mullen et al. 2004). However, isolation and *in vitro* cultivation of amoebae to fulfill Koch's postulates were not successful. Since *Neoparamoeba pemaquidensis* inhabited LIS prior to 1999, perhaps summer water temperatures in western LIS in 1999 provided ideal conditions for pathogen growth and immunodepression of lobsters (Mullen et al. 2005). The presence of *Paramoeba* sp. in lobsters was monitored for 3 years following the 1999 die-off; more than 800 lobsters were collected systematically throughout LIS and showed prevalence rates from 0 to 14 % (Mullen et al. 2005). Therefore, an endemic population of *Neoparamoeba pemaquidensis* continues to be present in the LIS.

1.2 Worldwide Paramoebiasis

Neoparamoeba spp. is the causative agent of paramoebiasis with important ecological and economic impacts in marine fisheries worldwide. Parasome-containing amoebae have been isolated in various hosts, and cause disease conditions ranging from internal infection in invertebrates to gill surface colonization in finfish (Figure 1.1).

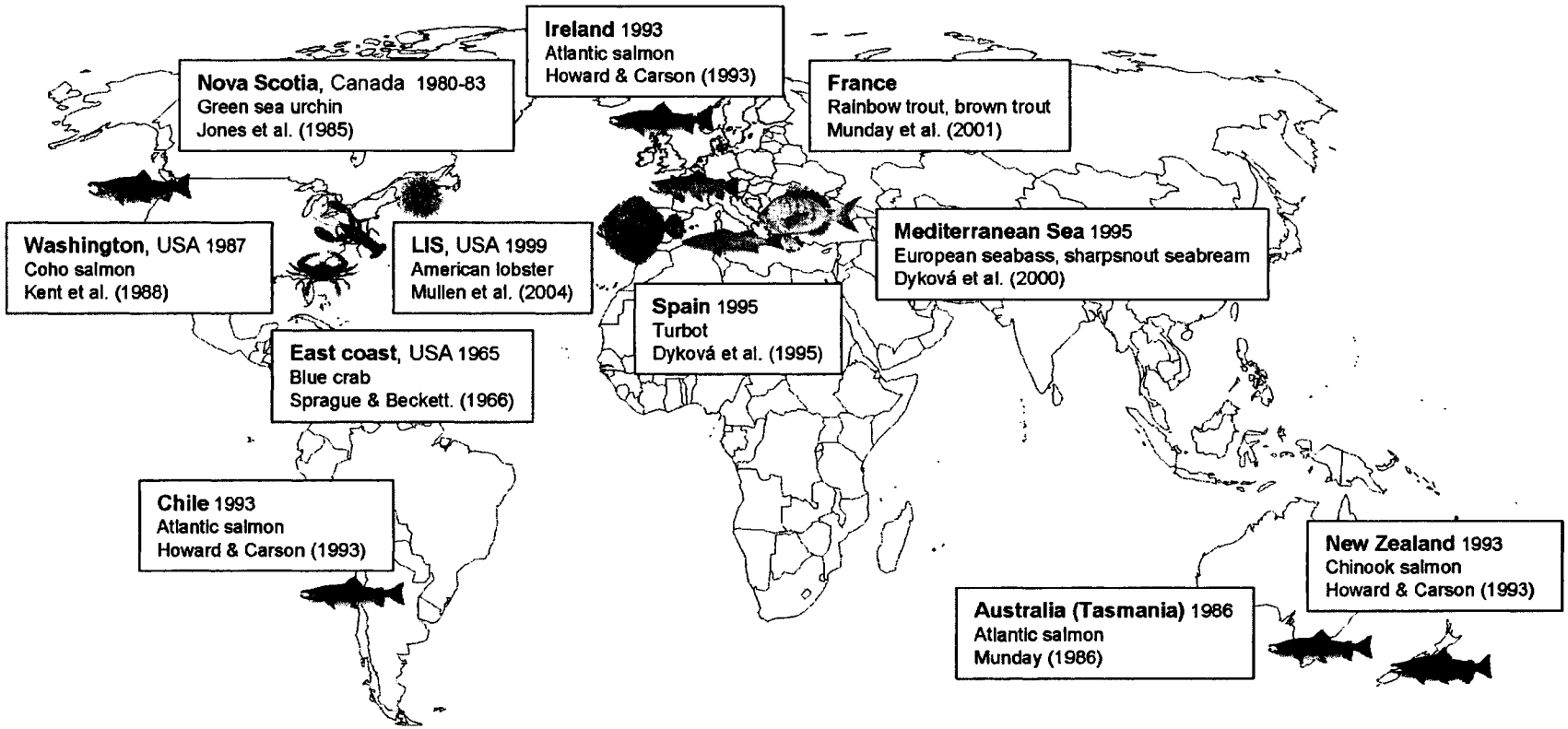


Figure 1.1. Worldwide distribution of paramoebiasis. *Neoparamoeba* spp. have been reported from all continents, except Africa and Asia, in cultured finfish and marine invertebrates. This amphizoic amoeba has also been isolated as a free-living organism from the marine environment.

1.2.1 "Gray Crab Disease" in blue crabs

In the late 1960's, recurrent epidemics in blue crabs, *Callinectes sapidus* occurred in high-salinity areas of Chincoteague and Chesapeake Bays (Maryland and Virginia) and along the east coast from Connecticut to Florida (Sprague & Beckett 1966, 1968, Lunz 1968, Sawyer 1969, Newman & Ward 1973, Johnson 1977). Sprague and Beckett (1966) first described the disease syndrome from peller (pre-molt) crabs in Virginian commercial shedding tanks during a peak period of mortality. The abdomens of infected crabs appeared gray with translucent appendages that contained cloudy hemolymph and watery tissue. Consequently, a crab dealer named the syndrome "gray crab disease". Microscopic examination revealed enormous numbers of amoeboid cells in the hemolymph that contained two nucleus-like bodies with quite different morphology. Initially considered as virus-infected hemocytes (Sprague & Beckett 1966), the amoeboid cells were later identified as a marine amoeba, *Paramoeba* sp. (Sprague & Beckett 1968). By morphological comparison with *Paramoeba eilhardi*, Schaudinn 1896, Sprague et al. (1969) identified and described the crab isolate as a new species, *Paramoeba perniciosa* Sprague, Beckett & Sawyer, 1969.

A preliminary report showed high prevalence of infection (35 %) in wild blue crab populations in the latter part of June and reduced prevalence (8 %) in July and August (Sawyer 1969). The portal of entry for *P. perniciosa* is assumed to be the mid-gut epithelium. Then the amoeba spreads systemically *via* the hemolymph and invades connective tissues and hemal spaces during the terminal stages of the infection. Pathological changes caused by systemic paramoebiasis include tissue displacement,

lysis of muscular tissues and hemocytes, and depletion of nutrient reserves within the host (Johnson 1977). Newman and Ward (1973) stated that blue crabs with amoebic parasitemia invariably died. However, warm waters and chemical pollution may have contributed to the mortalities. Pesticide pollution was considered potentially significant, particularly in view of a fire ant (*Solenopsis invicta*, Westwood 1840) control program using aerial spread of Mirex. This cyclodiene insecticide is very toxic to juvenile blue crabs but has fewer effects upon adults (Lowe at al. 1971).

No recent outbreaks of gray crab disease however, have been reported. The establishment of the new species *P. perniciosa* "without delay" by Sprague et al. (1969) was probably justified since no paramoeba had ever been associated previously with blue crab. However, the use of molecular biology techniques could help to compare *Paramoeba perniciosa* with the morphologically similar *Neoparamoeba pemaquidensis* (Page 1970).

1.2.2 Paramoebiasis of green sea urchins

The Atlantic Coast of Nova Scotia, Canada, experienced two major epidemics of paramoebiasis in green sea urchins, *Strongylocentrotus droebachiensis*, between 1980 and 1983 (Miller & Colodey 1983, Scheibling & Stephenson 1984) and between 1993 and 1995 (Scheibling & Hennigar 1997) that had a major impact on the ecology of this area (Scheibling 1984, 1986, Miller 1985). *Paramoeba invadens* Jones, 1985 was isolated from diseased echinoids (Jones & Scheibling 1985, Jones et al. 1985). Experimental investigations confirmed the etiologic nature of the agent by producing diseased individuals by injection and exposure transmissions (Scheibling & Stephenson

1984, Jones & Scheibling 1985). Furthermore, *P. invadens* was successfully re-isolated from infected echinoids whereas it was not isolated from healthy urchins (Jones & Scheibling 1985). Field observations and laboratory experiments confirmed that transmission and development of the disease occurred faster at higher temperatures (Scheibling & Stephenson 1984). This positive correlation between temperature and growth rate of the amoeba was confirmed with *in vitro* cultures of *P. invadens* (Jellett & Scheibling 1988). In conclusion, Koch's postulates were successfully fulfilled confirming *P. invadens* as the etiologic agent of urchin paramoebiasis.

Clinically, urchin paramoebiasis is characterized within 10 days post infection by the loss of attachment to the substratum of tube feet and failure to right itself after being inverted (Scheibling & Stephenson 1984, Jones & Scheibling 1985). Morphological and histological observations reported muscular degeneration, progressive loss of spines, gaping of the mouth and jaws, and reddish-brown discolouration of tissues (Jones et al. 1985). Most urchin tissues were infiltrated at low numbers with *P. invadens* (Jones et al. 1985). Amoebae could be easily isolated by culturing the radial nerves and coelomic fluid from vascular tissue (Jones & Scheibling 1985). Bacterial infections were reported in late stage paramoebiasis, likely as secondary invaders (Jones & Scheibling 1985).

Recently, O'Kelly (pers. comm.) isolated two strains of amoeba from a moribund sea urchin (UA 1 and UA 6) from the Gulf of Maine in the autumn 2002. The amoebae were identified as *Neoparamoeba pemaquidensis* using the partial sequence of the18S rRNA gene (98-99 % similarity with the Tasmanian strain PA027, Mullen et al.

2005). This suggests that the species name *Paramoeba invadens* may be a junior synonym of *Neoparamoeba pemaquidensis*.

1.2.3 Amoebic Gill Disease

Amoebic Gill Disease (AGD) was first reported from sea-caged Atlantic salmon, *Salmo salar* Linnaeus, 1758 and rainbow trout, *Oncorhynchus mykiss* Walbaum, 1792 soon after the establishment of salmon culture in Tasmania, Australia (Munday 1986). The Tasmanian etiologic agent was subsequently recognized as a member of the genus *Paramoeba* (Roubal et al. 1989). Similar pathogens were identified as *Paramoeba pemaquidensis* Page, 1970 in cultured Coho salmon, *Oncorhynchus kisutch* Walbaum, 1792 in Washington State (Kent et al. 1988), although Page (1987) had redescribed the genus as *Neoparamoeba*. Later, the amoebic organism was isolated and identified in Atlantic salmon from Ireland and Chile and in Chinook salmon, *Oncorhynchus tshawytscha* Walbaum, 1792 from New Zealand (Howard & Carson 1993).

Subsequently, outbreaks of AGD have been reported from most continents where intensive salmonid marine culture is practised. However, the disease has not yet been reported from Canada, Iceland, Scotland or Norway, probably because water temperatures are colder in these locations (Munday et al. 2001). Additional to salmonid species, AGD is a recurrent problem in cultured turbot, *Scophthalmus maximus* Linnaeus, 1758 from north-west Spain since 1995 (Dyková et al. 1995, 1998). Furthermore, only brief references have been made to AGD in European seabass, *Dicentrarchus labrax* Linnaeus, 1758 and sharpsnout seabream, *Diplodus puntazzo*

Cetti, 1777 (Dyková & Novoa 2001) and in wild fish (Foster & Percival 1988a, Nowak et al. 2000).

AGD has major impact mainly in Tasmanian aquaculture with significant losses and costs up to 20 % of production (Munday et al. 2001). During warm summers, peak mortalities in smolts reach 10 % per week with losses of 2-4 % per week in 1-2 kg fish and 1-2 % per week in fish over 2 kg (Foster & Percival 1988a). Clinical signs are lethargy and respiratory distress manifested as rising to the surface of the water and increased rate of opercular movement (Kent et al. 1988, Munday et al. 1990, Rodger & McArdle 1996). Salmon gills show white to grey multifocal patches associated with swollen tissues and excess mucus (Munday et al 2001).

The most consistent environmental factors associated with AGD are water temperature and salinity. Generally, outbreaks occurred at water temperature from 12 to 20 °C (Kent et al. 1988, Munday et al. 1990), although Douglas-Helders et al. (2001) reported AGD at 9.1-10.6 °C. In turbot, the maximum temperatures ranged from 14 to 18.8 °C (Dyková et al. 1998). Chronic infections reported in salmonids have been associated with high salinity (≥ 32 ‰) (Munday et al 1990). In contrast, in turbot AGD occurred at a constant salinity of 22 ‰ (Dyková et al. 1998). The recent description and identification of some turbot isolates as *Neoparamoeba branchiphila* (Dyková et al. 2005), may explain the differences in salinity.

Current treatment involves fish being bathed in oxygenated fresh water for up to 4 h (Foster & Percival 1988b), and is presently the most successful treatment for AGD (Parsons et al. 2001). Freshwater appears to significantly reduce the prevalence of mucoid patches on the gills and the presence of *Neoparamoeba* on the lesions (Parsons

et al. 2001). However, the cost of treatment is the major factor that contributes to economic losses associated with AGD (Munday et al. 2001). In the field, AGD prevalence is reduced for up to 21 days post freshwater bath (Clark & Nowak 1999); but total removal of the parasite is not achieved (Parsons et al. 2001, Clark et al. 2003), and surviving amoebae are capable of initiating recurrent AGD (Clark et al. 2000). Adaptation of amoebae to the bathing procedure may be due to the hardness of freshwater, requiring supplementary treatment to achieve total removal of *Neoparamoeba* (Roberts & Powell 2003).

AGD prevention is traditionally done by prophylactic freshwater baths or by utilizing low salinity sites for all or part of the salmonid marine culture phase (Munday et al. 2001). Since experimental serial passage infection appeared to amplify the virulence of the pathogen, management strategy requires an all-in all-out plan associated with fallowing, lower fish density and increased distance between cages (Munday et al. 2001).

1.3 Taxonomy and Diagnostics

1.3.1 Taxonomic history

1.3.1.1 Paramoeba, Schaudinn 1896

In 1896, the German protistologist F. Schaudinn described the genus *Paramoeba* which includes amoebae containing, in addition to the nucleus, a secondary DNA-rich body, the *Nebenkörper* (or parasome). The original type species, *Paramoeba eilhardi*

Schaudinn, 1896, was isolated from a marine aquarium at the Zoological Institute in Berlin. The precise description of the naked (no permanent external shell) lobose (fingerlike pseudopodia) amoeba defined the organism as a member of the subclass Gymnamoebia (phylum Rhizopoda, class Lobosea). Complexity of the amoeba taxonomy is well recognized and has led one researcher to call it: "one of the knottiest problems in zoology is the specific identification of naked...amoebas" (Bovee 1953). Before Schaudinn, Grassi (1881) described two species of parasome-containing amoebae that were testicular parasites of chaetognaths. These two species were considered members of the genus *Paramoeba* by Janicki (1912) and consequently named *Paramoeba pigmentifera* Grassi, 1881 and *Paramoeba chaetognathi* Grassi, 1881. Later, Poche (1913) created the family Paramoebidae to accommodate the parasome-containing amoebae. However, Chatton (1953) placed them in a new genus, *Janickina* Chatton, 1953. Hollande (1980), after detailed structural comparison, merged the two species of *Janickina* under the name *J. pigmentifera* Chatton, 1953.

Other free-living parasome-containing species have been described from the marine environment. De Faria (1922) described *Paramoeba schaudinni* de Faria, 1922 isolated from laboratory saltwater aquaria in which the water source was the bay of Rio de Janeiro. Although the isolate is no longer accessible, this amoeba is suspected to be a rediscovery of the species *P. eilhardi* (Page 1970).

Later, electron microscopy provided additional morphological features on the cell surface that facilitate descriptions of species. Grell and Benwitz (1970) discovered scales on the surface of *P. eilhardi*. *Janickina* spp. have a glycocalyx (surface coat) but no scales (Hollande 1980). Sprague et al. (1969) described the first parasitic *Paramoeba*

in North America, *Paramoeba perniciosa* Sprague, Beckett & Sawyer, 1969, isolated from diseased blue crabs. One year later, Page (1970) described two new free-living species from Maine: *Paramoeba pemaquidensis* Page, 1970 isolated in the intertidal zone in Pemaquid Beach; and *Paramoeba aestuarina* Page, 1970 isolated in the estuary of the Damariscotta River. Cann and Page (1982) discovered a glycocalyx on *P. pemaquidensis* and *P. aestuarina* cell membranes that were subdivided into hexagonal "glycostyles". Jones (1985) described an additional *Paramoeba* species, *Paramoeba invadens* Jones, 1985 isolated from diseased green sea urchins. Page (1987) created the genus *Neoparamoeba* to separate species with hexagonal glycostyles (*N. pemaquidensis*, type species of *Neoparamoeba*, and *N. aestuarina*) from *P. eilhardi*, which has scales on the cell surface. He transferred *Neoparamoeba* from Paramoebidae to the family Vexilliferidae Page, 1987, which includes two other amoeba genera lacking endosymbionts, *Pseudoparamoeba* Page, 1979 and *Vexillifera* Schaeffer, 1926. Page (1987), however, did not consider parasitic species.

Glycostyles are absent from the surface of the blue crab-borne amoeba (Perkins & Castagna 1971), and from the urchin-borne (Jones 1985) and lobster-borne amoebae (Mullen et al. 2004). The observation that *N. pemaquidensis* with glycostyles was formerly identified and characterized as parasitic on finfish gills (Kent et al. 1988, Elliot et al. 2001, Fiala & Dyková 2003, Wong et al. 2004) and without glycostyles in lobster (Mullen et al. 2004, Mullen et al. 2005), suggests that *Neoparamoeba* can down regulate the expression of glycostyles during internal parasitism. Consequently, the presence of glycostyles is not reliable for morphological diagnosis of invasive stages (Mullen et al. 2005). However, Dyková et al. (2005) demonstrated that morphological criteria are not

meaningful for amoeba taxonomy and consequently described a new species based on the 18S rRNA gene sequence, *Neoparamoeba branchiphila* Dyková et al., 2005.

Parasitic *Neoparamoeba* from lobsters, sea urchins and finfish cluster together in a single phylogenetic clade (Mullen et al. 2005), and are considered representatives of the same species (O'Kelly 2003, Dyková et al. 2005). This species, if it includes the blue crab pathogen, would be called *Neoparamoeba perniciosa* Sprague, Beckett & Sawyer, 1969; consequently *N. pemaquidensis* and *P. invadens* would be synonyms of *P. perniciosa* (O'Kelly 2003). Phylogenetic analysis (Peglar et al. 2003, Dyková et al. 2005) also suggests that the family Vexilliferidae (containing the genus *Neoparamoeba*) is a separate sister group of the family Paramoebidae (containing the genus *Paramoeba*), a clade that contains *Korotnevella* Goodkov, 1988 and *Vexillifera* Schaeffer, 1926 (Figure 1.2).

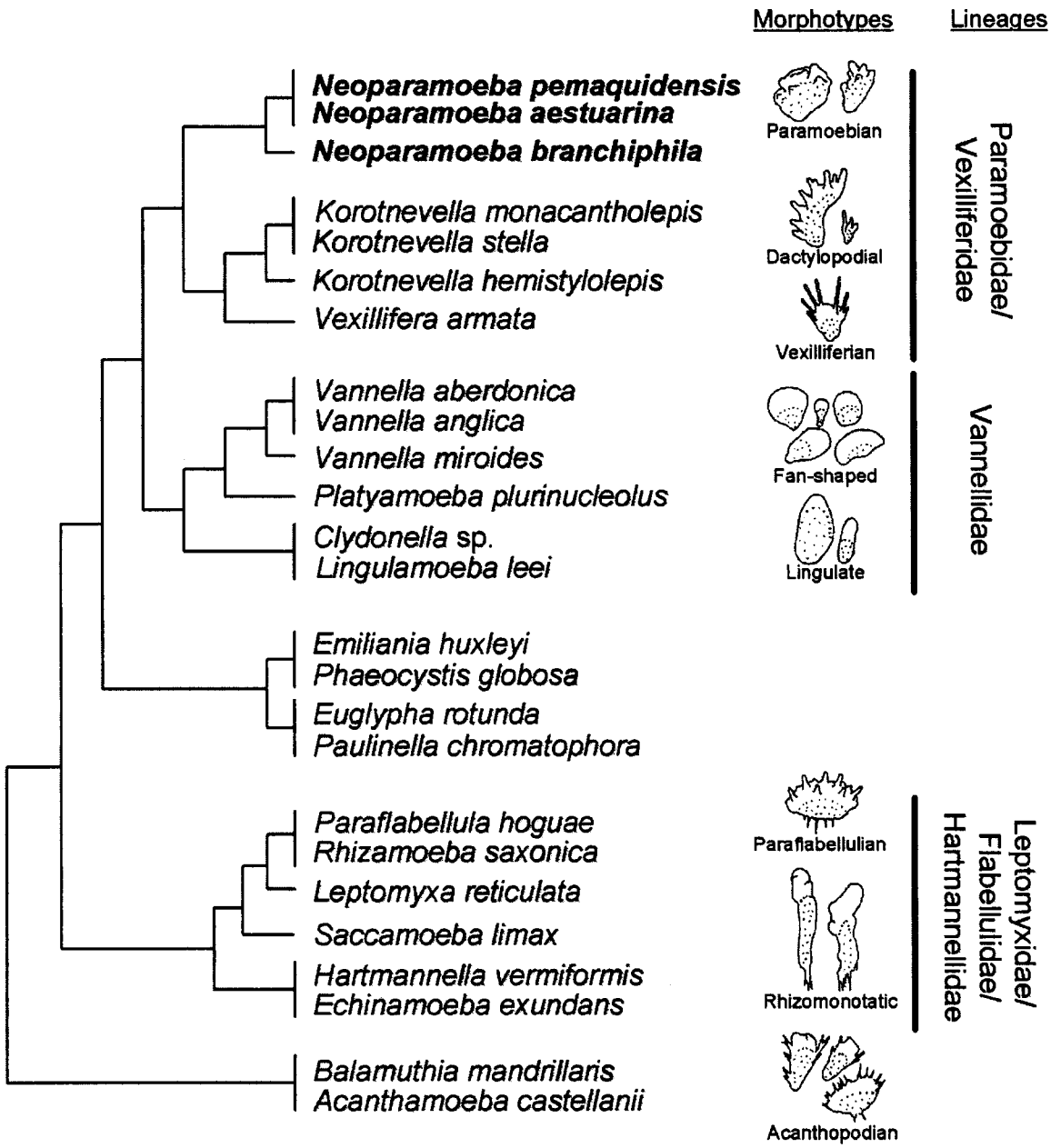


Figure 1.2. Phylogenetic tree of the Subclass Gymnamoebia using 18S rRNA sequences. Position of *Neoparamoeba* clade (in bold) within the Gymnamoebia lineage (adapted from Peglar et al. 2003, Dyková et al. 2005). Morphotypes of Gymnamoebia adapted from Smirnov & Goodkov 1999.

1.3.1.2 Endosymbiont

When Schaudinn (1896) described the amoeba *Paramoeba eilhardi*, he defined the *Nebenkörper* (a nuclear-like organelle) as the fundamental and characteristic organelle of the genus *Paramoeba*. The nuclear-like organelle was often observed juxtaposed to the nucleus of the amoeba but the author did not know its significance and identified it as a kind of plastid. Furthermore, Schaudinn described the life cycle of the marine amoeba including a biflagellate zoospore in which he recognized the *Nebenkörper*. Subsequently, the *Nebenkörper* was the subject of many investigations and different terminologies have been used. Janicki (1912) renamed the organelle the "*nucleus secondus*" since he considered it a true nucleus. Minchin (1922), perhaps by mistake, considered it as a "*Nebenkern*" a term usually applied to a specific manifestation of the chondriome in insect spermatids. De Faria et al. (1922) described a *Paramoeba schaudinni* species isolated from the Marine Aquarium of Rio de Janeiro, and introduced the term "paranucleus" to describe the peculiar organelle. Later, Janicki (1928), in the description of two new *Paramoeba* species, kept the name paranucleus.

Only Schaudinn (1896) observed the flagellispore stage. However, Hollande (1940) evaluated this stage in the life cycle of *P. eilhardi* by comparing the biflagellate spore and its *Nebenkörper* original description with *Cryptomonas dangeardi* and its "amphosome". Hollande concluded that Schaudinn's observations were probably influenced by the contamination of *Cryptomonas* cells in his culture of *P. eilhardi*. Chatton (1953) still described the amoeba life cycle with the flagellate stage but noted that only Schaudinn had observed this phase and that Hollande's arguments could be correct.

Chatton (1953) assigned a new genus *Janickina* to the family Paramoebidae Poche, 1913 to differentiate the two marine amoebae described by Janicki: *Janickina pigmentifera* and *Janickina chaetognathi*. In addition, Chatton termed the organelle "amphosome", perhaps misunderstanding Hollande's (1940) publication. The interpretation of the paranucleus as a true nucleus was supported for years as an accessory nucleus from the amoeba (Janicki 1928) or as a parasitic nucleus which lost its cytoplasmic membrane and organelles during symbiosis (Grell 1961). In 1966, Kudo adopted this hypothesis in his Protozoology text using the term "secondary nucleus" in reference to the *Nebenkörper*. Sprague et al. (1969) used the same term to describe the inclusion observed in the new species *Paramoeba perniciosa*, etiological agent of "gray crab disease" in blue crab, *Callinectes sapidus*.

Ammerman (cited in Hollande 1980) demonstrated that *P. eilhardi* will die when the *Nebenkörper* is eliminated from the amoeba by UV irradiation. The closely apposed position of secondary nucleus to the real amoeba nucleus suggests that all the genetic material of the cell was affected. When Page (1970) described two new species of *Paramoeba* from Maine (*P. pemaquidensis*, *P. aestuarina*), he supported the nuclear nature of the inclusion and suggested the term "parasome" but only in an English-language publication. Because of the lack of information and useful tools to solve the identification problem of the parasome, he proposed postponing the terminology until the nature of the "body" is settled.

Using the electron microscope, Grell and Benwitz (1970) redefined the nature of the organelle not as a single nucleus but as a cell. Similarly, Perkins and Castagna (1971) confirmed the interpretation of a "discrete organism, not an organelle" by

studying the ultrastructure of the *Nebenkörper* of *P. perniciosa*. They described the inclusion as "two eukaryotic nuclei separated by a prokaryotic-like nucleoid with cytoplasm" and suggested that the *Nebenkörper* was a symbiotic microorganism. Grell (1973) later confirmed this hypothesis based on ultrastructural data.

The presence of a complete endosymbiont or intracellular parasite was further supported by Hollande (1980), where he redefined the median segment (Mittelstück) of the *Nebenkörper* not as a prokaryotic nucleus but as a kinetoplast composed of dispersed DNA fibrils in a reticulated matrix surrounded by a double membrane. Based on the cell structure, he interpreted the *Nebenkörper* as a symbiont originating from kinetoplastid flagellates and proposed a new generic and specific name: *Perkinsiella amoebae*. However, the final recognition of the *Nebenkörper* as a kinetoplastid endosymbiont was clouded by two unfortunate circumstances. First the original cultures and type specimen *Janickina pigmentifera* and *Janickina chaetognathi* are no longer available in protist collections. Secondly, the genus *Perkinsiella* Kirkaldy, 1903 was previously defined to designate a genus containing three species of sugarcane planthopper from Australia (Kirkaldy 1903). Therefore, we cannot refer to the original specimens and the name of the kinetoplastid endosymbiont genus was already taken. The confusion continued when Dyková et al. (2000) renamed the parasome of amoebae from the genera *Paramoeba* and *Neoparamoeba* as *Perkinsiella amoebae* Like Organism (PLO).

Fortunately, based on the 18S rRNA gene sequence, Dyková et al. (2003) determined that PLOs are organisms related to the kinetoplastid *Ichthyobodo necator* Henneguy, 1883, an important flagellate parasite of fish gills. Moreover, they suggested

coevolution between the PLO and its *Neoparamoeba* host, observing the congruent phylogeny of both organisms (Dyková et al. 2003, Dyková & Lom 2004).

The phylogenetic origin of this new organism (Figure 1.3) provided better understanding of kinetoplastid phylogeny with creation of the new order Prokinetoplastida (including the PLO, *I. necator* and unnamed organisms from hydrothermal vents) within the class Kinetoplastea (Moreira et al., 2004). Whereas the origin of the PLO is clearer, the extent of coevolution of this symbiotic relationship with *Neoparamoeba* is undetermined. Detailed molecular investigations will likely provide further insight to the origin, coevolution and the biology of the endosymbiont parasite.

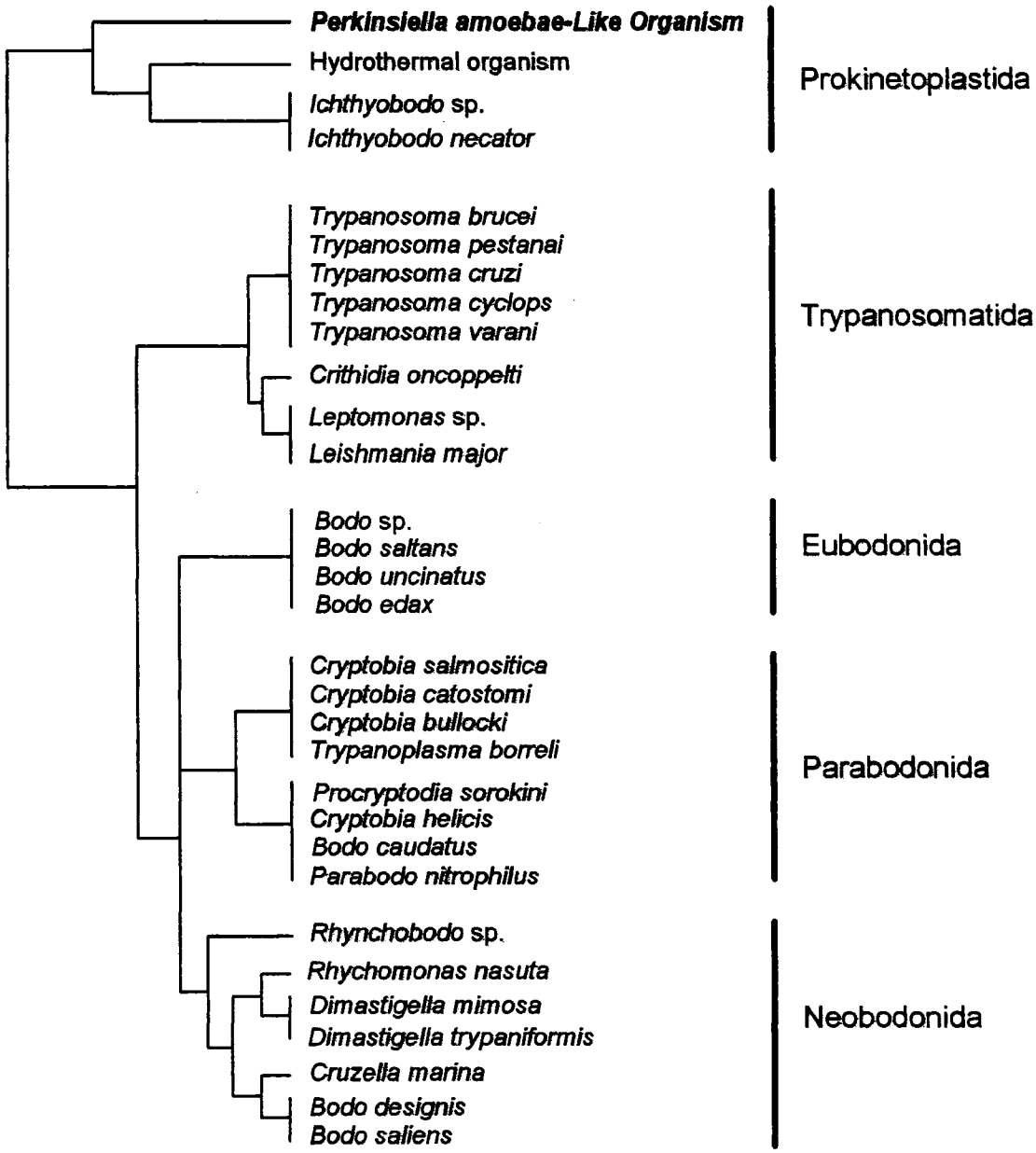


Figure 1.3. Phylogenetic tree of the Class Kinetoplastea using 18S rRNA sequences. Basal position of the *Perkinsiella* amoebae-Like Organism (in bold) within the five kinetoplastid orders (adapted from Moreira et al. 2004). The endosymbiont of *Neoparamoeba* sp. clusters with *Ichthyobodo necator* to create a basal clade (order Prokinetoplastida) sister group of the apical kinetoplastid phylum.

1.3.2 *Neoparamoeba* diagnostic methods

Traditional diagnostics of paramoebiasis have relied on the observation of gross clinical signs in the context of epidemiological analyses of outbreaks. For AGD, gill lesions are usually evaluated by counting white mucoid patches on the gills (Alexander 1991), and the gills are scored on the number of affected hemibranchs to determine the level of infection (Adams & Nowak 2003). However, the association between gross examination and the presence of *N. pemaquidensis* histologically is inconsistent in the field (Clark & Nowak 1999). The presumptive presence of *N. pemaquidensis* must be confirmed by complementary analyses including histology, immunological-based techniques or molecular biology.

1.3.2.1 Histopathology

The first rapid method to detect *N. pemaquidensis* microscopically is a simple gill smear stained with Quick Dip® (Fronine Pty Ltd, Riverstone, NSW, Australia). The stained wet mounts routinely revealed good analytical performance. Amoebae appear dark blue with darker blue and purple stained internal organelles (Zilberg et al. 1999). By examining gill arches with a dissecting microscope, the severity of the lesions could be assessed by calculating the percentage of affected filaments (Adams & Nowak 2001, Adams & Nowak 2003). Histological protocols for examination of gills are standard: Davidson's fixation, dehydration, embedding, sectioning and haematoxylin & eosin or Giemsa staining. Dissection and fixation need to be done rapidly to prevent gill

autolysis. The attachment of the amoeba causes characteristic cytopathology described by three progressive phases (Adams & Nowak 2003):

1. Initial attachment of amoebae to secondary lamellae is associated with localized host cellular alteration, including desquamation and oedema of surface epithelial cells. Thickening of secondary lamellae begins with hypertrophy and some hyperplasia of epithelial cells and oedema of the entire epithelium (Adams & Nowak 2003).

2. These regions progressed to more pronounced hyperplasia where fusion of secondary lamellae occurred simultaneously with oedema of the primary filament epithelium. The innate immune response is activated with infiltration of leucocytes migrating from the central venous sinus to the oedema (Adams & Nowak 2003).

3. Finally, lesions expand laterally with multifocal hyperplasia and lamellar fusions. Epithelial squamation and stratification at the lesion surface are associated with mucous cell recruitment, causing spongiotic appearance of the tissue. At this stage, several authors noted the development of interlamellar vesicles or channels often containing amoebae and inflammatory infiltrates in the supporting tissue (Kent et al. 1988, Roubal et al. 1989, Adams & Nowak 2001).

However, with the potential problem of gill autolysis and the likely presence of mixed amoeba gill infestations, the exact identity of amoebae is difficult to determine with histology. The precise identification of *N. pemaquidensis* requires the utilization of more specific methods.

1.3.2.2 Immunochemistry

To increase specificity, immunocytostaining was applied to gill smears and histological sections (Howard & Carson 1993). The indirect immunofluorescent antibody test (IFAT) uses polyclonal antisera from rabbits and goats previously immunized against *N. pemaquidensis* strain PA027. The IFAT, routinely used in Tasmania, was assumed highly effective (covalidated with histopathology) and considered as the "gold standard" (Zilberg et al. 1999). The specificity of the polyclonal antiserum has been assessed using a range of amoebae commonly found on gills associated with AGD: *Platyamoeba plurinucleolus* Page, 1968; *Platyamoeba* sp. Page, 1968; *Vanella* sp. Bovee, 1965; and *Flabellula* sp. Schaeffer, 1926 (Howard & Carson 1993). No cross reactivity was detected with these amoebae and the specificity of the antiserum was considered high.

Recently, Douglas-Helders at al. (2001) developed an immuno-dot blot test for the detection of *N. pemaquidensis* in non-lethal samples. Using the same polyclonal antiserum, the authors put digested mucus on membranes to test the samples with the antibodies. Verified by the correlation between immuno-dot blot and IFAT test (gold standard) and the correlation between IFAT and histopathology, Douglas-Helders at al. (2001) inferred that correlation exists between immuno-dot blot and histopathology and that the immuno-dot blot test was validated. The test was confirmed to be pathogen specific, sensitive, with strong repeatability (Douglas-Helders at al. 2001). These features and the ease of use made the immuno-dot blot test convenient for screening large number of samples. However, the antiserum cross-reacted with the closely related species *Neoparamoeba aestuarina* and *Pseudoparamoeba pagei* Page, 1979. Since these

amoeba species have not been isolated from gills affected with AGD (Howard & Carson 1993), the authors considered that the test possessed strong specificity to detect *N. pemaquidensis*. Recently, a new species belonging to the genus *Neoparamoeba* has been described using molecular techniques: *Neoparamoeba branchiphila* (Dyková et al. 2005). This amoeba has been isolated from gills of Atlantic salmon and turbot associated with episodes of AGD. Although *N. branchiphila* is highly suspected to be pathogenic, this has not yet been proven (Dyková et al. 2005). By only detecting *N. pemaquidensis*, the highly specific immunological tests concealed the discovery of *N. branchiphila*.

Moreover, Villavedra et al. (2005) compared the change of the antigenic profile during *in vitro* culture of *Neoparamoeba* sp. from amoebae freshly isolated from gills and old sub-cultured amoebae (PA027). Although the two isolates initially shared only two major antigens, the antigenic profile of the fresh isolate tended to vary over a 15 day period before attaining the same profile as the old cultures. Similarly, two *Neoparamoeba* sp. isolates cultured in different conditions (solid agar versus liquid medium) revealed differences in the antigenic profile whereas under the same conditions the antigenic profiles were very similar. Assuming that the antigenic profiles of *Neoparamoeba* sp. are constantly changing and that the antisera were obtained using *in vitro* cultured amoebae, this questions the analytical sensitivity of these immunological tests. Increases in genetic investigations of *N. pemaquidensis* have led to the development of new tools based on molecular biology with optimized sensitivity and specificity.

1.3.2.3 Molecular techniques

A nested two-step PCR targeting the 18S rRNA gene was developed for the detection of *N. pemaquidensis* at the same time as the IFAT (Elliot et al. 2001, Wong et al. 2004). However, the cross-specificity of the immunological tests and the changing antigenic profile of *Neoparamoeba* sp. were not yet known (Douglas-Helders at al. 2001, Villavedra et al. 2005). Since no one doubted the immunological tests, a molecular assay was setup only for detection in environmental samples where high numbers of cross-reacting organisms may be present. In the nested PCR, the first amplification step uses a *Neoparamoeba* genus specific primer (Np-Hxe23a1) coupled with an 18S rRNA gene universal reverse primer (Elliot et al. 2001, Wong et al. 2004). This primary amplification maximizes the concentration of target DNA template for the secondary PCR step. The second-round amplification uses an internally nested *N. pemaquidensis* specific primer set. The internal set comprises a *N. pemaquidensis* specific forward primer (fNp-Hxe23b1) and a *Neoparamoeba* genus specific reverse primer (rNp-Hx49) (Elliot et al. 2001, Wong et al. 2004). The second step increases the sensitivity of detection and the specificity of the assay (Elliot et al. 2001, Wong et al. 2004).

The specificity of the *N. pemaquidensis* primer set was tested against a panel of DNA from target and non-target organisms including *N. aestuarina*, *Pseudoparamoeba pagei* and *Paraflabellula hoguae* Sawyer, 1975 (Elliot et al. 2001, Wong et al. 2004). Although the threshold of PCR detection was not determined on field samples, the detection limit of the assay was estimated at approximately 40 amoeba cells in sterilized sea water. The major factors that affect the sensitivity of PCR detection in

environmental samples are the efficiency of DNA extraction from low numbers of the target organism in a complex biofilm matrix, and the possible presence of amplification inhibitory substances in the DNA preparation. Although two techniques based on filtered crude or culture enriched samples were developed to improve detection in environmental tests, the results from environmental studies remained inconsistent and too complex for interpretation (Elliot et al. 2001, Wong et al. 2004). Moreover, the nested PCR assay was unsuccessful in detecting *N. pemaquidensis* on fish gill samples (intact filaments and mucus scrapings). Among 15 fishes presenting AGD-like mucoid gill patches, only 4 (27 %) were positive by IFAT, 9 (60 %) by immunoblotting, and 2 (13 %) by PCR. There may be several reasons for the discrepancy between PCR and immunological tests. DNA extraction and amplification could be inhibited by excessive mucus or blood in the gill samples, or by the presence of numerous inhibitor enzymes from the autolyzed tissue (Wilson, 1997).

Moreover, regarding the description of *Neoparamoeba branchiphila* (see Dyková et al. 2005) combined with the high specificity of PCR tests and the low specificity of immunological tests (Douglas-Helders at al. 2001), divergent results between the two techniques might now be explained. Several methods of quantitative detection (real time PCR, flow cytometry) of *Neoparamoeba* sp. were developed for environmental samples but low sensitivity and imprecise quantification resulted in an unresolved relationship between AGD prevalence and the presence of *Neoparamoeba* sp. in net-pen or sediment samples (Nowak et al. 2005). Recently, another nested PCR protocol incorporating the 18S rRNA gene was developed for the LIS survey. The test generated a 165 bp product from Paramoebidae/Vexilliferidae amoebae (PV) without

cross-reactivity with genomic DNA from invertebrate hosts: lobster, blue crab and sea urchin (Mullen et al. 2005). The two-step PCR does not amplify templates from *Pseudoparamoeba pagei* or *Korotnevella hemistylolepis* and more importantly does not detect *N. pemaquidensis* strain ATCC 50172 (Mullen et al. 2005), a known AGD causing isolate (Kent et al. 1988). The lack of specificity and probably sensitivity of the test therefore requires caution when considering its incorporation into a screening or surveillance program.

The overall low correlation among these diagnostic tests confirmed that none is perfect, adapted or validated for paramoebiasis. The PCR results have however revealed that amoebae associated with AGD in three salmonid species farmed from four different countries USA (Coho salmon), New Zealand (Chinook salmon), Ireland and Australia (Atlantic salmon), belonged to the same species *Neoparamoeba pemaquidensis* (Elliot et al. 2001).

1.4 Rationale

The amoeboid protozoan *Neoparamoeba pemaquidensis* is a well known parasite involved in finfish gill infections (Munday et al. 2001) and in marine invertebrate mortalities (Sprague et al. 1969, Jones 1985, Mullen et al. 2005). This amphizoic protist was initially isolated as a free-living bacteriovorous amoeba from surface sediments (Page 1970). However, the life cycle and the biology of the pathogen remain uncertain.

A lack of understanding of the protist's epidemiology requires the development of efficient tools to answer critical questions that remain unresolved. Although *Neoparamoeba branchiphila* was recently associated with AGD (Dyková et al. 2005),

N. pemaquidensis is the primary target for investigations. Using conventional features and advanced techniques (morphology, antigenic profiles, SSU sequences), *N. pemaquidensis* can not be identified and characterized at a lower taxon level than the species level. The ability to recognize strains of *N. pemaquidensis* could help to further understand pathogenicity, host specificity, tissue tropism, outbreak history and geographical diversity of the parasite. The rationale of this research is to explore methods that allow the discrimination of *N. pemaquidensis* at the sub-species level.

1.5 Objectives

Molecular biology provides sensitive and specific techniques to detect and characterize organisms based on genetic information. The first step is to select a molecular marker with characteristics that fit with the final application. To assess the quality of a taxonomic marker, we must estimate the intra-taxon and inter-taxon variability. For example, based on the local alignment of the *Neoparamoeba* spp. 18S rRNA gene sequences available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide), the percentage of similarity among sequences within *N. pemaquidensis*, *aestuarina* and *branchiphila* (95.9-99.4 %, 96.9-98.1 %, 96-98.4 % respectively) was higher than the percentage of similarity among the three species (87.6-94.7 %). Consequently, Wong et al. (2004) reported that the 18S rRNA was an excellent species marker for *N. pemaquidensis* diagnostics, and Dyková et al. (2005) confirmed the gene to be appropriate for the discovery of species within the genus *Neoparamoeba*. Although the 18S rRNA gene showed attractive features at the species level, its intra-specific variability was not high

enough to differentiate the amoeba strains within a species (Dyková & Lom 2004). Therefore, the objectives of this study were to initiate an exploration phase to identify a hypervariable marker, then to evaluate its performance in differentiating isolates, and finally to integrate the chosen locus in the development of diagnostic tools.

1.6 References

Adams MB, Nowak B (2001) Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. J Fish Dis 24:535-542

Adams MB, Nowak BF (2003) Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. J Fish Dis 26:601-614

Aiken DE, Waddy SL (1986) Environmental influence on recruitment of the American lobster, *Homarus americanus* - a perspective. Can J Fish Aquat Sci 43:2258-2270

Alexander JM (1991) Treatment of amoebic gill disease: field trials, 1990/1991. In: Proceedings of the SALTAS Research and Review Seminar. Salmon Entreprise of Tasmania, Hobart, Tasmania 51-102

Bovee EC (1953) Morphological identification of free living Amoebida. Proc Iowa Acad Sci 60:599-615

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

Cawthorn RJ (1997) Overview of "bumper car" disease-impact on the North American lobster fishery. Int J Parasitol 27:167-172

Chang E (2004) Stress indicators in crustaceans: crustacean hyperglycaemic hormone and stress proteins. Second working meeting of LIS Lobster Research Initiative May 3-4 Groton, Connecticut

Chatton E (1953) Ordre des Amoebiens nus. In: Masson et Cie (ed) Traité de Zoologie. Paris 3-148

Clark A, Nowak BF (1999) Field investigations of amoebic gill disease in Atlantic salmon, *Salmo salar* L., in Tasmania. J Fish Dis 22:433-443

Clark G, Nowak BF, Powell, M (2000) Long term effects of freshwater bathing. AGD in the new millenium. Nowak BF (Ed.) Tasmanian Aquaculture and Fisheries Institute, Taroona, Tasmania

Clark G, Powell MD, Nowak B (2003) Effects of commercial freshwater bathing on reinfection of Atlantic salmon, *Salmo salar*, with amoebic gill disease. Aquaculture 219:135-142

Cobb SJ (1976) The American lobster: the biology of *Homarus americanus*. Rhode Island Sea Grant Marine Tech Rpt No. 49. 32 pp

CTDEP (1999) Monitoring data summaries from Long Island Sound water quality monitoring program survey. State of Connecticut Department of Environmental Protection.

CTDEP (2000) Impact of 1999 lobster mortalities in Long Island Sound. Marine Fisheries Office. Connecticut Department of Environmental Protection. Marine Fisheries Office, Old Lyme 46 pp

CTDEP (2006) The Long Island Sound Water Quality Monitoring Program. http://dep.state.ct.us/wtr/lis/monitoring/lis_page.htm

Cuomo C, Dogru D, Valente R, Draxler AFJ, Deshpande A, Howard D, Wieczorek D, Kapareiko D, Robohm RA, Sherrell RM, Varekamp E, Mullen TE, Buchholtz ten Brink M, Altabet M, Cooper S, Sangiorgi F, Wilson RE, Swanson RL, Waliser D, Lopez G, Cerrato R (2004) The role of the physical/chemical conditions in lobster mortality in Long Island Sound. Fourth Long Island Sound Lobster Health Symposium October 4th Stony Brook, New York

Cuomo C, Valente R, Dogru D (2005) Seasonal variations in sediment and bottom water chemistry of western Long Island Sound: implications for lobster mortality. J Shellfish Res 24:805-814

De Faria JG, da Cunha AM, Pinto C (1922) Estudos sobre Protozoarios do mar. Mem Inst Oswaldo Cruz 15:186-208

De Guise S, Maratea J, Perkins C (2004) Malathion immunotoxicity in the American lobster (*Homarus americanus*) upon experimental exposure. Aquat Toxicol 66:419-425

De Guise S, Maratea J, Chang ES, Perkins C (2005) Resmethrin immunotoxicity and endocrine disrupting effects in the American lobster (*Homarus americanus*) upon experimental exposure. J Shellfish Res 24:781-786

Douglas-Helders G, Carson J, Howard T, Nowak B (2001) Development and validation of a new dot blot test for the detection of *Paramoeba pemaquidensis* (Page) in fish. J Fish Dis 24:273-280

Dove ADM, Allam B, Powers JJ, Sokolowski MS (2005) A prolonged thermal stress experiment on the American lobster, *Homarus americanus*. J Shellfish Res 24:761-765

Draxler AFJ, Robohm RA, Wieczorek D, Kapareiko D, Pitchford S (2005) Effect of habitat biogeochemicals on survival of lobsters (*Homarus americanus*). J Shellfish Res 24:821-824

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, Casal JF (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. Dis Aquat Org 33:137-141

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, Novoa B (2001) Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*. Bull Eur Assoc Fish Pathol 21:40-44

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

Dyková I, Lom J (2004) Advances in the knowledge of amphizoic amoebae infecting fish. Folia Parasitol 51:81-97

Dyková I, Nowak BF, Crosbie PB, Fiala I, Peckova H, Adams MB, 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

Elliot NG, Wong F, Carson J (2001) Detection and abundance of *Paramoeba* species in the environment. FRDC Project 98/209, 1-68. Hobart. CSIRO Marine Research.

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

Foster CK, Percival S (1988a) Paramoebic gill disease. Occurrence of *Paramoeba* in Tasmania. SALTAS Aquanote No.15 Dover, Tasmania

Foster CK, Percival S (1988b) Treatment of paramoebic gill disease in salmon and trout. SALTAS Aquanote No.14 Dover, Tasmania

Gardner Pinfold Consulting Economists Ltd (2006) Benchmarking study on the Canadian lobster. Submitted to: Agriculture and Agri-Food Canada. 45 pp

Grassi GB (1881) Interni ad alcuni protisti endoparasitici etc. Atti Soc Ital Sc Nat 24

Grell KG (1961) Uben den "Nebenkörper" von *Paramoeba eilhardi* Schaudinn. Arch Protistenk 105:303-312

Grell KG, Benwitz G (1970) Ultrastrukturb mariner amoben I. *Paramoeba eilhardi* Schaudinn. Arch Protistenk 112:119-137

Grell KG (1973) Protozoologie. 3rd Edition, Berlin-Heidelberg-New York 362-364

Harding GC (1992) American lobster (*Homarus americanus* Milne Edwards): a discussion paper on their environmental requirements and the known anthropogenic effects on their populations. Can J Fish Aquat Sci 1887

Hollande A (1940) Le "*Nebenkorper*" 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

Howard T, Carson J (1993) Are there alternatives to freshwater treatment of AGD? In: Proceedings of the SALTAS Research and Development Review Seminar. Valentine P (ed), Tasmania 81-97

Howell P, Benway J, Giannini C, McKown K, Burgess R, Hayden J (2005) Long-term population trends in American lobster (*Homarus americanus*) and their relation to temperature in Long Island Sound. J Shellfish Res 24:849-857

Janicki C (1912) Untersuchungen an parasitischen arten der gattun *Paramoeba* Schaudinn (*P. pigmentifera* Grassi und *P. chaetognathi* Grassi). Verhand der Naturfoschenden-Gesselschaft in Basel 23:1-16

Janicki C (1928) Studien am genus *Paramoeba* Schaudinn. Zeits Wiss Zool 131:588-644

Jellett JF, Scheibling RE (1988) Effect of temperature and prey availability on growth of *Paramoeba invadens* in monoxenic culture. Appl Environ Microbiol 54:1848-1854

Johnson P (1977) Paramoebiasis in the blue crab, *Callinectes sapidus*. J Invertebr Pathol 29:308-320

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

Jones GM, Hebda AJ, Scheibling RE, Miller RJ (1985) Histopathology of the disease causing mass mortality of sea urchins (*Strongylocentrotus droebachiensis*) in Nova Scotia. J Invertebr Pathol 45:260-271

Jones GM, Scheibling RE (1985) *Paramoeba* sp. (Amoebida, Paramoebidae) as the possible causative agent of sea urchin mass mortality in Nova Scotia. J Parasitol 71:559-565

Karnofsky EB, Price HJ (1989) Dominance, territoriality, and mating in the lobster, *Homarus americanus*: a mesocosm study. Mar Behav Physiol 15:101-121

Kent ML, Sawyer T, Hedrick RP (1988) *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of Coho salmon *Onchorhynchus kisutch* reared in sea water. Dis Aquat Org 5:163-169

Kirkaldy GW (1903) Miscellanea Rhynchotalia. Entomologist 36:179-181

Kudo RR (1966) Protozoology. 5th edition. Thomas, Springfield 111 pp

Lavallée J (1999) Risk factors affecting post-capture health and productivity of impounded American lobsters (*Homarus americanus*) in Atlantic Canada. M.Sc. University of Prince Edward Island

Lawton P, Lavalli KL (1995) Postlarval, juvenile, adolescent, and adult ecology. In: Factor J.R. (ed) Biology of lobster, *Homarus americanus*. Academic Press, New York 47-88

Lowe JI, Parrish PR, Wilson AJ, Wilson PD, Duke TW (1971) Effects of mirex on selected estuarine organisms. Trans 36th N Amer Wildl Nat Resour Conf 171-186

Lunz GR (1968) In hearings before the subcommittee on fisheries and wildlife conservation of the Committee on Merchant Marine and Fisheries, House of Representatives. H. R. 18808 Ser. No.90-29. Commercial Fisheries Research Legislation

Mercaldo-Allen R, Kuropat CA (1994) Review of American lobster (*Homarus americanus*) habitat requirements and responses to contaminant exposures. Technical Memorandum. NMFS-NE-105, NOAA, NMFS, Department of Commerce 52 pp

Miller REL (2004) Applying water quality modeling technology to investigate the summer 1999 decline in Long Island Sound lobsters. Fourth Long Island Sound Lobster Health Symposium October 4th Stony Brook, New York

Miller REL, Wands JR, Chytalo KN, D'Amico RA (2005) Application of water quality modeling technology to investigate the mortality of lobsters (*Homarus americanus*) in western Long Island Sound during the summer of 1999. J Shellfish Res 24:859-864

Miller RJ (1985) Succession in sea-urchin and seaweed abundance in Nova Scotia, Canada. Mar Biol 84:275-286

Miller RJ, Colodey AG (1983) Widespread mass mortalities of the green sea-urchin in Nova Scotia, Canada. Mar Biol 73:263-267

Minchin EA (1922) An introduction to the study of Protozoa. Arnold, London 517 pp

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 TE, Russell S, Tucker MT, Maratea JL, Koerting C, Hinckley L, De Guise S, Frasca S, French RA, Burrage TG, Perkins C (2004) Paramoebiasis associated with mass mortality of American lobster *Homarus americanus* in Long Island Sound, USA. J Aquat Anim Health 16:29-38

Mullen TE, Nevis KR, O'Kelly CJ, Gast RJ, Frasca S (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 24:719-731

Munday BL (1986) Diseases of salmonids. In: Humphrey JD, Langdon JS (eds) Proceedings of the workshop on disease of Australian fish and shellfish. Department of Agriculture and Rural Affairs, Benalla, Victoria 127-141

Munday BL, Foster CK, Roubal FR, Lester RJG (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. (eds) Pathology in Marine Science. Academic Press, London 215-222

Munday BL, Zilberg D, Findlay V (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. J Fish Dis 24:497-507

Newman M, Ward GJr (1973) An epizootic of blue crabs, *Callinectes sapidus*, caused by *Paramoeba perniciosa*. J Invertebr Pathol 22:329-334

Nowak B, Douglas-Helders GM, Dawson D (2000) AGD-effects of environmental and husbandry factors. Nowak B. AGD in the new Millenium. Tasmanian Aquaculture and Fisheries Institute, Taroona, Tasmania.

Nowak B, Crosbie P, Adams M, Morrison R (2005) Relationship between the presence of *Neoparamoeba* sp. in the environment and Amoebic Gill Disease in cultured Atlantic salmon in Tasmania. XIth International Meeting on the Biology and Pathogenicity of Free-Living Amoebae. Ceske Budejovice, Czech Republic Folia Parasitol 52:12A

O'Kelly CJ (2003) Protist Image Data, *Paramoeba* and *Neoparamoeba*. http://megasun.bch.umontreal.ca/protists/paramoeba/summary.html

Page FC (1970) Two new species of *Paramoeba* from Maine. J Protozool 17:421-427

Page FC (1987) The classification of 'naked' amoeba (phylum Rhizopoda). Arch Protistenk 133:199-217

Parsons H, Nowak B, Fisk D, Powell MD (2001) Effectiveness of commercial freshwater bathing as treatment against amoebic gill disease in Atlantic salmon. Aquaculture 195:205-210

Pearce J, Balcom N (2005) The 1999 Long Island Sound Lobster mortality event: Findings of the comprehensive research initiative. J Shellfish Res 24:691-697

Peglar MT, Amaral Zettler LA, Anderson OR, Nerad TA, Gillevet PM, Mullen TE, Frasca S Jr, Silberman JD, O'Kelly CJ, Sogin ML (2003) Two new small-subunit ribosomal RNA gene lineages within the subclass Gymnamoebia. J Eukaryot Microbiol 50:224-232

Perkins FO, Castagna M (1971) Ultrastructure of the Nebenkorper or "secondary nucleus" of the parasitic amoeba *Paramoeba perniciosa* (Amoebida, Paramoebidae). J Invertebr Pathol 17:186-193

Pisani D, Poling LL, Lyons-Weiler M, Hedges SB (2004) The colonization of land by animals: molecular phylogeny and divergence times among arthropods. BMC Biol 19:1.

Poche F (1913) Das system der protozoa. Arch Protistenk 30:125-321

Powers J, Lopez G, Cerrato R, Dove A (2004) Effects of thermal stress on Long Island Sound lobsters, *Homarus americanus*. Second working meeting of LIS Lobster Research Initiative May 3-4 Groton, Connecticut

Roberts SD, Powell MD (2003) Reduced total hardness of fresh water enhances the efficacy of bathing as a treatment for amoebic gill disease in Atlantic salmon, *Salmo salar* L. J Fish Dis 26:591-599

Robohm RA, Draxler AFJ, Wieczorek D, Kapareiko D, Pitchford S (2005) Effects of environmental stressors on disease susceptibility in American lobsters: a controlled laboratory study. J Shellfish Res 24:773-779

Rodger HD, McArdle JF (1996) An outbreak of amoebic gill disease in Ireland. Vet Rec 139:348-349

Roubal FR, Lester RJG, Foster CK (1989) Studies on cultured and gill-attached *Paramoeba* sp. (Gymnamoebae, Paramoebidae) and the cyto-pathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. J Fish Dis 12:481-492

Sackton J (2004) Pricing and market factors affecting the Canadian lobster fishery. Prepared for Maritimes Fishermen's Union and Martime Lobster Processor's Co-operativve. Seafood Datasearch http://www.seafood.com 32 pp

Sawyer T (1969) Preliminary study on the epizootiology and host-parasite relationship of *Paramoeba* sp. in the Blue crab, *Callinectes sapidus*. Proceedings of the National Shellfisheries Association 59:60-64

Schaudinn F (1896) Der zeugungskreis von *Paramoeba eilhardi* nov. gen. nov. sp. Sitzungsberichte der Koniglich Preussiscken Akademie der Wissensckaften zu Berlin31-41

Scheibling RE (1984) Echinoids, epizootics and ecological stability in the rocky subtidal off Nova-Scotia, Canada. Helgoländer Meeresunters 37:233-242

Scheibling RE (1986) Increased macroalgal abundance following mass mortalities of sea urchins (*Strongylocentrotus droebachiensis*) along the Atlantic coast of Nova Scotia. Oecologia 68:186-198

Scheibling RE, Stephenson RL (1984) Mass mortality of *Strongylocentrotus droebachiensis* (Echinodermata, Echinoidea) off Nova-Scotia, Canada. Mar Biol 78:153-164

Scheibling RE, Hennigar AW (1997) Recurrent outbreaks of disease in sea urchins *Strongylocentrotus droebachiensis* in Nova Scotia: evidence for a link with large-scale meteorologic and oceanographic events. Mar Ecol Prog Ser 152:155-165

Smirnov AV, Goodkov AV (1999) An illustrated list of basic morphotypes of Gymnamoebia (Rhizopoda, Lobosea). Protistology 1:20-29

Smolowitz R, Chistoserdov AY, Hsu A (2005) A description of the pathology of epizootic shell disease in the American lobster, *Homarus americanus*, H. Milne Edwards 1837. J Shellfish Res 24:749-756

Sprague V, Beckett RL (1966) A disease of blue crabs (*Callinectes sapidus*) in Maryland and Virginia. J Invert Pathol 8:287-289

Sprague V, Beckett RL (1968) Note on the nature of the etiological agent of "gray crab" disease. J Invert Pathol 11:503

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

Squires HJ (1990) Decapod crustacea of the Atlantic coast of Canada. Can J Fish Aquat Sci 221

Stewart LL (1972) The seasonal movement, population dynamics, and ecology of the lobster, *Homarus americanus* (Milne-Edwards) off Ram Island, Connecticut. Ph.D. University of Connecticut

Stewart JE, Arie B, Zwicker BM, Dingle JR (1969) Gaffkemia, a bacterial disease of the lobster, *Homarus americanus*: effects of the pathogen, *Gaffkya homari*, on the physiology of the host. Can J Microbiol 15:925-932

Villavedra M, McCarthy K, To J, Morrison R, Crosbie P, Broady K, Raison RL (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

Walker AN, Bush P, Wilson T, Chang ES, Miller T, Horst MN (2005) Metabolic effects of acute exposure to methoprene in the American lobster, *Homarus americanus*. J Shellfish Res 24:787-794

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741-3751

Wilson RE, Swanson RL, Waliser D (2004) Summary points concerning prevailing water conditions. Second working meeting of LIS Lobster Research Initiative May 3-4 Groton, Connecticut

Wilson RE, Swanson RL (2005) A perspective on bottom water temperature anomalies in Long Island Sound during the 1999 lobster mortality event. J Shellfish Res 24:825-830

Wilson RE, Crowley HA, Brownawell BJ, Swanson RL (2005) Simulations of transient pesticide concentrations in Long Island Sound for late summer 1999 with a high resolution coastal circulation model. J Shellfish Res 24:865-875

Wong FY, Carson J, Elliott NG (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

Zilberg D, Nowak B, Carson J, Wagner T (1999) Simple gill smear staining for diagnosis of amoebic gill disease. Bull Eur Ass Fish Pathol 19:186-189

Zulkosky AM, Ruggieri JP, Terracciano SA, Brownawell BJ, McElroy AE (2005) Acute toxicity of resmethrin, malathion and methoprene to larval and juvenile American lobsters (*Homarus americanus*) and analysis of pesticide levels in surface waters after Scourge (TM), Anvil (TM) and Altosid (TM) application. J Shellfish Res 24:795-804

Chapter II: MICROHETEROGENEITY AND COEVOLUTION: AN EXAMINATION OF rDNA SEQUENCE CHARACTERISTICS IN *NEOPARAMOEBA PEMAQUIDENSIS* AND ITS PROKINETOPLASTID ENDOSYMBIONT

2.1 Introduction

The amphizoic marine amoeba *Neoparamoeba pemaquidensis* (Page 1970) Page, 1987 is the etiological agent of Amoebic Gill Disease (AGD) in sea-farmed salmonids (Kent et al. 1988, Munday et al. 1990, Munday et al. 1993, Roubal et al. 1989), and non-salmonid fish hosts (Dyková et al. 1995, Dyková et al. 1998, Dyková et al. 1999, Fiala & Dyková 2003). In addition, there is evidence that *Neoparamoeba pemaquidensis* causes paramoebiasis in American lobster (Mullen et al. 2004, Mullen et al. 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 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 (Grell & Benwitz 1970, Page 1987, Perkins & Castagna 1971, Cann & Page 1982). Consequently, some parasome-containing amoebae were removed from *Paramoeba* and moved to other genera (*Janickina*, Chatton 1953; *Neoparamoeba*, Page 1987). *Neoparamoeba* species belong to a separate lineage of amoebae, recently recognized at the molecular level (Fiala & Dyková 2003, Peglar et al. 2003), but the relationships of

the other parasome-containing amoebae to each other and to other Gymnamoebia have not yet been elucidated.

The structure and reproduction of the parasome have been examined many times (Schaudinn 1896, Janicki 1912, de Faria et al. 1922, Minchin 1922, Janicki 1928, Hollande 1940, Chatton 1953, Grell 1961, Kudo 1966, Grell 1968, Sprague et al. 1969, Grell & Benwitz 1970, Page 1970, Perkins & Castagna 1971). However, the exact origin and biological significance of this structure has proven 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 et al. (2000) renamed the endosymbiont of amoebae from the genera *Paramoeba* and *Neoparamoeba* as *Perkinsiella 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 et al. 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 *Ichthyobodo necator* Related Organism (IRO). Because of the difficulties in separating *Neoparamoeba* species morphologically and ultrastructurally, there has been 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 (Elliot et al. 2001, Dyková et al. 2003, Fiala & Dyková 2003, Peglar et al. 2003, Wong et al. 2004, Dyková et al. 2005; Mullen et al. 2005). The 18S rRNA gene is relatively well conserved and is 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 (Hillis & Dixon 1991) (Figure 2.0) that have been used to examine both intra-specific and inter-strain variation, as well as intra-genomic variability in various organisms. 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 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. 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 ITS 1 and ITS 2 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 ITS region intra-specific variability and estimated the level of microheterogeneity of nuclear and IROs sequences from six *Neoparamoeba pemaquidensis* isolates. Additionally, informative sites obtained from sequences allowed for parallel phylogenetic studies, which has led to a

better understanding of the interactions between *N. pemaquidensis* and its endosymbiont.

2.2 Materials and Methods

2.2.1 Amoeba isolates & cultures

Six isolates of *Neoparamoeba pemaquidensis* and one of *Neoparamoeba aestuarina* (Page, 1970) Page, 1987 were obtained from private and public culture collections (Table I). The two CCAP isolates were grown in MY75S agar medium at room temperature (19-22 °C). ATCC isolates were cultured in "ATCC medium 994" agar medium at room temperature (19-22 °C) bacterized (Page 1983) with *Klebsiella pneumoniae*. Urchin amoebae 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 (19-22 °C).

2.2.2 Genomic DNA extraction

Amoebae were detached from the agar using 2 ml of sterile sea water 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, Ontario, Canada). DNA concentration was determined spectrophotometrically and quality was assessed by electrophoretic separation in a 0.8 % agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

Table I. *Neoparamoeba* spp. and respective endosymbiont *Ichthyobodo necator* Related Organism isolates information. AGD: Amoebic Gill Disease. CCAP: Culture Collection of Algae and Protozoa. UA: Urchin amoeba. ATCC: American Type Culture Collection. IRO: *Ichthyobodo necator* Related Organism.

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

2.2.3 Amplification & sequencing of Internal Transcribed Spacers regions

The ITS region of *Neoparamoeba* spp. was amplified using universal eukaryote primers NLF 1624/20/SSU rDNA (5'-TTTGYACACACCGCCCGTCG-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 et al. 1994) (Figure 2.0). Approximately 10-50 ng of genomic DNA was amplified in a 50-µl reaction containing 10 pmol of each primer NLF 1624/20/SSU rDNA and NLR 204/21 in the presence of the following reagents (Fermentas International Inc., Burlington, Ontario, Canada): 200 µM of each dNTP (A, G, C and T), 1.5 mM $MgCl_2$, 10X PCR Buffer (10 mM Tris-HCl, pH = 8.8, 50 mM KCl and 0.8 % 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, Ontario, 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'-GCGCACTACAATGACAAAGTG-3') positioned on the 3' end of the 18S rRNA gene, and an universal eukaryote reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), positioned on the 5' end of the 28S rRNA gene (Ristaino et al. 1998) (Figure 2.0). Each 50-µl reaction included 100 ng of genomic

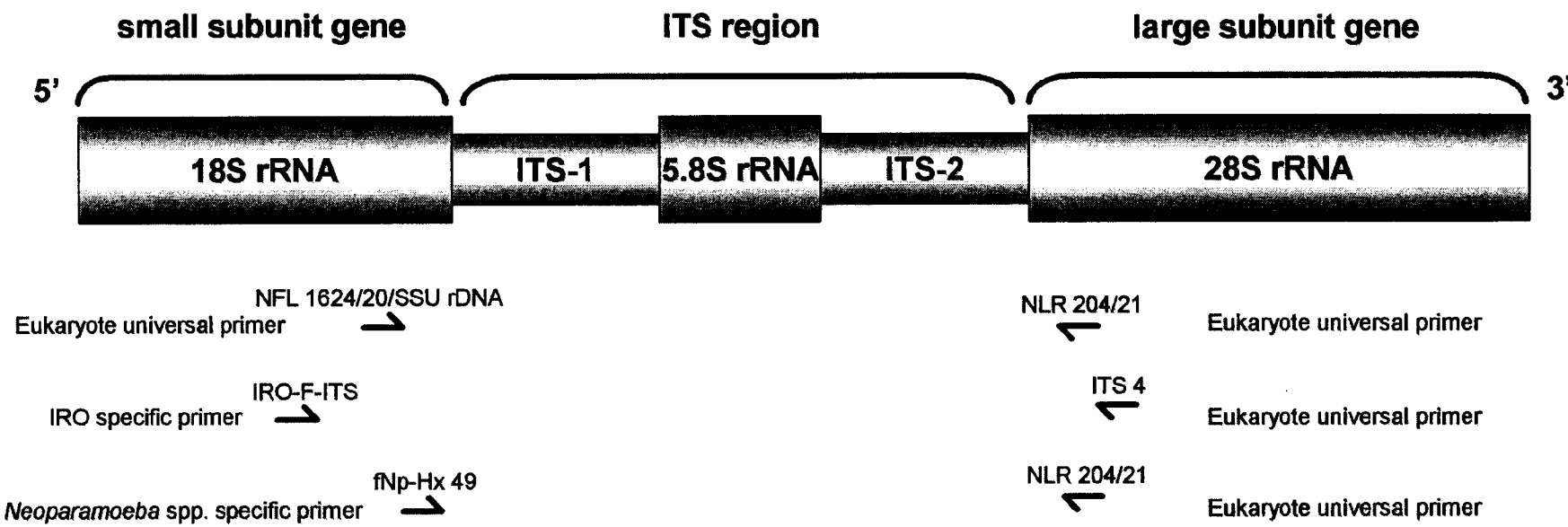


Figure 2.0. Eukaryote ribosomal RNA transcriptional unit map with relative position of primer sets used for ITS region amplification. rRNA: ribosomal RNA. ITS: Internal Transcribed Spacer.

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 pCR 2.1 using the TOPO TA Cloning® Kit (Invitrogen Canada Inc., Burlington, Ontario, 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 in both direction using M13 F & R primers on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems Inc., Foster City, California, USA) at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, Ontario, Canada).

2.2.4 ITS regions analysis

ITS region sequences were assembled by alignment using BioEdit (Hall 1999). 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 the18S 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 and the 18S and 28S ends. Estimations of the nucleotide diversity (Pi) and respective standard deviation (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 bp, step size: 10 bp) on the total length of the obtained sequences. The Arlequin software (Schneider et al. 2000) was used to perform a hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), using the Kimura 2-parameter distance method and considering the inter-strain level (4 defined strains: CCAP, UA, ATCC 30735 and ATCC 50172), the inter-isolate within strain level (2 isolates, CCAP 1560/4&5, within strain CCAP and 2 isolates, UA1 and UA6, within strain UA), and the intra-isolate level. Phylogenies were constructed with MEGA-2 software (Molecular Evolutionary Genetics Analysis-2) (Kumar et al. 2001) using neighbor-joining (Kimura 2-parameter model with gaps and missing data handled by complete deletion) and maximum parsimony. Statistical evaluation of phylogenetic tree branching order was bootstrap-resampled 1,000 times (Felsenstein 1985). A host:parasite coevolution test, using ParaFit (Legendre et al. 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 co-evolution through a test of significance incorporating 9999 random permutations.

2.3 Results

2.3.1 *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 (Table II). The GenBank accession numbers are the following (Table II): *Neoparamoeba pemaquidensis* CCAP 1560/4 clones # 1 to 8 (DQ167506 to DQ167513), CCAP 1560/5 clones # 1 to 8 (DQ167514 to DQ167521), UA1 clones # 1 to 8 (DQ167522 to DQ167529), UA6 clones # 1 to 8 (DQ167530 to DQ167537), ATCC 30735 clones # 1 to 8 (DQ167538 to DQ167545), and ATCC 50172 clones # 1 to 8 (DQ167546 to DQ167553).

2.3.1.1 CCAP isolates

The total length of the eight CCAP 1560/4 sequences varied from 748 to 752 base pairs (bp) with an intra-isolate nucleotide diversity (Pi) of 0.0201. For the eight CCAP 1560/5 sequences, the total length was 746 to 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 Jukes and Cantor (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 (respectively P = 0.25 and P = 0.21).

2.3.1.2 UA isolates

The total length of the eight UA1 sequences varied from 734 to 740 bp with a Pi of 0.0296. For the eight UA6 sequences, the total length was 731 to 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 (respectively P = 0.94 and P = 0.58). 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.

2.3.1.3 ATCC isolates

The total length of the eight ATCC 30735 sequences varied from 734 to 739 bp with an estimated Pi of 0.0216. For the eight ATCC 50172 sequences, the total length was 770 to 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 to the CCAP strain (Da(JC) = 0.086); and 31 when compared to the UA strain (Da(JC) = 0.057). ATCC 50172 sequences had 24 fixed nucleotide differences when compared to the CCAP strain (Da(JC) = 0.044); and 54 when compared to 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 ITS 1 and ITS 2 regions compared to the low levels found within the ribosomal DNA genes (Figure 2.1.A).

2.3.1.1 AMOVA

The molecular analysis of variance confirmed that most of the variation came from inter-strain variability (76.6 %), but also noteworthy was the intra-isolate variability (23.8 %) (Table III). 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). 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.

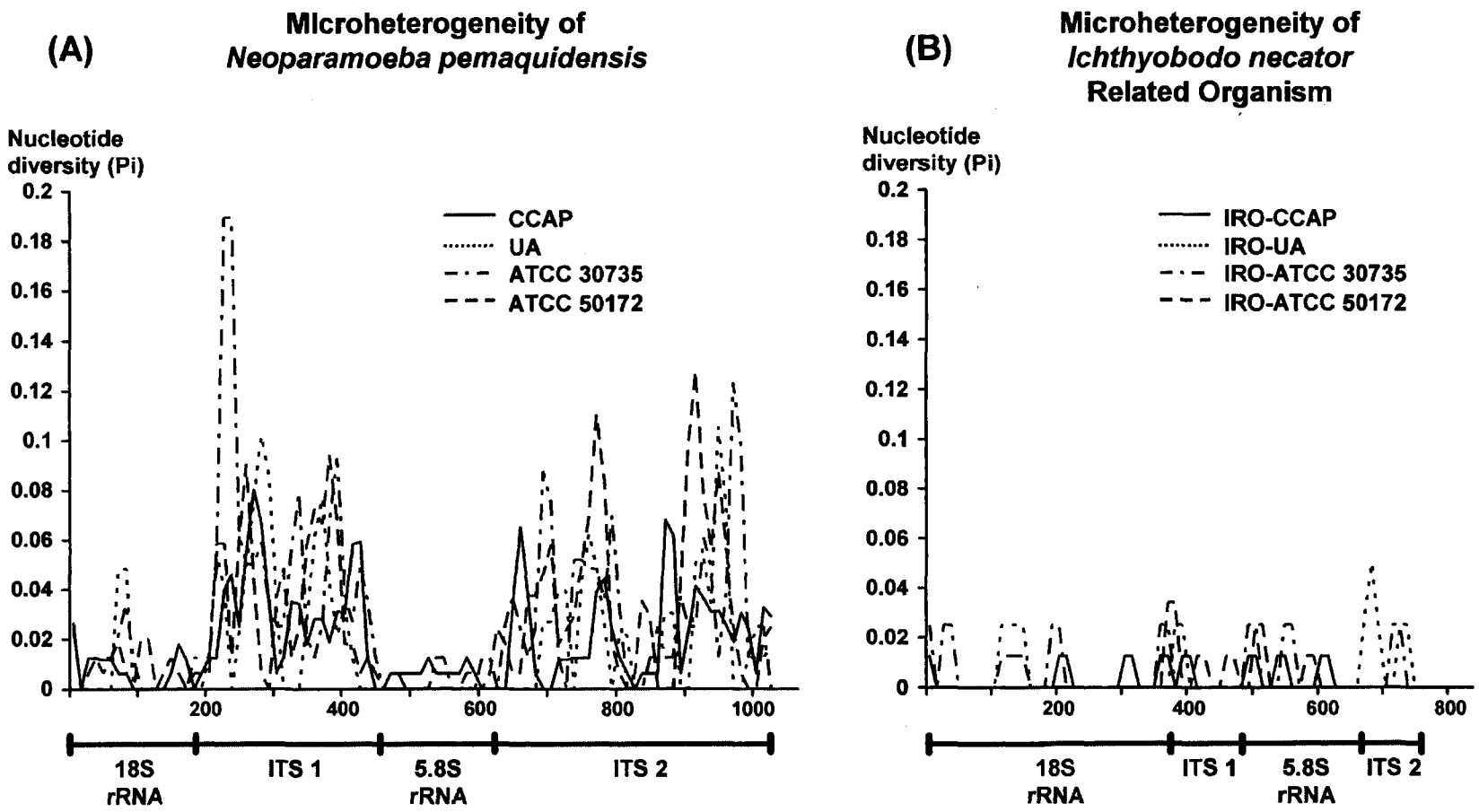


Figure 2.1. Microheterogeneity variation along the ITS regions of *Neoparamoeba pemaquidensis* and respective *Ichthyobodo necator* Related Organism strains. (A) Four *Neoparamoeba pemaquidensis* strains (1032 nucleotide alignment). (B) Four *Ichthyobodo necator* Related Organism strains (753 nucleotide alignment). The X axis represents the nucleotide position in the alignment. Sliding window approach: 20 nucleotide length and 10 nucleotide steps.

2.3.2 *Ichthyobodo necator* Related Organism ITS region nucleotide variability

Four clones of the IROs' ITS region were sequenced from PCR amplicons generated from each of six different *N. pemaquidensis* isolates, yielding a total of 24 ITS sequences (Table II). The GenBank accession numbers are the following (Table II): IRO-CCAP 1560/4 clones # 1 to 4 (DQ167481 to DQ167484), IRO-CCAP 1560/5 clones # 1 to 4 (DQ167485 to DQ167488), IRO-UA1 clones # 1 to 4 (DQ167489 to DQ167492), IRO-UA6 clones # 1 to 4 (DQ167493 to DQ167496), IRO-ATCC 30735 clones # 1 to 4 (DQ167497 to DQ167500), and IRO-ATCC 50172 clones # 1 to 4 (DQ167501 to DQ167504).

2.3.2.1 CCAP isolates

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 IRO sequences from the two CCAP isolates, no fixed nucleotide difference was observed (Da(JC) = 0). Therefore, 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 Pi's (respectively P = 0.62 and P = 0.70).

2.3.2.2 UA isolates

The total length of the four IRO-UA1 sequences varied from 369 to 371 bp with an estimated Pi of 0.0054. For the four IRO-UA6 sequences, the total length was 370 to

371 bp and the Pi was 0.0054. No significant difference was observed between the two intra-isolate nucleotide diversities (P = 1). Based on the alignment of the eight IRO sequences from the two UA isolates, no fixed nucleotide difference was observed (Da(JC) = - 0.0003). Therefore, based on the ITS region, 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 Pi's (respectively P = 0.95 and P = 0.94). 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.

2.3.2.3 ATCC isolates

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 IRO sequences from the two ATCC isolates, 29 fixed nucleotide differences were observed. The Da(JC) was estimated at 0.087. The two IROs were considered different and represent separate strains. The IRO-ATCC 30735 sequences also shared 21 fixed nucleotide differences when compared to the IRO-CCAP strain (Da(JC) = 0.067); and 33 when compared to the IRO-UA strain (Da(JC) = 0.102). The IRO-ATCC 50172 sequences had 18 fixed nucleotide differences when compared to the IRO-CCAP strain (Da(JC) = 0.051); and 32 when compared to 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 (Figure 2.1.B).

2.3.2.1 AMOVA

The molecular analysis of variance 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 III). 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.

2.3.3 *Neoparamoeba aestuarina* ITS region nucleotide variability

Two clones of the ITS region were sequenced from PCR amplicons generated from the *N. aestuarina* isolate (ATCC 50806) 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 (DQ167554 and DQ167505 respectively).

Table II. Internal Transcribed Spacer sequences for *Neoparamoeba pemaquidensis* and respective endosymbiont *Ichthyobodo necator* Related Organism isolates and strains (Pi: Nucleotide Diversity; SD: Standard deviation of the estimate).

Source	# Clones sequenced	ITS region Total Length	ITS region Pi (SD)	ITS 1 Pi (SD)	5.8S Pi (SD)	ITS 2 Pi (SD)	Accession Numbers
Neoparamoeba pemaquidensis							
CCAP 1560/4[1]	8	748-752 bp	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 bp	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 bp	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 bp	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 bp	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 bp	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 bp	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 bp	0.0313 (0.00307)^{C}	0.0463 (0.00658)^{B}	0.0034 (0.00242)$^{B,C, D}$	0.0325 (0.00359)^{B}	DQ167546-53
***Ichthyobodo necator* Related Organism**							
IRO-CCAP 1560/4[1]	4	357 bp	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 bp	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 bp	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 bp	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 bp	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 bp	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 bp	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 bp	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 ($P < 0.05$)

Table III. Analysis of Molecular Variance (AMOVA) of *Neoparamoeba pemaquidensis* ITS sequences (A) and of *Ichthyobodo necator* Related Organism (B) ITS sequences. The hierarchical structure of the analysis is the same for both organisms; 4 strains: CCAP, UA, ATCC 30735 and ATCC 50172; within the strain CCAP two isolates: CCAP 1560/4 and CCAP 1560/5; within the strain UA two isolates: UA1 and UA6.

A. *Neoparamoeba pemaquidensis* ITS region

Source of variation	df	Sum of Squares	Variance components	Percentage of variation
Among strain	3	1551.81	43.75	76.6
Among isolates within a strain	2	23.62	-0.22	-0.4
Within an isolate (microheterogeneity)	42	570.50	13.58	23.8
Total	47	2145.94	57.10	100

B. *Ichthyobodo necator* Related Organism ITS region

Source of variation	df	Sum of Squares	Variance components	Percentage of variation
Among strain	3	334.92	19.16	95.1
Among isolates within a strain	2	1.87	-0.02	-0.1
Within an isolate (microheterogeneity)	18	18.25	1.01	5.0
Total	23	355.04	20.15	100

2.3.4 *Neoparamoeba* spp. phylogenetic analysis

The alignment of 828 nucleotides from 49 *Neoparamoeba* spp. ITS region sequences was assessed by neighbor joining analysis that incorporated a Kimura 2-parameter model (Figure 2.2.A). The neighbor joining consensus tree rooted with an *N. aestuarina* outgroup represents the branching order among the four *N. pemaquidensis* strains (Figure 2.2.A). Maximum parsimony analysis produced a tree with similar branching order and bootstrap support values (within brackets) (Figure 2.2.A). *Neoparamoeba pemaquidensis* sequences were separated into two distinct sister groups. Within these groups, all clones from a single strain were grouped together and formed a cluster supported by high bootstrap values (96-100 %). The sister groups, UA and ATCC 30735 strains, were consistently supported by high bootstrap values (72-84 %). The CCAP and ATCC 50172 strains formed a monophyletic group supported by high bootstrap support values (98-99 %). The general branching structure of the tree did not indicate a phylogeographic pattern.

2.3.5 *Ichthyobodo necator* Related Organism phylogenetic analysis

The alignment of the 383 nucleotides from the 25 IRO ITS region sequences was analyzed by neighbor joining incorporating a Kimura 2-parameter model (Figure 2.2.B). The neighbor joining consensus tree, rooted with an IRO-*N. aestuarina* outgroup represents the phylogenetic relationship among the four IRO-*N. pemaquidensis* strains (Figure 2.2.B). Maximum parsimony analysis produced a tree with similar branching order but with different bootstrap support values (within brackets) (Figure 2.2.B). All clones from a single strain consistently grouped together and formed a cluster validated

by high bootstrap support values (90-100 %). The IRO-UA strain sequences produced a well supported monophyletic group (bootstrap support value of 99 %) (Figure 2.2.B). The IRO-CCAP and IRO-ATCC 50172 strain sequences clustered together to form a sister group supported by bootstrap values of 71-84 %. 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 %). Consequently, using a 70 % cutoff value, this node was collapsed generating an unresolved trifurcation (Figure 2.2.B).

2.3.6 Host/Parasite coevolution test

Genetic distances based on the Kimura 2-parameter model were computed from the 6 aligned *N. pemaquidensis* isolate ITS consensus sequences and compared with the genetic distances computed from the 6 aligned IRO ITS consensus sequences. The ParaFit test indicated that there was a global relationship between the host and parasite (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 non rejected ($P = 0.30$).

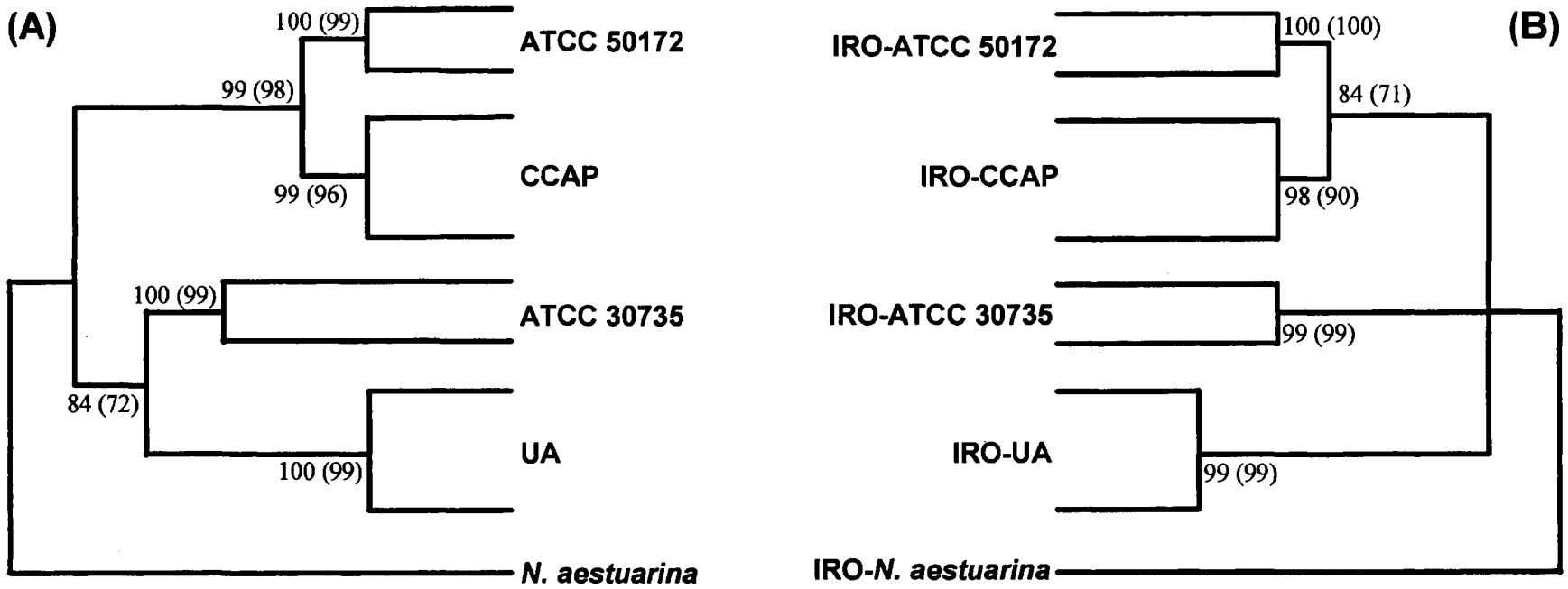


Figure 2.2. Simplified version of the phylogenetic analysis of *Neoparamoeba pemaquidensis* and respective endosymbiont *Ichthyobodo necator* Related Organism based on ITS sequences (adapted from Appendix 18, p. 159). (A) *Neoparamoeba pemaquidensis* Neighbor-joining phylogram based on the alignment of 828 nucleotides. (B) *Ichthyobodo necator* Related Organism Neighbor-joining phylogram based on the alignment of 383 nucleotides. Values at nodes represent the percentages of bootstrap replications: Neighbor-joining and Maximum Parsimony (within brackets), only values equal to or greater than 70 % are shown. Each strain includes the cluster of all cloned sequences.

2.4 Discussion

2.4.1 Intraspecific polymorphism

Evaluation of the *Neoparamoeba 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 the ability to differentiate isolates.

2.4.2 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, but most of it occurred in the ITS 1 and ITS 2 regions (Figure 2.1.A). Approximately 24 % of the ITS region total intra-specific variation observed in *N. pemaquidensis* was explained by microheterogeneity (Table III). Dyková et al. (2005) reported surprisingly high divergence levels among cloned 18S rDNA sequences (microheterogeneity) with 16 to 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 other amoebae: *Entamoeba* sp. (Som et al. 2000), *Naegleria* sp. (De Jonckheere 2004), and *Acanthamoeba* (Stothard et al. 1998). However, significant intra-specific polymorphism was studied in the marine alveolate protozoan genus *Perkinsus* (Brown et al. 2004, *Perkinsus marinus*). 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 ITS 1 locus. The microheterogeneity among the *N. pemaquidensis* isolates varied from 0.0201 to 0.0313 for the entire ITS region with the highest variation in ITS 1 (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 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 artifact (Tindall and Kunkel 1988; Pääbo et al. 1990). However, using the same PCR reagents, we obtained low or non-existent microheterogeneity within the IRO sequences (Table III and Figure 2.1.B). Therefore, biased PCR is not likely 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 contained several different isolates. Although all initial cultures were considered clonal, we tested this 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 P = 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.

Finally, our results suggest that the assumption of concerted evolution, as normally perceived to operate in eukaryotic cells (Dover 1982; Elder and Turner 1995), is not totally appropriate for *Neoparamoeba*. 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 et al. (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 result from complex adaptation of the amoeba and its endosymbiont to a wide range of life styles and environments.

2.4.3 Coevolution

The topologies of the *N. pemaquidensis* and IRO phylogenetic trees are almost but not completely congruent. The dissymmetry between the two consensus trees is based on the unresolved trifurcation in the IRO phylogeny. The lack of congruence does not refute the hypothesis of coevolution. The fragility of IRO-ATCC 30735 branch suggests a lack of parsimony-informative sites within IRO sequences. 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. Additionally 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 may not extend to other parasome-containing amoebae. O'Kelly et al. (2001) noted that *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. eihardi*. 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. Additionally, *Paramoeba* and *Neoparamoeba* may represent two stages in the development of an obligate symbiotic relationship between two lineages of free-living heterotrophic protists. They would therefore, provide an excellent model in which to study genomic integration.

2.5 References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Brown GD, Hudson KL, Reece KS (2004) Multiple polymorphic sites at the ITS and ATAN loci in cultured isolates of *Perkinsus marinus*. J Eukaryot Microbiol 51:312-320

Cann JP, Page FC (1982) Fine-structure of small free-living *Paramoeba* (Amebida) and taxonomy of the genus. J Mar Biol Assn 62:25-43

Chatton E (1953) Ordre des Amoebiens nus. In: Masson et Cie (ed) Traité de Zoologie. Paris 3-148

De Faria JG, da Cunha AM, Pinto C (1922) Estudos sobre Protozoarios do mar. Mem Inst Oswaldo Cruz 15:186-208

De Jonckheere JF (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, Figueras A, Novoa B (1995) Amoebic gill infection of turbot, *Scophthalmus maximus*. Folia Parasitol 42:91-96

Dyková I, Figueras A, Novoa B, Casal JF (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. Dis Aquat Org 33:137-141

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, Lukes J (2003) *Perkinsiella amoebae*-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo* . Eur J Protistol 39:37-52

Dyková I, Lom J (2004) Advances in the knowledge of amphizoic amoebae infecting fish. Folia Parasitol 51:81-97

Dyková I, Nowak BF, Crosbie PB, Fiala I, Peckova H, Adams MB, 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 JF, Turner BJ (1995) Concerted evolution of repetitive DNA-sequences in Eukaryotes. Q Rev Biol 70:297-320

Elliot NG, Wong F, Carson J (2001) Detection and abundance of *Paramoeba* species in the environment. FRDC Project 98/209, 1-68. Hobart. CSIRO Marine Research.

Excoffier L, Smouse PE, Quattro JM (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 characterization of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus*. Dis Aquat Org 55:11-16

Gower JC (1966) Some distant properties of latent root and vector methods used in multivariate analysis. Biometrika 53:325-338.

Grell KG (1961) Uben den "Nebenkorper" von *Paramoeba eilhardi* Schaudinn. Arch Protistenk 105:303-312

Grell KG (1968) Protozoologie. 2nd edition. Springler, Berlin

Grell KG, Benwitz G (1970) Ultrastrukturb mariner amoben I. *Paramoeba eilhardi* Schaudinn. Arch Protistenk 112:119-137

Hall TA (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 DM, Dixon MT (1991) Ribosomal DNA - Molecular evolution and phylogenetic inference. Q Rev Biol 66:410-453

Hollande A (1940) Le "*Nebenkorper*" 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). Verhand der Naturfoschenden-Gesselschaft in Basel 23:1-16

Janicki C (1928) Studien am genus *Paramoeba* Schaudinn. Zeits Wiss Zool 131:588-644

Jones GM (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 ML, Sawyer T, Hedrick RP (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 GW (1903) Miscellanea Rhynchotalia. Entomologist 36:179-181

Kudo RR (1966) Protozoology. 5th edition. Thomas, Springfield 111 pp

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244-1245

Legendre P, Legendre L (1998) Numerical Ecology. 2nd English edition. Elsevier Science BV, Amsterdam 870 pp

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

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

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71:491-499

Minchin EA (1922) An introduction to the study of Protozoa. Arnold, London 517 pp

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 TE, Russell S, Tucker MT, Maratea JL, Koerting C, Hinckley L, De Guise S, Frasca S, French RA, Burrage TG, Perkins C (2004) Paramoebiasis associated with mass mortality of American lobster *Homarus americanus* in Long Island Sound, USA. J Aquat Anim Health 16:29-38

Mullen TE, Nevis KR, O'Kelly CJ, Gast RJ, Frasca S (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 24:719-731

Munday BL, Foster CK, Roubal FR, Lester RJG (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. (eds) Pathology in Marine Science. Academic Press, London 215-222

Munday BL, Lange K., Foster CK, Lester RJG, 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 254-257

O'Kelly CJ, Peglar MT, Black MND, Sawyer TK, Nerad TA (2001) *Korotnevella hemistylolepis* n. sp and *Korotnevella monacantholepsis* n. sp (Paramoebidae), two new scale-covered mesohaline amoebae. J Eukaryot Microbiol 48:655-662

Pääbo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265:4718-4721

Page FC (1970) Two new species of *Paramoeba* from Maine. J Protozool 17:421-427

Page FC (1983) Marine gymnamoebae. Institute of Terrestrial Ecology, Natural Environment Research Council. Culture Centre of Algae and Protozoa, Cambridge UK. 54 pp

Page FC (1987) The classification of 'naked' amoeba (phylum Rhizopoda). Arch Protistenk 133:199-217

Peglar MT, Amaral Zettler LA, Anderson OR, Nerad TA, Gillevet PM, Mullen TE, Frasca S Jr, Silberman JD, O'Kelly CJ, Sogin ML (2003) Two new small-subunit ribosomal RNA gene lineages within the subclass Gymnamoebia. J Eukaryot Microbiol 50:224-232

Perkins FO, Castagna M (1971) Ultrastructure of the Nebenkorper or "secondary nucleus" of the parasitic amoeba *Paramoeba perniciosa* (Amoebida, Paramoebidae). J Invertebr Pathol 17:186-193

Ristaino JB, Madritch M, Trout CL, Parra G (1998) PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. Appl Environ Microbiol 64:948-954

Roubal FR, Lester RJG, Foster CK (1989) Studies on cultured and gill-attached *Paramoeba* Sp (Gymnamoebae, Paramoebidae) and the cyto-pathology of Paramoebic 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 RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. Int J Parasitol 30:723-728

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

Stothard DR, Schroeder-Diedrich JM, Awwad MH, Gast RJ, Ledee DR, Rodriguez-Zaragoza S, Dean CL, Fuerst PA, Byers TJ (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

Tindall KR, Kunkel TA (1988) Fidelity of DNA-synthesis by the *Thermus aquaticus* DNA-Polymerase. Biochemistry 27:6008-6013

Van der Auwera G, Chapelle S, De WR (1994) Structure of the large ribosomal subunit RNA 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 RL (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 FY, Carson J, Elliott NG (2004) 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. Dis Aquat Organ 60:65-76

Chapter III: DEVELOPMENT OF COMPLEMENTARY MOLECULAR DIAGNOSTICS TO CHARACTERIZE *NEOPARAMOEBA PEMAQUIDENSIS* PAGE, 1987 AT THE SUB-SPECIES LEVEL

3.1 Introduction

Neoparamoeba pemaquidensis Page, 1987 is an 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, Dyková et al. 1998, Dyková et al. 1999, Dyková et al. 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, Mullen et al. 2005) and green sea urchins, *Strongylocentrotus droebachiensis* (Jones 1985, as *Paramoeba invadens*, 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 amoeba nucleus. Recent molecular evidence places members of the genus *Neoparamoeba* in a separate lineage of amoebae with unresolved associations with other parasome-containing amoebae and other 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 us to reconsider the endosymbiont that

we have more appropriately renamed *Ichthyobodo necator* Related Organism (IRO) (Chapter II).

Several diagnostic methods have been developed for the identification of *N. pemaquidensis*. The isolation and culture of amoebae and histopathology represent primary techniques based on morphological features but 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: 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 *Neoparamoeba 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 the first genetic marker studied in *Neoparamoeba* species (Elliot et al. 2001, Fiala & Dyková 2003, Peglar et al. 2003, Wong et al. 2004, Dyková et al. 2005, Mullen et al. 2005). Recognized as a useful specific marker for *Neoparamoeba* species (Dyková et al. 2005), the 18S rRNA sequences had high levels of similarity (98.1 % to 99 %) among sequences from different isolates of *N. pemaquidensis* (Wong et al. 2004). Two nested PCR diagnostics tools based on the 18S rRNA gene were developed to detect and identify the pathogen. The first is specific for *N. pemaquidensis* but relatively inefficient when used with

environmental and host derived samples (Elliot et al. 2001, Wong et al. 2004). The second nested PCR generates a 165 base pairs product from Paramoebidae/Vexilliferidae amoebae (Mullen et al. 2005). Intriguingly, it does not amplify templates from *Pseudoparamoeba pagei*, *Korotnevella hemistylolepis* and more importantly from *N. pemaquidensis* strain ATCC 50172 (Mullen et al. 2005). The lack of specificity and sensitivity of both tests requires a level of caution when used as a diagnostic test. Both assay methods however, are still unable to discriminate at the subspecies level due to the low degree of intraspecific variability of the 18S rRNA marker.

Characterization of *N. pemaquidensis* at the subspecies level may help resolve some underlying inconsistencies that may shed light on the epidemiology of *Neoparamoeba* infections in different marine environments. First, *N. pemaquidensis* is widely distributed over a range of varying coastal habitats and hosted by a diverse assemblage of marine organisms (Cann & Page 1982); it is unknown, whether *Neoparamoeba* detected in tissue or recovered from finfish originated from, or have the potential to infect, invertebrates in geographically nearby locales. Second, isolates of *N. pemaquidensis* have been characterized as pathogenic from infected fish (e.g. ATCC 50172) and others as environmental from the marine ecosystem (e.g. CCAP 1560/5); it is undetermined if environmental strains can become pathogenic. Third, *N. pemaquidensis* is an external parasite in finfish gill infections (Adams et al. 2004) but an internal parasite in lobsters (Mullen et al. 2004, 2005); therefore, it has yet to be determined if particular strains of *Neoparamoeba* have specific host tissue tropisms. Several *N. pemaquidensis* isolates were previously identified as the same species

although they originated from widely different geographic locales (Fiala & Dyková 2003); however, no diagnostic feature has been reported that can discriminate amoeba isolates based on geographic origin. Subspecies identification of *N. pemaquidensis* through a broadly applicable diagnostic marker could have direct applications in disease monitoring, surveillance and epidemiologic 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 *N. pemaquidensis* ITS region showed qualitative and quantitative inter-strain variability that was mainly localized to the more variable regions of the Internal Transcribed Spacer 1 (ITS 1) and Internal Transcribed Spacer 2 (ITS 2). Unfortunately, detailed investigations of intra-strain variability revealed the existence of very high levels of microheterogeneity in the same regions (Chapter II). The existence of this intragenomic variability potentially precluded the use of the *N. pemaquidensis* ITS region as a diagnostic marker. The endosymbiont (IRO) ITS region however, revealed qualitative and quantitative inter-strain variability among the IRO isolates and showed low to non-existent levels of microheterogeneity (Chapter II). Since the IRO is intimately associated and coevolves with the amoeba host (Chapter II), the endosymbiont ITS region may provide an alternative target for *N. pemaquidensis* diagnostic test development.

The purpose of this study was to develop a molecular diagnostic tool based on restriction fragment length polymorphism (RFLP) of the *N. 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 episode of AGD in Atlantic salmon, *Salmo salar* from the west coast of North America (Washington State, USA).

3.2 Materials and Methods

3.2.1 Amoeba isolates

Four isolates of *Neoparamoeba pemaquidensis* Page, 1987 were obtained from private and public culture collections (Table IV). Each isolate refers to one of the four initial strains defined within *N. pemaquidensis* (Chapter II). The CCAP 1560/4 isolate, representing the strain CCAP, was grown in MY75S agar medium at room temperature (19-22 °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 (19-22 °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*. The culture of *Neoparamoeba aestuarina* Page, 1987 was maintained in liquid 'ATCC medium 994' at room temperature (19-22 °C).

Amoebae were isolated from an AGD episode 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 clonal cultures were established in solid "ATCC medium 994" bacterized with *Klebsiella*

Table IV. *Neoparamoeba* spp. reference isolates and respective endosymbiont *Ichthyobodo necator* Related Organism isolates information. AGD: Amoebic Gill Disease. CCAP: Culture Collection of Algae and Protozoa. UA: Urchin Amoeba. ATCC: American Type Culture Collection. Acc. No.: GenBank accession numbers. NA: Non Applicable outgroup. *According to the *Neoparamoeba pemaquidensis* strain definitions in Chapter II (p.57 & 61).

Reference isolate	Identification	Strain*	Acc. No.
CCAP 1560/4	*Neoparamoeba pemaquidensis*	CCAP	DQ167506-13
UA 6	*Neoparamoeba pemaquidensis*	UA	DQ167530-37
ATCC 30735	*Neoparamoeba pemaquidensis*	ATCC 30735	DQ167538-45
ATCC 50172	*Neoparamoeba pemaquidensis*	ATCC 50172	DQ167546-53
ATCC 50806	*Neoparamoeba aestuarina*	NA	DQ167554
IRO-CCAP 1560/4	*Ichthyobodo necator* Related Organism	IRO-CCAP	DQ167481-84
IRO-UA 6	*Ichthyobodo necator* Related Organism	IRO-UA	DQ167493-96
IRO-ATCC 30735	*Ichthyobodo necator* Related Organism	IRO-ATCC 30735	DQ167497-500
IRO-ATCC 50172	*Ichthyobodo necator* Related Organism	IRO-ATCC 50172	DQ167501-504
IRO-ATCC 50806	*Ichthyobodo necator* Related Organism	NA	DQ167505

pneumoniae. The first isolate, AVCLSC-001, was flattened, irregularly fan-shaped and a parasome 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, a denser intracellular compartment, and no parasome was observed.

3.2.2 Genomic DNA extraction

Amoebae were detached from the agar using 2 ml of sterile sea water spread directly on plates; cell suspensions were collected by centrifugation for 5 min at 6,500 *g* at room temperature. DNA was extracted using the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Ltd, Oakville, Ontario, Canada). 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.

3.2.3 Amplification & sequencing of Internal Transcribed Spacer regions

The ITS region of *Neoparamoeba pemaquidensis* was amplified using 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) (Figure 2.0, p.52) and a 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) (Figure 2.0, p.52). Approximately 10-50 ng of genomic DNA was 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, Baie d'Urfé, Québec, Canada): 200 μM of each dNTP (A, G, C and T), 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 a MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories Inc., Toronto, Ontario, Canada) 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 a specific ITS forward primer IRO-F-ITS (5'-GCGCACTACAATGACAAAGTG-3') positioned on the 3' end of the 18S rDNA gene (Figure 2.0, p.52), and an universal eukaryote reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), positioned on the 5' end of the 28S rDNA gene (Ristaino et al. 1998) (Figure 2.0, p.52). 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 $\mu g\ ml^{-1}$ ethidium bromide.

3.2.4 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 IV) (Chapter II). For each isolate, a consensus sequence was constructed and analyzed by restriction mapping using BioEdit software (Hall 1999). A single restriction endonuclease *Ase*I was

selected to discriminate *Neoparamoeba* ITS region PCR products; and two 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).

3.2.5 Restriction Fragment Length Polymorphism

A 5 μL aliquot of *Neoparamoeba* PCR amplicon was digested with 5 U of the restriction enzyme *Ase*I (New England Biolabs, Pickering, Ontario, Canada) as directed by the manufacturer in a final volume of 20 μL at 37 °C for either one or three hours. Similarly, an aliquot of 8 μL of IRO PCR amplicon was digested with 5 U of *Ngo*MIV and 2 U of *Ale*I (New England Biolabs, Pickering, Ontario, Canada) as directed by the manufacturer in a final volume of 20 μL at 37 °C for either one or three hours. Following incubation, the digested products were electrophoresed on a 2.0 % agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

3.2.6 Species confirmation

The identities of the unknown amoeba 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'-AACCTGGTTGATCCTGCCAGT-3'), and Medlin B (5'-TGATCCTTCTGCAGGTTCACCT-3') (Medlin et al. 1988). Approximately 10-50 ng of genomic DNA was amplified using PCR beads as previously described, under the following conditions: an initial denaturation at 94 °C for 1 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at

55 °C for 30 s, and extension at 72 °C for 1 min 30 s. Final extension was at 72 °C for 10 min. If the ITS sequence was first successfully amplified during the test, we only sequenced the ITS region. Amplified SSU and ITS products were cloned directly into plasmid pCR 2.1 using the TOPO TA Cloning® Kit (Invitrogen Canada Inc., Burlington, Ontario, Canada). Plasmids containing inserts were isolated and purified from recombinant *E. coli* using the GenElute™ Plasmid Mini-Prep Kit (Sigma-Aldrich Ltd). Plasmid inserts were sequenced in both direction using M13 F & R primers on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems Inc., Foster City, California, USA) at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, Ontario, Canada). Sequences from each amoeba were identified by a BLAST sequence similarity search (Basic Local Alignment Search Tool, Altschul et al. 1997).

3.3 Results

3.3.1 *Neoparamoeba* PCR-RFLP

The paired primers fNp-Hx49 rDNA and NLR 204/21 yielded an approximately 850 to 900 bp PCR product from the four *N. pemaquidensis* reference isolates; CCAP 1560/4, UA 6, ATCC 30735, ATCC 50172 (Figure 3.0.A). The detection of a similar sized but lighter band for the *N. aestuarina* isolate confirmed the expected cross-specificity of the forward primer fNp-Hx49 rDNA previously described by Wong et al. (2004). The AGD outbreak episode isolate AVCLSC-001 was successfully amplified

with an approximately 900 bp size band; whereas the AVCLSC-002 isolate was not (Figure 3.0.A).

The restriction patterns of the PCR products generated by *Ase*I enzyme for the different incubation times are presented in Figure 3.0.B; and the number and calculated size of the restriction fragments are shown in Table V. After 1 h incubation, distinct cleavage patterns were observed from each of the *N. pemaquidensis* reference isolates and from the *N. aestuarina* isolate. By comparison with the expected bands (Table V), we noted the presence of residual undigested or partially digested bands for all the isolates except for ATCC 30735. After 3 h incubation, distinct cleavage patterns were observed from each of the *N. pemaquidensis* reference isolates and from the *N. aestuarina* isolate. We again noted residual undigested or partially digested bands for most of the isolates. The cleavage patterns of the amplified isolate, AVCLSC-001, matched the ATCC 50172 pattern, including the undigested band. The AVCLSC-001 isolate was consequently identified and characterized as an isolate of the *N. pemaquidensis* ATCC 50172 strain.

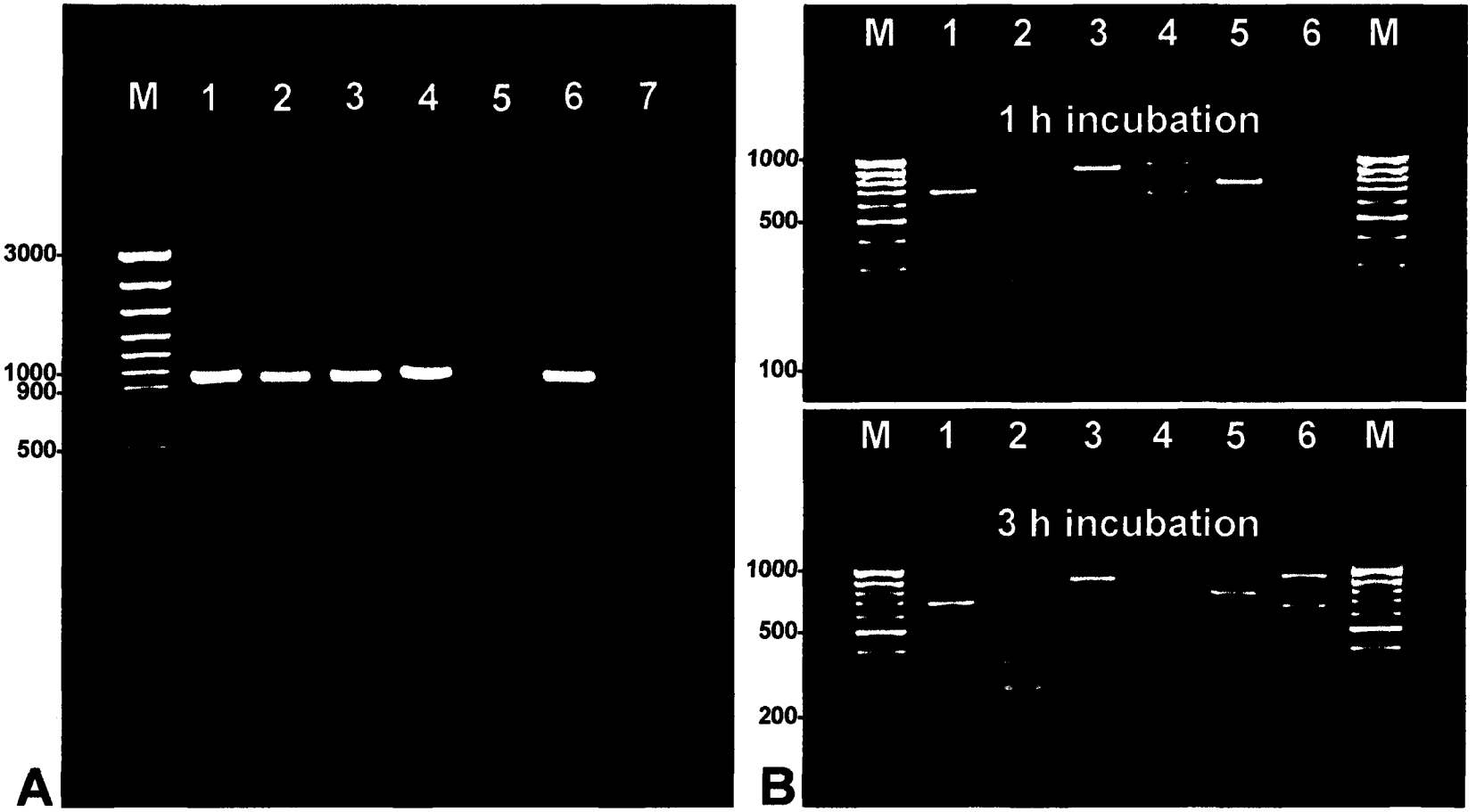


Figure 3.0. Agarose gel electrophoresis of *Neoparamoeba* spp. ITS amplicons (A), **and restriction pattern of *Neoparamoeba* spp. ITS amplicons digested with *Ase*I after 1 and 3 h incubation** (B). Source of templates for *Neoparamoeba* spp. PCR, Lanes; 1, CCAP 1560/4; 2, UA 6; 3, ATCC 30735; 4, ATCC 50172; 5, ATCC 50806; 6, AVCLSC-001; 7, AVCLSC-002. Lane M, DNA ladder. (A) Gel features; 1.0 % agarose, GeneRuler™ 100bp DNA Ladder Plus, 80Volts, 1 h. (B) Gel features; 2.0 % agarose, GeneRuler™ 100bp DNA Ladder, 80Volts, 50 min.

3.3.2 *Ichthyobodo necator* Related Organism PCR-RFLP

The paired primers IRO-F-ITS and ITS4 successfully yielded an approximately 750 bp PCR product from each IRO isolate from the four *N. pemaquidensis* reference isolates (CCAP 1560/4, UA6, ATCC 30735, ATCC 50172) (Figure 3.1.A). The detection of a similar sized band for IRO-*N. aestuarina* confirmed amplification by the IRO universal forward primer IRO-F-ITS. The tested template AVCLSC-001 was also successfully amplified with an approximately 750 bp size band; whereas the AVCLSC-002 isolate was not (Figure 3.1.A).

The restriction patterns of the PCR products generated by *Ale*I and *Ngo*MIV enzymes for the different incubation times are presented in Figure 3.1.B; the number and calculated size of the restriction fragments are shown in Table V. After 1 h incubation, distinct cleavage patterns were observed from each of the IRO-*Neoparamoeba pemaquidensis* reference isolates and from the IRO-*N. aestuarina* isolate. By comparison with the expected bands (Table V), we noted the presence of residual undigested bands for IRO-ATCC 50172 and IRO-ATCC 50806 isolates, and two partially undigested bands for IRO-CCAP 1560/4. After 3 h incubation, distinct cleavage patterns were observed from each of the IRO-*N. pemaquidensis* reference isolates and from the IRO-*N. aestuarina* isolate; we noted the presence of a single light undigested band for the IRO-ATCC 50172 isolate. The cleavage pattern of the episode isolate, IRO-AVCLSC 001, was identical to the IRO-ATCC 50172 pattern, including the undigested band.

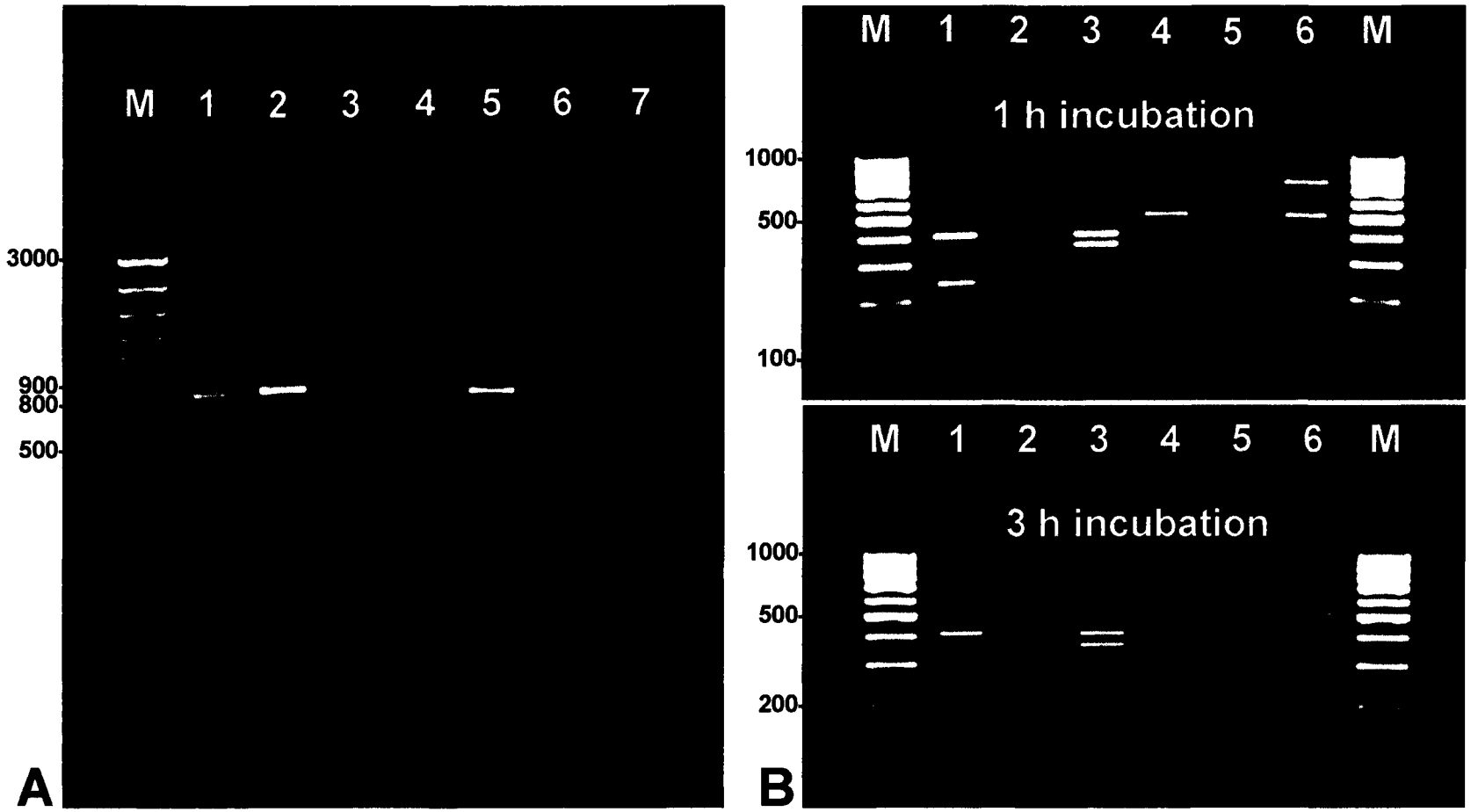


Figure 3.1. Agarose gel electrophoresis of *Ichthyobodo necator* Related Organism (IRO) ITS amplicons (A), **and restriction pattern of IRO ITS amplicons digested with *Ale*I and *Ngo*MIV after 1 and 3 h incubation** (B). Source of templates for IRO PCR, Lanes; 1, CCAP 1560/4; 2, UA 6; 3, ATCC 30735; 4, ATCC 50172; 5, ATCC 50806; 6, AVCLSC-001; 7, AVCLSC-002. Lane M, DNA ladder. (A) Gel features; 1.0 % agarose, GeneRuler™ 100bp DNA Ladder Plus, 80Volts, 1 h. (B) Gel features; 2.0 % agarose, GeneRuler™ 100bp DNA Ladder, 80Volts, 45 min.

Table V. *Neoparamoeba* spp. and respective endosymbiont *Ichthyobodo necator* Related Organism (IRO) Restriction Fragment Length Polymorphism (RFLP) patterns. Parallel comparison of number (No.) and size of restriction fragments obtained after NEBcutter 2.0 simulation (expected), or after 1 h and 3 h incubation of PCR products. *Neoparamoeba* spp. amplicons were digested with *Ase*I endonuclease and IRO amplicons with *Ale*I and *Ngo*MIV. [a] light band. [b] double bands.

	Expected		1 hour incubation		3 hour incubation	
Isolate	No. of fragments	Sizes of fragments in base pairs	No. of fragments	Approximate sizes of fragments in base pairs	No. of fragments	Approximate sizes of fragments in base pairs
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	-	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	-	3	725 / 500 / 250	3	725[a] / 500 / 250

3.3.3 Species confirmation

After positive ITS region amplification, the AVCLSC-001 isolate was confirmed to be *Neoparamoeba pemaquidensis* and more specifically was identical to ATCC 50172 using the ITS region sequences. The AVCLSC-001 amoeba ITS region sequence (DQ660492) has 96 % to 97.1 % similarity with GenBank sequences DQ167530 to DQ167553; and the AVCLSC-001 IRO ITS region sequence (DQ660493) has 99.6 % to 99.9 % similarity with GenBank sequences DQ167501 to DQ167504. 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 gene 5' end) and the isolate was identified as the leptomyxid amoeba *Paraflabellula hoguae* Sawyer, 1975 (99.4 % similarity with GenBank sequences AF293899 and AY277797).

3.4 Discussion

Despite the ubiquity of *Neoparamoeba pemaquidensis* and its important role as a pathogen in commercial finfish aquaculture, there are several outstanding questions associated with 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. The present confusion may be compounded because currently available diagnostic methods used to identify *N. pemaquidensis* (see Elliot et al. 2001, Munday et al. 2001, Wong et al. 2004, Mullen et al. 2005) cannot differentiate isolates at the subspecies level. In this study, we developed and evaluated the usefulness of a diagnostic method, based on Restriction Fragment Length Polymorphism (RFLP), of the ITS regions from both *N. pemaquidensis* and its

associated endosymbiont *Ichthyobodo necator* Related Organism (IRO), 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 diagnostic tool. However, the *N. pemaquidensis* ITS region contained significant intra-genomic variability (consequently intra-isolate and intra-strain), that was earlier recognized as microheterogeneity (Chapter II). This microheterogeneity within the ITS region introduced the dilemma of a potential 'moving target' for the marker (i.e. the potential 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 diagnostic value of the ITS region as a marker) (Chapter II). The reduced to absent microheterogeneity within the IRO-ITS and the intimate association of the endosymbiont and its amoeba host made the IRO-ITS an potential useful alternative target for *N. pemaquidensis* diagnostics.

The ITS region PCR-RFLP was successfully used to separate the four distinct strains of *N. pemaquidensis* using either amoeba or IRO derived markers. Faint extra bands however, were present in all sample lanes following restriction enzyme digestion of one and three hours. 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 conditions revealed that microheterogeneity could account for appearing or disappearing restriction sites,

dependent on the nature of the amplified ITS region copy. Therefore, the final representation of amplified ITS alleles may reflect a biased random amplification of initial heterogenous alleles from the genomes.

The effectiveness of the ITS region PCR-RFLP diagnostic test was evaluated using amoebae isolated from Atlantic salmon during an AGD episode in Washington State (USA) in the fall 2004. DNA from isolate AVCLSC-001 was successfully amplified using both *Neoparamoeba* and IRO region ITS primer sets and subsequently confirmed the amoeba as identical to *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 associated IRO. *Paraflabellula hoguae* has previously been isolated together with *N. pemaquidensis* from diseased fish gills (Elliot et al. 2001; Wong et al. 2004). Therefore, it may be interesting to further investigate the potential role of this non-IRO carrying amoeba during AGD.

Interestingly, twenty years after *N. pemaquidensis* ATCC 50172 (originally deposited as *Paramoeba pemaquidensis* in 1987) was isolated from the infected gills of coho salmon, *Oncorhynchus kisutch,* reared in sea water 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 region of both organisms was stable and represents an excellent geographical marker for the *N. pemaquidensis* isolate. This scenario of a geographic isolate clearly illustrates the critical importance of sub-species markers in the context of disease monitoring and surveillance.

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 one 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 associated with AGD (Dyková et al. 2005). This primer would not anneal to the *N. branchiphila* 18S rRNA gene as the same region contains seven non matching nucleotides (Dyková et al. 2005).

After the primers are confirmed, we recommend that any new isolate must be amplified and subsequently sequenced to select the best discriminative restriction endonuclease(s) according to the pool of isolates evaluated. The discriminative power of using complementary markers from both the host and endosymbiont will rise as the collection of *Neoparamoeba* isolates 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 an useful internal control and an acceptable alternative target. Nevertheless, we recognize that 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) (Chapter II).

In the present study, the required quantity of genomic DNA was five to ten times higher for IRO amplification than for the amoeba host. Consequently, we suggest using both markers in series or in parallel: first, the *Neoparamoeba* ITS region to maximize the analytical sensitivity of the detection; and secondly, 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. Apparently, at least two factors have been found to 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). Optimization therefore, is required for any direct diagnostic method from tissues and environmental samples to ensure analytical sensitivity. Hiney and Smith (1998) described test optimization in a four step procedure including three laboratory-based studies and a final field-based study which corresponds to the validation phase. The laboratory-based studies use experimental systems based on the qualitative, quantitative and reliability properties of the test by increasing the complexity of the DNA template matrix: *in vitro* test, sterile seeded microcosm and non-sterile incurred mesocosm. For example, a method to isolate parasitic amoebae from host tissues should be developed to rapidly, conveniently and efficiently separate high number of amoeba from host material and/or from any mucoid or autolysis products.

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

3.5 References

Adams MB, Ellard K, Nowak BF (2004) Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon, *Salmo salar* L. J Fish Dis 27:151-161

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Beszteri B, Acs E, Medlin LK (2005) Ribosomal DNA sequence variation among sympatric strains of the *Cyclotella meneghiniana* complex (Bacillariophyceae) reveals cryptic diversity. Protist 156:317-333

Brown GD, Hudson KL, Reece KS (2004) Multiple polymorphic sites at the ITS and ATAN loci in cultured isolates of *Perkinsus marinus*. J Eukaryot Microbiol 51:312-320

Cann JP, Page FC (1982) Fine-structure of small free-living *Paramoeba* (Amoebida) and taxonomy of the genus. J Mar Biol Ass UK 62:25-43

Caraguel CGB, O'Kelly CJ, Legendre P, Frasca SJr, Gast RJ, Després BM, Cawthorn RJ, Greenwood SJ. Microheterogeneity and coevolution: an examination of rDNA sequences characteristics in *Neoparamoeba pemaquidensis* and its prokinetoplastid endosymbiont. J Eukaryot Microbiol (submitted)

Douglas-Helders M, Carson J, Howard T, Nowak B (2001) Development and validation of a new dot blot test for the detection of *Paramoeba pemaquidensis* (Page) in fish. J Fish Dis 24:273-280

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, Casal JF (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. Dis Aquat Organ 33:137-141

Dyková I, Figueras A, Novoa B (1999) Epizoic amoebae from the gills of turbot *Scophthalmus maximus*. Dis Aquat Organ 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, Novoa B (2001) Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*. Bull Eur Ass Fish Pathol 21:40-44

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

Dyková I, Nowak BF, Crosbie PB, Fiala I, Peckova H, Adams MB, 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

Elliot NG, Wong F, and Carson J (2001) Detection and abundance of *Paramoeba* species in the environment. FRDC Project 98/209, CSIRO Marine Research, Hobart, p 1-68

Fiala I, Dyková I (2003) Molecular characterization of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus*. Dis Aquat Org 55:11-16

Hall TA (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

Hiney MP, Smith PR (1998) Validation of polymerase chain reaction-based techniques for proxy detection of bacterial fish pathogens: framework, problems and possible solutions for environmental applications. Aquaculture 162:41-68

Howard T, Carson J (1993) Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. In: Valentine P (ed) Proceedings of the SALTAS research and development review seminar. Salmon Enterprises of Tasmania Pty Ltd Dover, Tasmania, pp 69-80

Jones GM (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 ML, Sawyer T, Hedrick RP (1988) *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of Coho salmon *Oncorhynchus kisutch* reared in sea water. Dis Aquat Org 5:163-169

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71:491-499

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 TE, Russell S, Tucker MT, Maratea JL, Koerting C, Hinckley L, De Guise S, Frasca S, French RA, Burrage TG, Perkins C (2004) Paramoebiasis associated

with mass mortality of American lobster *Homarus americanus* in Long Island Sound, USA. J Aquat Anim Health 16:29-38

Mullen TE, Nevis KR, O'Kelly CJ, Gast RJ, Frasca S (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 24:719-731

Munday BL, Lange K., Foster CK, Lester RJG, Handlinger J. (1993) Amoebic gill disease of sea-caged salmonids in Tasmanian waters. Tasmanian Fisheries Res 28: 14-19

Munday BL, Zilberg D, Findlay V (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. J Fish Dis 24:497-507

Page F (1970) Two new species of *Paramoeba* from Maine. J Protozool 17:421-427

Peglar MT, Amaral Zettler LA, Anderson OR, Nerad TA, Gillevet PM, Mullen TE, Frasca S Jr, Silberman JD, O'Kelly CJ, Sogin ML (2003) Two new small-subunit ribosomal RNA gene lineages within the subclass Gymnamoebia. J Eukaryot Microbiol 50:224-232

Ristaino JB, Madritch M, Trout CL, Parra G (1998) PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. Appl Environ Microbiol 64:948-954

Ruggiero MV, Procaccini G (2004) The rDNA ITS region in the lessepsian marine angiosperm *Halophila stipulacea* (Forssk.) Aschers. (Hydrocharitaceae): intragenomic variability and putative pseudogenic sequences. J Mol Evol 58:115-121

Van der Auwera G, Chapelle S, De WR (1994) Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. FEBS Lett 338:133-136

Wong FY, Carson J, Elliott NG (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

Zilberg D, Gross A, Munday BL (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. J Fish Dis 24:79-82

Chapter IV: GENERAL DISCUSSION

4.1 General Discussion

The American lobster fishery is an essential activity for the regional economy of the eastern provinces in Canada and coastal communities along the eastern seaboard of the USA (Sackton 2004, Gardner Pinfold 2006). The sustainability of this industry and the associated communities depends directly on the status of the lobster resource. A tragic example of this dependence was the collapse of the lobster industry in LIS during fall 1999 (Pearce & Balcom 2005). The local lobster population experienced major mass mortality associated with a reduction up to 99% of landings (CTDEP 2000).

In response to lobster mortalities, lobstermen lobbied local governments to quickly establish funding which resulted in a considerable amount of research being established to discover the causes and consequences of the die-off (Pearce & Balcom 2005). The collective research effort concluded that the lobster mortality was multifactorial. The combination of climatic events and human impact induced significant stress and immunosuppression in lobsters which resulted in increased susceptibility to infectious diseases (Pearce & Balcom 2005). Although Koch's postulates were not fulfilled, the amphizoic amoeba, *Neoparamoeba pemaquidensis*, was consistently identified in large numbers of limp and dying lobsters (Mullen et al. 2004, Mullen et al. 2005). However, it still remains uncertain whether *N. pemaquidensis* is a definitive parasite or an opportunistic pathogen of lobster (Pearce & Balcom 2005).

As a consequence of the LIS lobster die-off, current research continues to focus on the prevention of such catastrophes in other regions of north east America.

Significant effort has also concentrated on accumulating information on all aspects of the biology of *N. pemaquidensis*. However, currently no geographic distribution data of the amoeba are available and since it can exist as a free living protist, it potentially could be found in any marine environment.

Neoparamoeba pemaquidensis is a well known finfish parasite. Numerous diagnostic methods have been developed based on morphologic features (Page 1987), immunologic assays (Howard & Carson 1993, Douglas-Helders at al. 2001) and genetic markers (Fiala & Dykova 2003, Peglar et al. 2003). However, none of these techniques is able to differentiate *N. pemaquidensis* at a level that would allow one to distinguish an amoeba isolated from salmon or lobster or from environmental isolates. The development of sensitive and specific diagnostic tools that would allow the characterization of the amoeba at the sub-species level was considered important for providing insight into understanding the epidemiology of this perplexing amoeba.

In the current study, the hyper-variable ITS regions from *N. pemaquidensis* and IRO were confirmed as two complementary sub-species markers. Both targets presented enough inter-strain variability to be integrated into the development of a diagnostic tool. However, the *N. pemaquidensis* ITS region also revealed intra-genomic variability (microheterogeneity) that could have negated the usefulness of this DNA region as an efficient marker. The ITS region was chosen as a potential diagnostic target as it is part of the ribosomal RNA multigene family that is characterized by multiple tandemly repeated copies within the genome (Hillis and Dixon 1991). Furthermore, it has been assumed and accepted that nucleotide sequence homogeneity among rDNA copies is maintained within individuals and even within species by concerted evolution (Dover

1982). The phenomenon of concerted evolution is generally believed to result from numerous DNA repair and replication mechanisms that incorporate unequal crossing over, replication transposition, gene amplification or gene conversion (Elder and Turner 1995).

The discovery of intragenomic variation in *N. pemaquidensis* therefore, represented an important deviation from the expected eukaryotic pattern of concerted evolution. This finding questioned the dogma of concerted evolution and provided further support for the microheterogeneity that was previously observed in other protists e.g. *Perkinsus marinus* (see Brown et al. 2004), *Acanthamoeba* sp. (see Visvesvara et al. 2005). Although the microheterogeneity discovered in *N. pemaquidensis* did not interfere with the application of the ITS PCR-RFLP, it may decrease the analytical specificity of amplification (Chapter III).

Previously, many researchers apparently overlooked these sequence heterogeneities and only a few reported a potential microheterogeneity pattern in *N. pemaquidensis* (Dyková et al. 2005, Mullen et al. 2005). Since the level of microheterogeneity in 18S rRNA sequences is lower, we assumed that previous studies considered that PCR or sequencing artifacts were the cause of the heterogeneity. Kanagawa (2003) recognized that multi-template PCR were susceptible to four potential categories of artifacts; PCR bias, random events, heteroduplexes and chimera formation. In the current study, only polymerase error and chimera synthesis were considered as plausible explanations for the observed microheterogeneity.

Polymerase error rates per nucleotide for the *Taq* enzyme have been reported at as low as 10^{-5} for base substitution and 10^{-6} for frameshift errors (Tindall & Kunkel

1988). Referring to the higher levels of nucleotide diversity found within the *N. pemaquidensis* ITS sequences, the *Taq* errors would not be a credible explanation. Furthermore, using the same PCR reagents, we obtained low or non-existent microheterogeneity within the IRO's ITS region sequences (Figure 2.0.B, p.57), and microheterogeneity only explains 5 % of the total ITS region variation in IRO sequences (Table III, p. 62). The hypothesis of low DNA synthesis fidelity (point mutation) and processivity (deletion mutation) by the polymerase is not sufficient to explain the totality of the observed microheterogeneity.

Chimeric template artifacts are created by recombination during PCR by a process referred to as "jumping PCR" (Pääbo et al. 1990). Jumping PCR occurs frequently in highly repetitive sequences such as rDNA, which share substantial sequence similarity and are present in high-copy number. Therefore, rDNA sequences can generate complex recombinant patterns. This would be alleviated if the levels of sequence homogenization by concerted evolution are high and thus the effects of "jumping PCR" would be minimized (Cronn et al. 2002). Therefore, the explanation of "jumping PCR" causing microheterogeneity could be accepted if the starting DNA templates were heterogenous. If the initial rDNA copies are heterogenous, regular PCR will also reveal this polymorphism. In both biased and unbiased PCR, the initial heterogeneity is real whereas a biased PCR could minimally result in overestimates of microheterogeneity. Furthermore, heterogenous templates could result from non-clonal cultures of *N. pemaquidensis* that contained more than one isolate. Although all initial cultures were clonal, the hypothesis of non-clonality was tested and rejected with no

significant difference in nucleotide diversities between the initial and newly-cloned isolates.

Finally, the observed microheterogeneity could be explained by failure of concerted evolution, suggesting that the diverse mechanisms of homogenization of rDNA copies may be partially non-functional in *N. pemaquidensis*. Surprisingly this would also mean that the nuclear genome of *N. pemaquidensis* and the IRO genome have different degrees of concerted evolution with respect to the rRNA genes. The presence of non-shared microheterogeneity between the amoeba and its endosymbiont could lead to further speculation on the dynamics of cytoplasmic symbioses and the associated mechanisms of genomic integration between two eukaryotes. Therefore, *Neoparamoeba* represents an interesting model organism of the mutual interactions between symbiotic genomes.

The global coevolution pattern found between *N. pemaquidensis* and the IRO during the current investigation of the ITS region corroborated previous observations of Dyková et al. (2003) and Dyková and Lom (2004). Additionally studies should be extended to include both *N. aestuarina* and *N. branchiphila* to verify if the observed pattern can be generalized to the entire genus *Neoparamoeba*. Confirmation of the coevolution pattern is consistent with the little that we know of the biology of the *Neoparamoeba*/IRO association. 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).

Further taxonomic clarification is required for both *Neoparamoeba* and its associated endosymbiont. First, the use of *Perkinsiella amoebae* Like Organism (PLO)

to name the endosymbiont does not follow the priority of taxonomic nomenclature and consequently was changed in the current study to *Ichthyobodo necator* Related Organism (Chapter II) to more appropriately reflect its phylogenetic origins (Dyková et al. 2003, Moreira et al. 2004). However, this denomination will probably need to be changed for a complete scientific name according to the International Code of Zoological Nomenclature (Four Edition 1999).

Second, morphological, immunologic and genetic comparisons of amoebae from blue crab, sea urchin, salmon and lobsters are required. Recently, O'Kelly (pers. comm.) isolated two amoebae from a moribund sea urchin and identified them as *N. pemaquidensis*. Assuming that these urchin amoebae are identical to the isolate initially described by Jones (1985), this suggests that the species name *Paramoeba invadens* Jones, 1985 is a junior synonym of *Neoparamoeba pemaquidensis* (Page 1970) Page, 1987. However, *Paramoeba perniciosa* Sprague, Beckett & Sawyer, 1969 has not yet been compared with other agents of paramoebiasis. If the blue crab pathogen species is determined to be identical to *N. pemaquidensis*, then *N. pemaquidensis* would be a synonym of *P. perniciosa*.

This study successfully identified two subspecies markers for *N. pemaquidensis* and consequently developed a PCR-RFLP able to differentiate amoeba strains from pure cultures. This assay and those previously published, include some inconveniences and require further optimization. However, the discriminative power of this method has several applications that will certainly increase our knowledge of *Neoparamoeba* biology. The structure and distribution of *N. pemaquidensis* populations need to be defined relative to geographic locales, lifestyles (free-living vs. parasitic) or hosts of the

protozoa. In lobster paramoebiasis, many unknown factors could be examined with this new method. First, do parasitic *N. pemaquidensis* from LIS lobsters belong to the same strain as free-living *N. pemaquidensis* from the LIS environment? Additionally, do *N. pemaquidensis* from LIS belong to the same geographic strain as the *N. pemaquidensis* from Maine or the Maritime provinces of Canada? Finally, do parasitic *N. pemaquidensis* from lobsters belong to the same strain that parasitize salmon? At present, none of these questions can be answered since amoebae from lobsters or the LIS environment are not readily accessible for comparison.

In the present study, the AGD episode isolate AVCLSC-001 was identified by both ITS PCR-RFLP assays as *N. pemaquidensis* ATCC 50172. Therefore, this AGD isolate has been found in two different finfish species from the same relative geographic area (Chapter III). These first observations present both ITS regions as geographical markers for *N. pemaquidensis*. However, this assumption needs to be confirmed by a large scale study that includes an extensive collection of amoeba isolates.

The discriminative power of the *N. pemaquidensis* and IRO ITS regions could be used to develop *in situ* hybridization probes. The ability to localize *Neoparamoeba* in histological sections would be critical to identify progressive stages of the infection within host tissues. Since *N. pemaquidensis* was described as an external parasite in vertebrate gill infections (Adams et al. 2004) and as an internal parasite in invertebrates (Mullen et al. 2004), the hypothesis of specific tissue tropisms and modes of pathogenesis of the amoeba could be further explored with development of *in situ* probes.

In conclusion, the development of specific and sensitive diagnostics for identifying *N. pemaquidensis* at the subspecies level has provided a useful tool for increasing our understanding of the biology of *Neoparamoeba.* The principal challenge will be to refine the application of the DNA markers to answer some of the outstanding questions pertinent to paramoebiasis research.

4.2 References

Adams MB, Ellard K, Nowak BF (2004) Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon, *Salmo salar* L. J Fish Dis 27:151-161

Brown GD, Hudson KL, Reece KS (2004) Multiple polymorphic sites at the ITS and ATAN loci in cultured isolates of *Perkinsus marinus*. J Eukaryot Microbiol 51:312-320

Cronn R, Cedroni M, Haselkorn T, Grover C, Wendel JF (2002) PCR-mediated recombination in amplification products derived from polyploid cotton. Theor Appl Genet 104:482-489

CTDEP (2000) Impact of 1999 lobster mortalities in Long Island Sound. Marine Fisheries Office. Connecticut Department of Environmental Protection. Marine Fisheries Office, Old Lyme 46 pp

Douglas-Helders G, Carson J, Howard T, Nowak B (2001) Development and validation of a new dot blot test for the detection of *Paramoeba pemaquidensis* (Page) in fish. J Fish Dis 24:273-280

Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111-117

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

Dyková I, Lom J (2004) Advances in the knowledge of amphizoic amoebae infecting fish. Folia Parasitol 51:81-97

Dyková I, Nowak BF, Crosbie PB, Fiala I, Peckova H, Adams MB, 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 JF, Turner BJ (1995) Concerted evolution of repetitive DNA-sequences in eukaryotes. Q Rev Biol 70:297-320

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

Gardner Pinfold Consulting Economists Ltd (2006) Benchmarking study on the Canadian lobster. Submitted to: Agriculture and Agri-Food Canada. 45 pp

Hillis DM, Dixon MT (1991) Ribosomal DNA - molecular evolution and phylogenetic inference. Q Rev Biol 66:410-453

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

Howard T, Carson J (1993) Are there alternatives to freshwater treatment of AGD? In: Proceedings of the SALTAS Research and Development Review Seminar. Valentine P (ed), Tasmania 81-97

International Commission on Zoological Nomenclature (1999) International Code of Zoological Nomenclature. Fourth Edition. Published by the International Trust for Zoological Nomenclature 1999 c/o The Natural History Museum - Cromwell Road - London SW7 5BD – UK. www.iczn.org/iczn/index.jsp

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

Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reaction (PCR). J Biosci Bioeng 96:317-323

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 TE, Russell S, Tucker MT, Maratea JL, Koerting C, Hinckley L, De Guise S, Frasca S, French RA, Burrage TG, Perkins C (2004) Paramoebiasis associated with mass mortality of American lobster *Homarus americanus* in Long Island Sound, USA. J Aquat Anim Health 16:29-38

Mullen TE, Nevis KR, O'Kelly CJ, Gast RJ, Frasca S (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 24:719-731

Pääbo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265:4718-4721

Page FC (1970) Two new species of *Paramoeba* from Maine. J Protozool 17:421-427

Page FC (1987) The classification of 'naked' amoeba (phylum Rhizopoda). Arch Protistenk 133:199-217

Pearce J, Balcom N (2005) The 1999 Long Island Sound Lobster mortality event: findings of the comprehensive research initiative. J Shellfish Res 24:691-697

Peglar MT, Amaral Zettler LA, Anderson OR, Nerad TA, Gillevet PM, Mullen TE, Frasca S Jr, Silberman JD, O'Kelly CJ, Sogin ML (2003) Two new small-subunit ribosomal RNA gene lineages within the subclass Gymnamoebia. J Eukaryot Microbiol 50:224-232

Sackton J (2004) Pricing and market factors affecting the Canadian lobster fishery. Prepared for Maritime Fishermen's Union and Martime Lobster Processor's Co-operativve. Seafood Datasearch http://www.seafood.com 32 pp

Tindall KR, Kunkel TA (1988) Fidelity of DNA-synthesis by the *Thermus aquaticus* DNA-polymerase. Biochemistry 27:6008-6013

Visvesvara GS, Booton G, Sriram R (2005) Epidemiologic, serologic and molecular identification of *Acanthamoeba* and *Balamuthia* amoebas isolated from brain, lungs, sinus and skin tissues of 14 humans and brain tissues of two dogs and an orangutan. XIth International Meeting on the Biology and Pathogenicity of Free-Living Amoebae. Ceske Budejovice, Czech Republic Folia Parasitol 52:12A

Neoparamoeba pemaquidensis X 1

Neoparamoeba pemaquidensis - DNA extraction

using GenElute™ Mammalian Genomic DNA Miniprep Kit

(Adapted from GenElute™ Mammalian Genomic DNA Miniprep Kit Manual)

1. Harvest Cells. Pellet up from 1 to 5 mL sea water suspended cells for 5 min at 6500 *g*; remove the culture medium completely and discard.

Note: Cell suspensions must be aliquotted into1.5 or 2 ml microcentrifuge tubes.

2. Resuspend cells. Re-suspend the pellet thoroughly in 200 μL of Re-suspension Solution. If previously frozen, allow the cell pellet to thaw slightly before resuspending.

Optional RNase A treatment: If RNA-free genomic DNA is required, add 20 μL of RNase A Solution and incubate for 2 minutes at room temperature.

3. Lyse cells. Put 30 to 40 mg of tissue within a microtube of 2 mL. Add 20 μL of the Proteinase K solution (20 mg/mL) to the sample, followed by 200 μL of Lysis Solution Tissue. Vortex thoroughly (about 15 seconds), and incubate at 55°C to complete digestion. A homogeneous mixture is essential for efficient lysis.

4. Column preparation. Add 500 μL of the Column Preparation Solution to each pre-assembled GenElute Miniprep Binding Column (with a red o-ring, not to be confused with other GenElute kits) and centrifuge at 12000 *g* for 1 minute at room temperature. Discard flow-through liquid.

Note: The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.

5. Prepare for binding. Add 200 μL of Ethanol (95-100%) to the lysate; mix thoroughly by vortexing 5-10 seconds. A homogeneous solution is essential.

6. Load lysate. Transfer the entire contents of the tube (650 μL) into the treated binding column from Step 4. Use a wide bore pipette tip to reduce shearing the DNA when transferring contents into the binding column. Centrifuge at > 6500 g for 1 minute at room temperature. Discard the collection tube containing the flowthrough liquid and place the binding column in a new 2 ml collection tube.

7. First wash. Prior to first use, dilute the Wash Solution Concentrate with ethanol as described under Preparation Instructions. Add 500 μL of Wash Solution to the binding column and centrifuge for 1 minute at >6500 *g* at room temperature. Discard the collection tube containing the flow-through liquid and place the binding column in a new 2 ml collection tube.

8. Second wash. Add another 500 μL of Wash Solution to the binding column; centrifuge for 3 minutes at maximum speed (17000 g) at room temperature to dry the binding column. The binding column must be free of Ethanol before eluting the DNA. Centrifuge the column for one additional minute at maximum speed if residual ethanol is seen. You may empty and re-use the collection tube if you need this additional centrifugation step. Finally, discard the collection tube containing the flow-through liquid and place the binding column in a new 2 ml collection tube.

9. Elute DNA. Pipette 200 μL of the Elution Solution directly into the center of the binding column; centrifuge for 1 minute at > 6500 g at room temperature to elute the DNA. To increase the elution efficiency, incubate for 5 minutes at room temperature after adding the Elution Solution, then centrifuge. Label and store the DNA at -20°C.

Note: Avoid freezing and thawing, which causes breaks in the DNA strand. The Elution Solution will help stabilize the DNA at these temperatures.

APPENDIX 2

Neoparamoeba pemaquidensis - PCR

using universal Eukaryote 18S rRNA primers

Reagents:

- MBI Fermentas Cat#EP0402 includes *Taq* polymerase 5 u/μl, $MgCl_2$ 25 mM and dNTPs 10mM.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks Medlin A (21 bs) AAC CTG GTT GAT CCT GCC AGT
  Medlin B (22 bs) TGA TCC TTC TGC AGG TTC ACC T

PCR conditions:

		Final concentration
10X PCR buffer	10 μl	
dNTP mix (10 mM)	2.5 μl	200 μM
Primer Medlin A (1 μM)	10 μl	10 pmol
Primer Medlin B (1 μM)	10 μl	10 pmol
$MgCl_2$ (25 mM)	6 μl	1.5 mM
Taq	0.25 μl	
DNA	X	
H_2O	up to 100 μl (Use Sigma water only!)	

When running several samples, prepare a mix with:
10X PCR buffer
dNTP
both primers
$MgCl_2$
Taq

Prepare enough for all the samples +1. Don't forget to always run a negative control and also a positive control when available.

- Transfer 38.75 μl / PCR tube
- Add H_2O in each tube
- Add DNA sample in each tube.

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> 18S-Univ

Denaturation:	94°C	1 min
30 to 35 cycles:		
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1.5 min
Last extension	72°C	10 min

Total time: ~ 2h10

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 3

Neoparamoeba pemaquidensis - PCR Beads

using universal Eukaryote 18S rRNA primers

Reagents:

- 0.5 ml tubes

- Amersham Biosciences Ready-To-Go PCR Beads: PuReTaq™ DNA polymerase 2.5 units, 10 mM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP, and stabilizers including BSA: final volume 25 μl.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks Medlin A (21 bs) AAC CTG GTT GAT CCT GCC AGT
  Medlin B (22 bs) TGA TCC TTC TGC AGG TTC ACC T

PCR conditions:

		Final concentration
10X PCR buffer	Beads	
dNTP mix	Beads	200 μM
Primer Medline A (1 μM)	2.5 μl	2.5 pmol
Primer Medline B (1 μM)	2.5 μl	2.5 pmol
$MgCl_2$	Beads	1.5 mM
Taq	Beads	2.5 units
DNA	X (usually 1 μL)	
H_2O	up to 25 μl (Use Sigma water only!)	

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> UNIV-18S **COVER LID: ON**

Denaturation:	94°C	1 min
30 to 35 cycles:		
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1.5 min
Last extension	72°C	10 min

Total time: ~ 2h10

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 4

Neoparamoeba pemaquidensis - PCR

using universal Eukaryote ITS primers

Reagents:

- MBI Fermentas Cat#EP0402 includes Taq polymerase 5 μl/μl, $MgCl_2$ 25 mM and dNTPs 10mM.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks ITS A1 (20 bs) TTT GYA CAC ACC GCC CGT CG
  ITS B1 (21 bs) ATA TGC TTA ART TCA GCG GGT

PCR conditions:

		Final concentration
10X PCR buffer	5 μl	
dNTP mix (10 mM)	1 μl	200 μM
Primer A1 (1 μM)	5 μl	5 pmol
Primer B1 (1 μM)	5 μl	5 pmol
$MgCl_2$ (25 mM)	3 μl	1.5 mM
Taq	0.25 μl	
DNA	X	
H_2O	up to 50 μl (Use Sigma water only!)	

When running several samples, prepare a mix with:
10X PCR buffer
dNTP
both primers
$MgCl_2$
Taq
Prepare enough for all the samples +1. Don't forget to always run a negative control and also a positive control when available.

- Transfer 19.25 μl / PCR tube
- Add H_2O in each tube
- Add DNA sample in each tube

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> ITS-UNIV

Denaturation:	94°C	2.5 min
25 cycles:		
Denaturation	94°C	1 min
Annealing	55°C	30 sec
Extension	72°C	1 min
Last extension	72°C	10 min

Total time: ~ 1h38

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 5

Neoparamoeba pemaquidensis - PCR Beads

using universal Eukaryote ITS primers

Reagents:

- 0.5 ml tubes

- Amersham Biosciences Ready-To-Go PCR Beads: PuReTaq™ DNA polymerase 2.5 units, 10 mM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP, and stabilizers including BSA: final volume 25 μl.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks ITS A1 (20 bs) TTT GYA CAC ACC GCC CGT CG
  ITS B1 (21 bs) ATA TGC TTA ART TCA GCG GGT

PCR conditions:

		Final concentration
10X PCR buffer	Beads	
dNTP mix	Beads	200 μM
Primer ITS A1 (1 μM)	2.5 μl	2.5 pmol
Primer ITS B1 (1 μM)	2.5 μl	2.5 pmol
$MgCl_2$	Beads	1.5 mM
Taq	Beads	2.5 units
DNA	X (usually 1 μL)	
H_2O	up to 25 μl (Use Sigma water only!)	

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> UNIV-ITS **COVER LID: ON**

Denaturation:	94°C	2.5 min
30 cycles:		
Denaturation	94°C	1 min
Annealing	55°C	30 sec
Extension	72°C	1 min
Last extension	72°C	10 min

Total time: ~ 1h55

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 6

Neoparamoeba pemaquidensis - PCR Beads

using *Neoparamoeba* spp. specific ITS primers

Reagents:

- 0.5 ml tubes

- Amersham Biosciences Ready-To-Go PCR Beads: PuReTaq™ DNA polymerase 2.5 units, 10 mM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP, and stabilizers including BSA: final volume 25 μl.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks fNP-Hx49 (21 bs) GGG TAG AGC GAG TTT GTT GTG
  ITS B1 (21 bs) ATA TGC TTA ART TCA GCG GGT

PCR conditions:

		Final concentration
10X PCR buffer	Beads	
dNTP mix	Beads	200 μM
Primer f (1 μM)	2.5 μl	2.5 pmol
Primer r (1 μM)	2.5 μl	2.5 pmol
$MgCl_2$	Beads	1.5 mM
Taq	Beads	2.5 units
DNA	X (usually 1 μL)	
H_2O	up to 25 μl (Use Sigma water only!)	

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> UNIV-ITS **COVER LID: ON**

Denaturation:	94°C	2.5 min
30 cycles:		
Denaturation	94°C	1 min
Annealing	50°C	30 sec
Extension	72°C	1 min
Last extension	72°C	10 min

Total time: ~ 1h55

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 7

Neoparamoeba pemaquidensis - PCR

using IRO specific ITS primer

Reagents:

- MBI Fermentas Cat#EP0402 includes Taq polymerase 5 μl/μl, $MgCl_2$ 25 mM and dNTPs 10mM.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks PLO-f-ITS2 (20 bs) GAC GTG CTT CAT CAA AGC AC
  ITS B2 (20 bs) TCC TCC GCT TAT TGA TAT GC

PCR conditions:

		Final concentration
10X PCR buffer	5 μl	
dNTP mix (10 mM)	1 μl	200 μM
Primer PLO-f-ITS2 (1 μM)	5 μl	5 pmol
Primer ITS B2 (1 μM)	5 μl	5 pmol
$MgCl_2$ (25 mM)	3 μl	1.5 mM
Taq	0.25 μl	
DNA	X (usually 10 μl)	
H_2O	up to 50 μl (Use Sigma water only!)	

When running several samples, prepare a mix with:
10X PCR buffer
dNTP
both primers
$MgCl_2$
Taq
Prepare enough for all the samples +1. Don't forget to always run a negative control and also a positive control when available.

- Transfer 19.25 μl / PCR tube
- Add H_2O in each tube
- Add DNA sample in each tube.

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> PLO-ITS

Denaturation:	94°C	2.5 min
30 cycles:		
Denaturation	94°C	1 min
Annealing	**50**°C	30 sec
Extension	72°C	1 min
Last extension	72°C	10 min

Total time: ~ 1h55

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 8

Neoparamoeba pemaquidensis - PCR Beads

using IRO specific ITS primers

Reagents:

- 0.5 ml tubes

- Amersham Biosciences Ready-To-Go PCR Beads: PuReTaq™ DNA polymerase 2.5 units, 10 mM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP, and stabilizers including BSA: final volume 25 μl.

- MBI Fermentas Cat#SM0311 GeneRuler 1Kb DNA ladder 0.5 mg/ml

- MBI Fermentas Cat#R0611 6X Loading dye

- Sigma Molecular Biology Grade Water W4502

- Sigma Agarose TypI Low EEO A6013

- Primers 1 μM stocks PLO-f-ITS2 (20 bs) GAC GTG CTT CAT CAA AGC AC
  ITS B2 (20 bs) TCC TCC GCT TAT TGA TAT GC

PCR conditions:

		Final concentration
10X PCR buffer	Beads	
dNTP mix	Beads	200 μM
Primer PLO-f-ITS2 (1 μM)	2.5 μl	2.5 pmol
Primer ITS B2 (1 μM)	2.5 μl	2.5 pmol
$MgCl_2$	Beads	1.5 mM
Taq	Beads	2.5 units
DNA	X (usually 5 μL)	
H_2O	up to 25 μl (Use Sigma water only!)	

Thermocycler conditions:

Thermocycler: MJ Research, PTC-200 (Peltier Thermal Cycler)
Program: <CHARLE> PLO-ITS **COVER LID: ON**

Denaturation:	94°C	2:30 min
30 cycles:		
Denaturation	94°C	1 min
Annealing	**50**°C	30 sec
Extension	72°C	1 min
Last extension	72°C	10 min

Total time: ~ 1h55

Run a 0.8% agarose gel electrophoresis with each sample (including the GeneRuler™ 1Kb DNA ladder):

- 1 μl of DNA
- 1 μl of 6X Loading dye
- 4 μl of H_2O

APPENDIX 9

Neoparamoeba pemaquidensis - Cloning

using TOPO TA Cloning®Kit
(Kit for cloning *Taq* polymerase-amplified PCR products: K4500-40
TOP10 chemically competent, pCR® 2.1-TOPO®)

(Adapted from the *TOPO TA Cloning®Kit* Manual)

A. Producing PCR Products

It is important to properly design your PCR primers to ensure that you obtain the product you need for your studies. Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your PCR product. **Remember that your PCR product will have single 3' adenine overhangs.**
Do not add 5' phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®2.1-TOPO®
You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

If you wish to use a mixture containing Taq polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3' A-overhangs on the PCR product.
If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3' A-overhangs.

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3' adenylated.

DNA Template	10-100 ng
10 X PCR Buffer	5 μl
50 mM dNTPs	0.5 μl
Primers (100-200 ng each)	1 μM each
Sterile water	49 μl add to a final volume
Taq Polymerase (1 unit/μl)	1 μl
Total Volume	50 μl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, refer to the Note below.

Note: If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before. Take special care to avoid sources of nuclease contamination. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.,* 1990).

B.Setting Up the TOPO® Cloning Reaction (Transforming Chemically Competent *E. coli*)

For TOPO® Cloning and transformation into chemically competent *E. coli,* adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM MgCl2 in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M MgClz) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

The table below describes how to set up your TOPO® Cloning reaction (6 μl) for eventual transformation into chemically competent TOP10 One Shot® E. *coli.*

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent E.*coli*
Fresh PCR product	0.5 to 4 μl
Salt Solution	1 μl
Dilute Salt Solution	**NO**
Sterile Water	add to a total volume of 5 μl
TOPO® vector	1 μl
Final Volume	6 μl

* Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine sub cloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to Transforming Competent Cells.

Note: You may store the TOPO® Cloning reaction at -20°C overnight.

C. Chemical Transforming Competent Cells

Once you have performed the TOPO® Cloning reaction, you will transform your pCR®2.1-TOPO® construct into the competent E. *coli* provided with your kit. Two protocols are provided to transform One Shot® chemically competent E. *coli.*

In addition to general microbiological supplies *(e.g.* plates, spreaders), you will need the following reagents and equipment.

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction
- S.O.C. medium (included with the kit)
- LB plates containing 50 μg / ml ampicillin
- 40 mg/ml X-gal in dimethylformamide (DMF)
- 42°C water bath
- 37°C shaking and non-shaking incubator

For each transformation, you will need **one** vial of competent cells and **two** selective plates.

- Equilibrate a water bath to 42°C
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes (see important note below).
- Spread 40 μl of 40 mg/ ml X-gal on each LB plate and incubate at 37°C until ready for use.

Thaw **on ice** 1 vial of One Shot® cells for each transformation.

If you are performing the rapid chemical transformation protocol, it is essential that you **prewarm** your LB plates containing 50-100 μg/ ml ampicillin prior to spreading.

One Shot® Chemical Transformation Protocol

1. Add 2 μl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction into a vial of One Shot® Chemically Competent E. *coli* and mix gently. Do not mix by pipetting up and down.

2. Incubate on ice for 5 to 30 minutes.
Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 μl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 μl from each transformation on a **prewarmed** selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 μl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones). Do not pick dark blue colonies.

Rapid One Shot® Chemical Transformation Protocol

An alternative protocol is provided below for rapid transformation of One Shot® chemically competent E. *coli*. This protocol is only recommended for transformations using ampicillin selection.

Note: It is essential that LB plates containing ampicillin are prewarmed prior to spreading.

1. Add 4 μl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction into a vial of One Shot® Chemically Competent E. *coli* and mix gently. Do not mix by pipetting up and down.
2. Incubate on ice for 5 minutes.
3. Spread 50 μl of cells on a **prewarmed** LB plate containing 50-100 μg/ml ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones). Do not pick dark blue colonies.

D. Analyzing Transformants

Analyzing Positive Clones

1. Take the 10 white or light blue colonies and culture them overnight in LB medium containing 50 μg / ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing.
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation. The M13 Forward (-20) and M13 Reverse primers are included to help you sequence your insert. Refer to the pCR®2.1-TOPO® map for sequence surrounding the TOPO TA Cloning® site. For the full sequence of either vector, refer to our Web site (www.invitrogen.com).

Analyzing Transformants by PCR

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use either the M13 Forward (-20) or the M13 Reverse primer and a primer that hybridizes within your insert. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol is provided below for your convenience. Other protocols are suitable.

Materials Needed:

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020) Appropriate forward and reverse PCR primers (20 μM each)

1. For each sample, aliquot 48 μl of PCR SuperMix High Fidelity into a 0.5 ml micro centrifuge tube. Add 1 μl each of the forward and reverse PCR primers.
2. Pick 10 colonies and resuspend them individually in 50 μl of the PCR cocktail from Step 1, above. Don't forget to make a patch plate to preserve the colonies for further analysis.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out on LB plates containing 50 μg / ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 μg / ml ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

APPENDIX 10

Neoparamoeba pemaquidensis - MiniPrep

using GenElute™ Plasmid Miniprep Kit

(Adapted from GenElute™ Plasmid Miniprep Kit Manual)

1. Harvest cells.

Pellet 3 ml of culture for low recombinant *E. coli* culture by centrifugation. The optimal volume of culture to use depends upon the plasmid and culture density. For best yields, follow the instructions in the note below. Transfer the appropriate volume of the recombinant *E. coli* culture to a microcentrifuge tube and pellet cells at 17,000 g for 1 minute. Discard the supernatant.

Note: For best results with recombinant E. coli grown in LB (Luria Broth), use 1-3 ml of culture for copy plasmids. With recombinant E. coli grown in rich media such as ***TB*** *(Terrific Broth) or 2X YT, use only 1 ml of culture. Higher volumes can cause a reduction in yield.*

2. Resuspend cells.

Prior to first time use, be sure to add the appropriate volume of the RNaseA Solution to the Resuspension Solution (store at 4 °C). Completely resuspend the bacterial pellet with 200 μL of the Resuspension Solution. Vortex or pipette up and down to thoroughly resuspend the cells until homogeneous. Incomplete resuspension will result in poor recovery.

3. Lyse cells.

Lyse the resuspended cells by adding 200 μL of the Lysis Solution. Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. **Do not vortex.** Harsh mixing will shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA. **Do not allow the lysis reaction to exceed 5 minutes**. Prolonged alkaline lysis may permanently denature supercoiled plasmid DNA that may render it unsuitable for most downstream applications.

4. Neutralize.

Precipitate the cell debris by adding 350 μL of the Neutralization/Binding Solution. Gently invert the tube 4-6 times. Pellet the cell debris by centrifuging at 17,000 g for 10 minutes. Cell debris, proteins, lipids, SDS, and chromosomal DNA should fall out of solution as a cloudy, viscous precipitate. Recentrifuge the supernatant before proceeding to step 6.

5. Prepare Column.

Insert a GenElute Miniprep Binding Column into a provided microcentrifuge tube, if not already assembled. Add 500 μL of the Column Preparation Solution to each miniprep column and centrifuge at **12,000 g** for 1 minute. Discard the flow-through liquid.

Note: The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.

6. Load cleared lysate.

Recentrifuge (10 minutes at 17,000 g) the supernatant before proceeding to switch the orientation of the microtube. Transfer the cleared lysate (750 μL) from step 4 to the column prepared in step 5 and centrifuge at 17,000 g for 1 minute. Discard the flow-through liquid.

7. Optional wash (use only with EndA+ strains).

Add 500 μL of the Optional Wash Solution to the column. Centrifuge at 17,000 g for 1 minute. Discard the flow-through liquid.

Note: When working with bacterial strains containing the wild-type EndA+ gene, such as HB101, JM101, and the NM and PR series, the Optional Wash step is necessary to avoid nuclease contamination of the final plasmid DNA product.

8. Wash column.

Prior to first time use, be sure to add ethanol to the concentrated Wash Solution. Add 750 μL of the diluted Wash Solution to the column. Centrifuge at 17,000 *g* for 1 minute. The column wash step removes residual salt and other contaminants introduced during the column load. Discard the flow-through liquid and centrifuge again at maximum speed for 1 to 2 minutes without any additional Wash Solution to remove excess ethanol.

9. Elute DNA.

Transfer the column to a fresh collection tube. Add 50 μL of Elution Solution or molecular biology reagent water to the column. For DNA sequencing and other enzymatic applications, use water or 5 mM Tris-HCl, pH 8.0, as an eluant. **Wait 5 minutes** on the bench and centrifuge at 17,000 g for 1 minute. The DNA is now present in the elution and is ready for immediate use or storage at -20 °C.

Results

Recovery and purity may be determined by spectrophotometric analysis. The ratio of absorbance at 260 nm to 280 nm *(A260/A280)* should be 1.7 to 1.9. The size and quality of DNA may be determined by agarose gel electrophoresis or pulsed field electrophoresis.

APPENDIX 11

MY75S

(CCAP recipe for medium for *Neoparamoeba pemaquidensis*)

Natural seawater filtered (.2 μm)	750 ml
Deionized water	250 ml
Malt Extract (Oxoid L39)	0.1 g
Yeast extract (Oxoid L21)	0.1 g
Bacteriological Agar	15.0 g

Dissolve on a hot plate. Autoclave. Bring to 50°C before pouring the plates (approximately 18 ml per plate). Store the plates in the fridge. Plates must be dried in the biosafety cabinet for several hours before use.

APPENDIX 12

L1 medium (Guillard & Hargraves 1993)
for sea urchins *Neoparamoeba* from Bigelow Laboratory (C. O'Kelly)

To 950 mL filtered seawater (.2 μm) add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
1 mL	$NaNO_3$	75 g/L dH_2O	8.83×10^{-4} M
1 mL	$NaH_2PO_4 . H_2O$	5 g/L dH_2O	3.63×10^{-5} M
1 mL	L1 trace metal solution	(see recipe below)	-
0.5 mL	f/2 vitamin solution	(see recipe below)	-

Make final volume up to 1 L with filtered seawater (0.2 μm) and autoclave.

For plates, add 15 g/L agar and dissolve on a hotplate. Autoclave at 121 °C for 25 min. Bring to 50 °C before pouring the plates (approximately 18 ml per plate). Store the plates in the fridge. Plates must be dried in the biosafety cabinet for several hours prior to use.

L1 Trace Metal Solution (Guillard and Hargraves 1993)

To 950 mL **dH_2O** add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
3.15 g	$FeCl_3 . 6H_2O$	-	1.17×10^{-5} M
4.36 g	$Na_2EDTA . 2H_2O$	-	1.17×10^{-5} M
0.25 mL	$CuSO_4 . 5H_2O$	2.45 g/L dH_2O	1×10^{-8} M
3 mL	$Na_2MoO_4 . 2H_2O$	19.9 g/L dH_2O	9×10^{-8} M
1 mL	$ZnSO_4 . 7H_2O$	22 g/L dH_2O	8×10^{-8} M
1 mL	$CoCl_2 . 6H_2O$	10 g/L dH_2O	5×10^{-8} M
1 mL	$MnCl_2 . 4 H_2O$	180 g/L dH_2O	9×10^{-7} M
1 mL	H_2SeO_3	1.3 mg/L dH_2O	1×10^{-8} M
1 mL	$NiSO_4 . 6H_2O$	2.7 g/L dH_2O	1×10^{-8} M
1 mL	Na_3VO_4	1.84 g/L dH_2O	1×10^{-8} M
1 mL	K_2CrO_4	1.94 g/L dH_2O	1×10^{-9} M

Make final volume up to 1 L with filtered seawater (0.2 μm).

f/2 Vitamin Solution
(Guillard & Ryther 1962, Guillard 1975)

To 950 mL dH_2O add:

Quantity	Compound	Stock Solution	Molar Concentration in Final Medium
1.0 mL	Vitamin B_{12} (cyanocobalamin)	1.0 g/L dH_2O	3.7×10^{-10} M
10.0 mL	Biotin	0.1 g/L dH_2O	2.1×10^{-9} M
200.0 mg	Thiamine : HCl	-	3×10^{-7} M

Make final volume up to 1 L with dH_2O. Filter sterilized into plastic vials and store in refrigerator.

Note: Vitamin B_{12} and biotin are obtained in a crystalline form. When preparing the vitamin B_{12} stock solution, allow for approximately 11% water of crystallization (for each 1.0 mg of Vitamin B_{12}, add 8.9 ml dH_2O). When preparing the biotin stock solution, allow for approximately 4% water of crystallization (for each 1.0 mg of biotin, add 9.6 ml dH_2O).

APPENDIX 13

ATCC 994

(ATCC recipe for medium for *Neoparamoeba pemaquidensis*)

Artificial Seawater	1 L
Malt Extract (Oxoid L39)	0.1 g
Yeast Extract (Oxoid L21)	0.1 g
Bacteriological Agar	15.0 g

Dissolve on a hot plate with stirring. Autoclave at 121 °C for 25 minutes. Bring to 50 °C before pouring the plates (approximately 18 ml per plate). Store the plates in the fridge. Plates must be dried in the biological cabinet for several hours before use.

APPENDIX 14

LB (Luria-Bertani)

(medium for cloning, TOPO TA Cloning®Kit)

Tryptone	10.0 g
NaCl	10.0 g
Yeast extract (Oxoid L21)	5.0 g
Agar	15 g
Deionized water	1 L
Antibiotic	50 mg
pH= 7.0 (NaOH)	

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g Agar in 950 mL deionized water.
2. Adjust the pH to 7.0 with NaOH and bring the volume up to 1 liter with deionized water.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add the antibiotic ampicillin or kanamycin (50 μg/mL), and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C in the dark.

APPENDIX 15
Amoeba cultures

Maintenance of Cultures:

Label	Name	Origin	Medium	"Food"	Temperature
ATCC 30735	*N. pemaquidensis*	ATCC	994 agar	*Klebsiella pneumoniae*	RT
ATCC 50172	*N. pemaquidensis*	ATCC	994 agar	*Klebsiella pneumoniae*	RT
ATCC 50806	*N. aestuarina*	ATCC	994 liquid	No	RT
UA1	*N. pemaquidensis*	Bigelow Laboratory	L1 agar	*Enterobacter aerogenes*	16 °C
UA6	*N. pemaquidensis*	Bigelow Laboratory	L1 agar	*Enterobacter aerogenes*	16 °C
CCAP 1560/4	*N. pemaquidensis*	CCAP	MY75S agar	No	RT
CCAP 1560/5	*N. pemaquidensis*	CCAP	MY75S agar	No	RT
AVCLSC-001	*N. pemaquidensis*	AGD outbreak	994 agar	No	16 °C
AVCLSC-002	*P. hoguae*	AGD outbreak	994 agar	*Klebsiella pneumoniae*	16 °C

Healthy cells look bright and shiny under the microscope, having a roughly spherical shape. Cultures die from the agar cube outwards and dead cells appear as very bright clumps, having an irregular shape. Cultures typically require passaging every one to three weeks and it is prudent to keep several plates of the culture in case one happens to die out.

Methods

1. Streak out each media plate with the appropriate bacterial culture, as required.

   Note: The *K. pneumoniae* culture tends to be gooey and difficult to spread, so it may require extensive spreading to achieve a somewhat uniform culture. Do not inoculate too heavily since too many bacteria tend to inhibit the growth of the amoebae. *K. pneumoniae* and *E. aerogenes* grow on blood agar plates at 37 °C; a refrigerated culture (no older than one month) can be used for streaking out the media plates.

2. Incubate the plate containing the bacteria at 37 °C for 8-16 hours. Allow the plate to equilibrate to room temperature before transferring the amoebae.

3. Examine the old culture and look for an area of healthy amoebae (typically at the edge of the culture most distant from the agar cube). Draw a square approximately 1 cm^2 on the bottom of the plate surrounding the area of healthy cells.

4. In the biohood place a scalpel with a #22 surgical steel blade and a pair of bent tweezers in 70% ethanol. Remove the scalpel from the 70% ethanol and flame it to remove any residual ethanol. After the scalpel has cooled, cut through the agar following the outline of the square.

5. Remove the tweezers from the 70% ethanol and flame them. After they have cooled, use the tweezers to remove the cut cube from the old plate. Invert the cube on the centre of a new media plate so that the surface containing the amoebae is in contact with the agar surface of the new plate.

6. Seal the plates containing the amoebae with a strip of parafilm and incubate at the appropriate temperature, right side up.

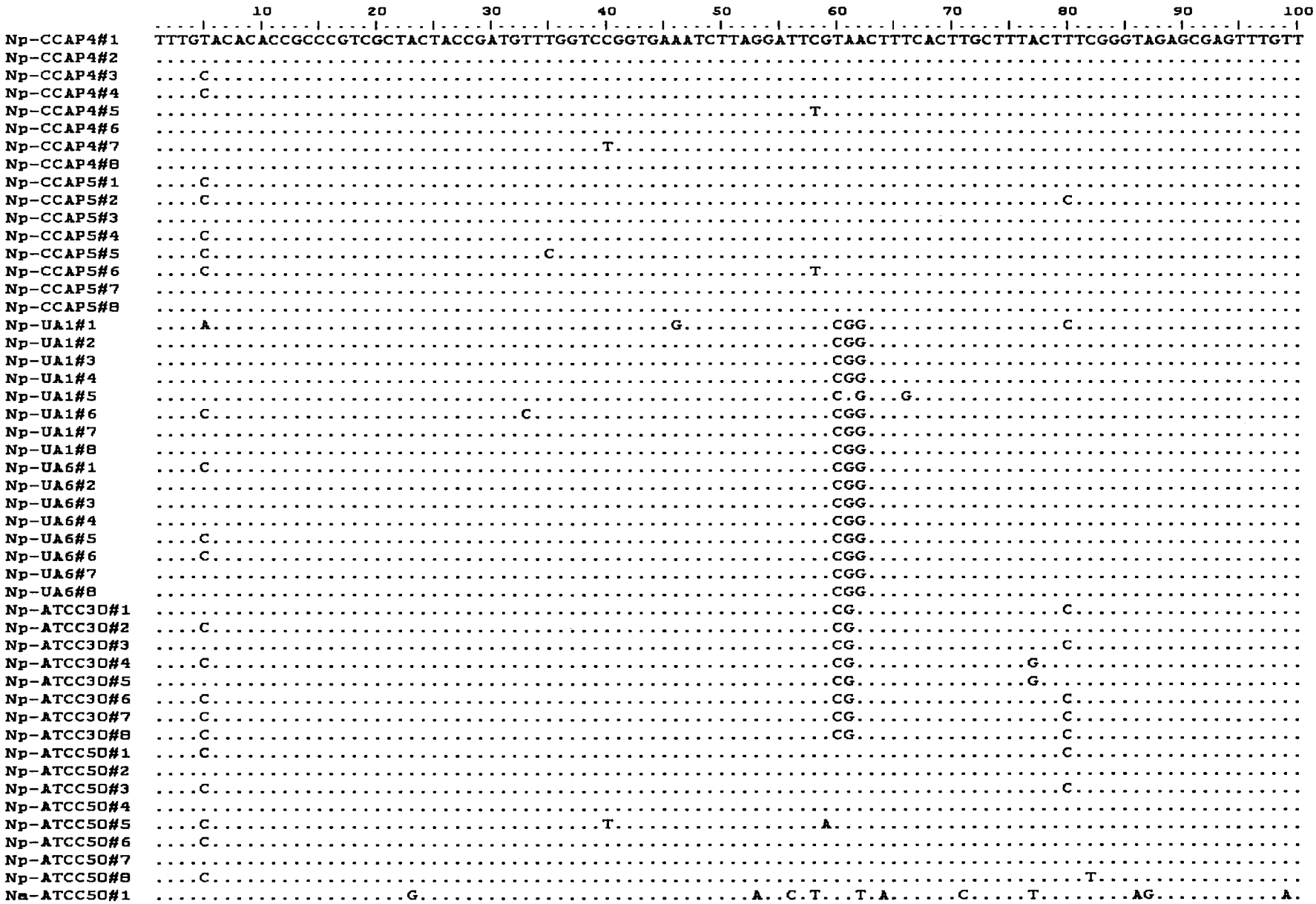
7. Monitor the amoebae every few days to ensure that the culture is growing well.

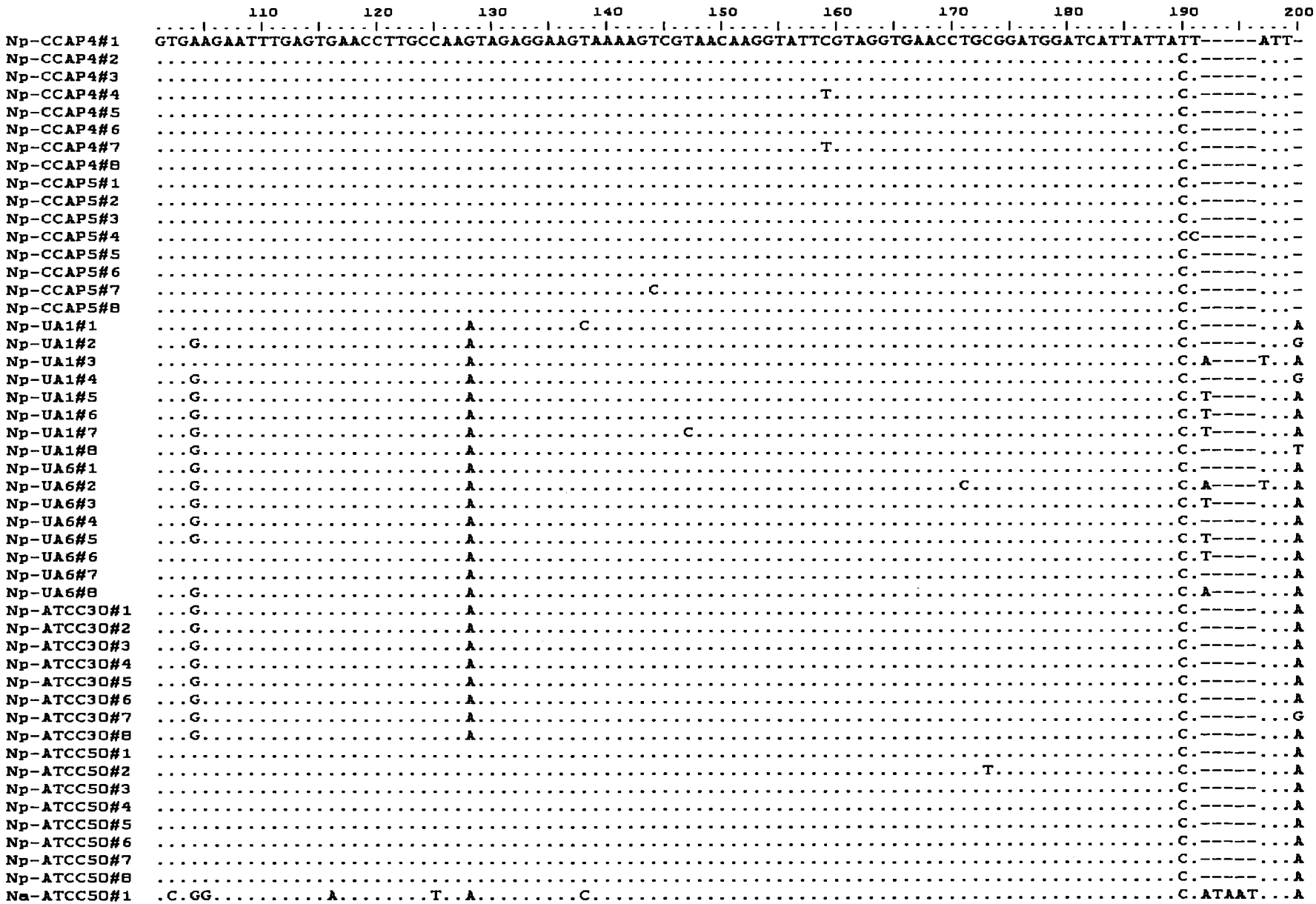
APPENDIX 16

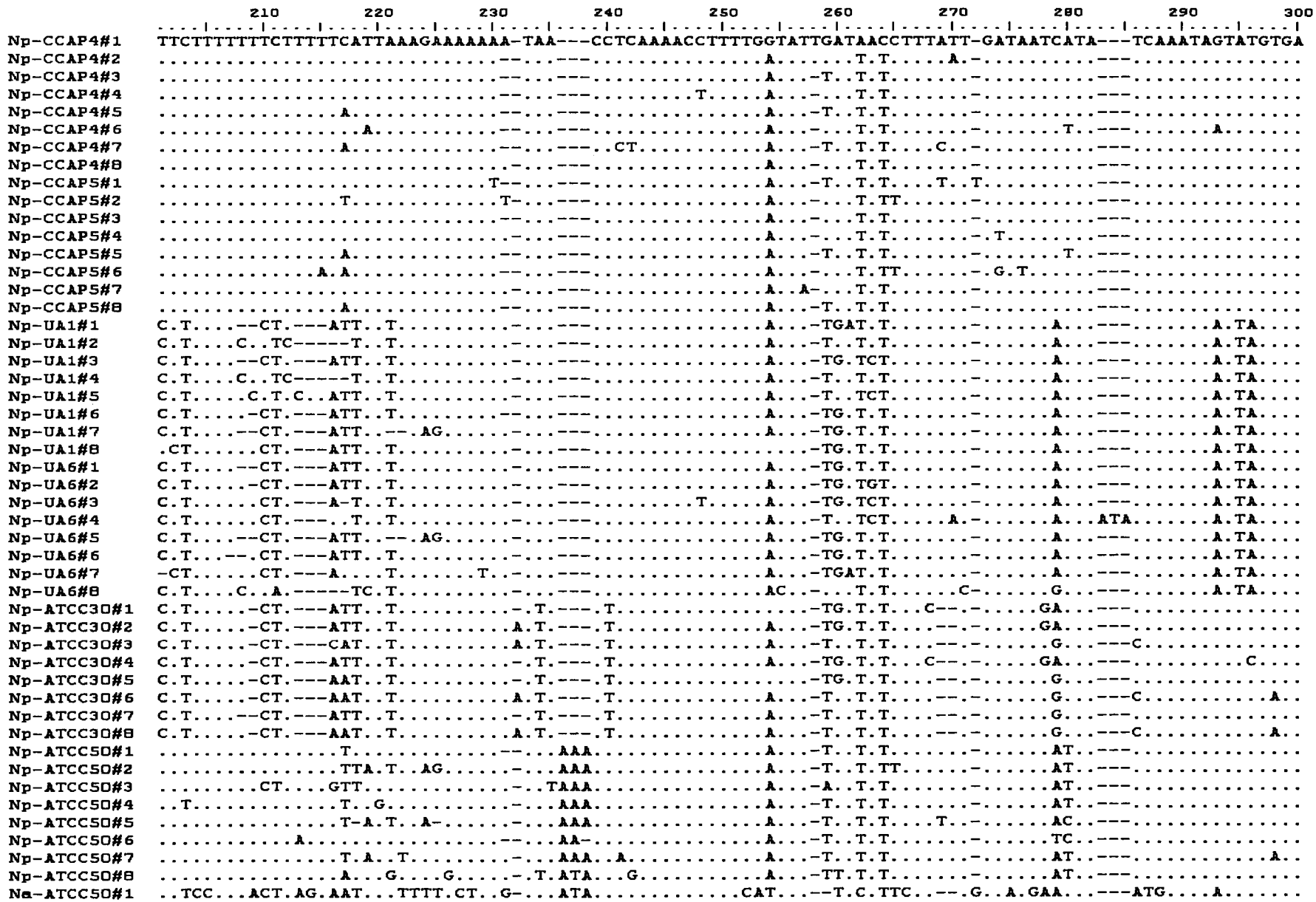
Neoparamoeba pemaquidensis ITS region alignment

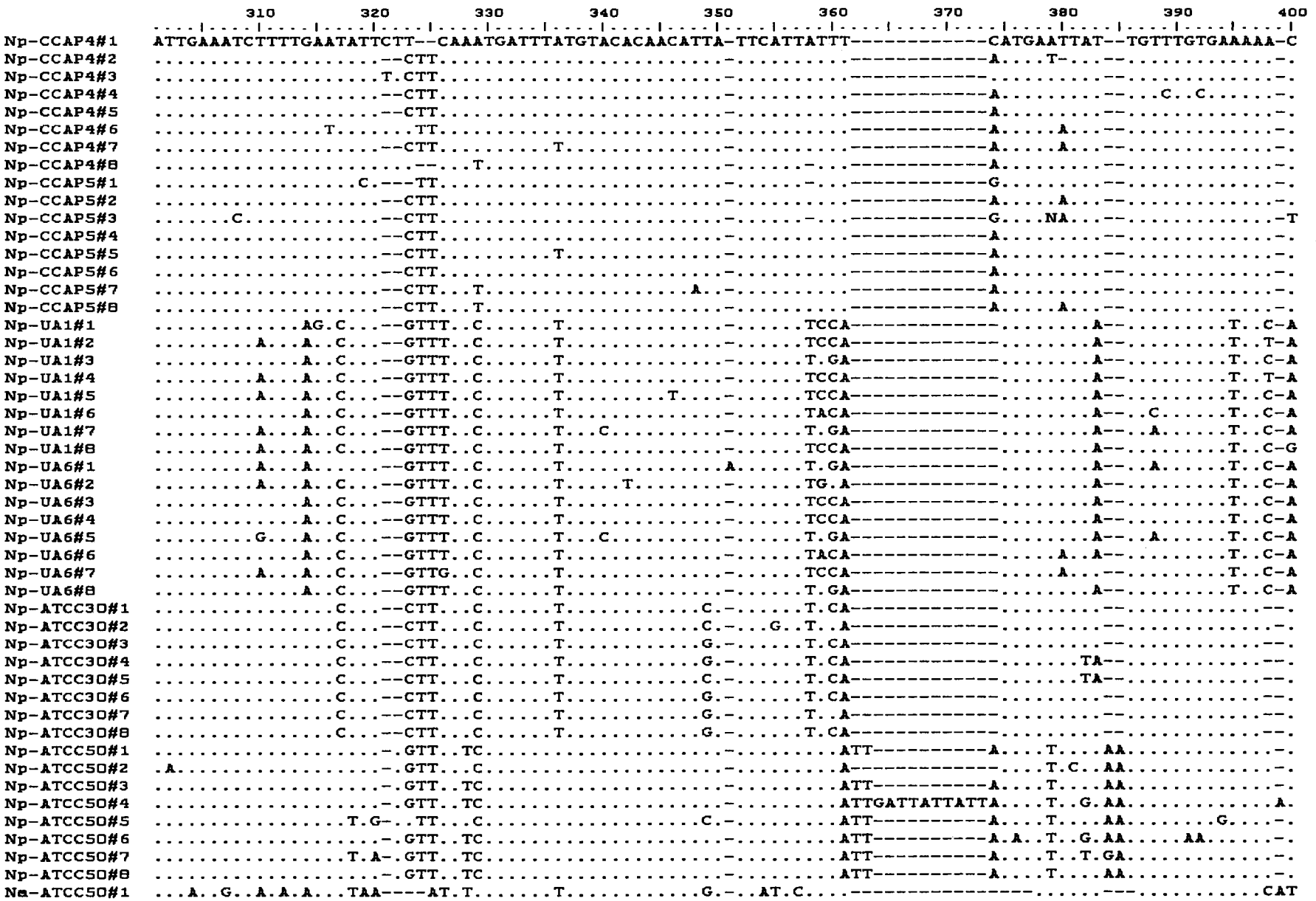
A total of 49 sequences including
eight cloned sequences for each *Neoparamoeba pemaquidensis* isolates
and one cloned sequence for the *Neoparamoeba aestuarina* isolate (outgroup)

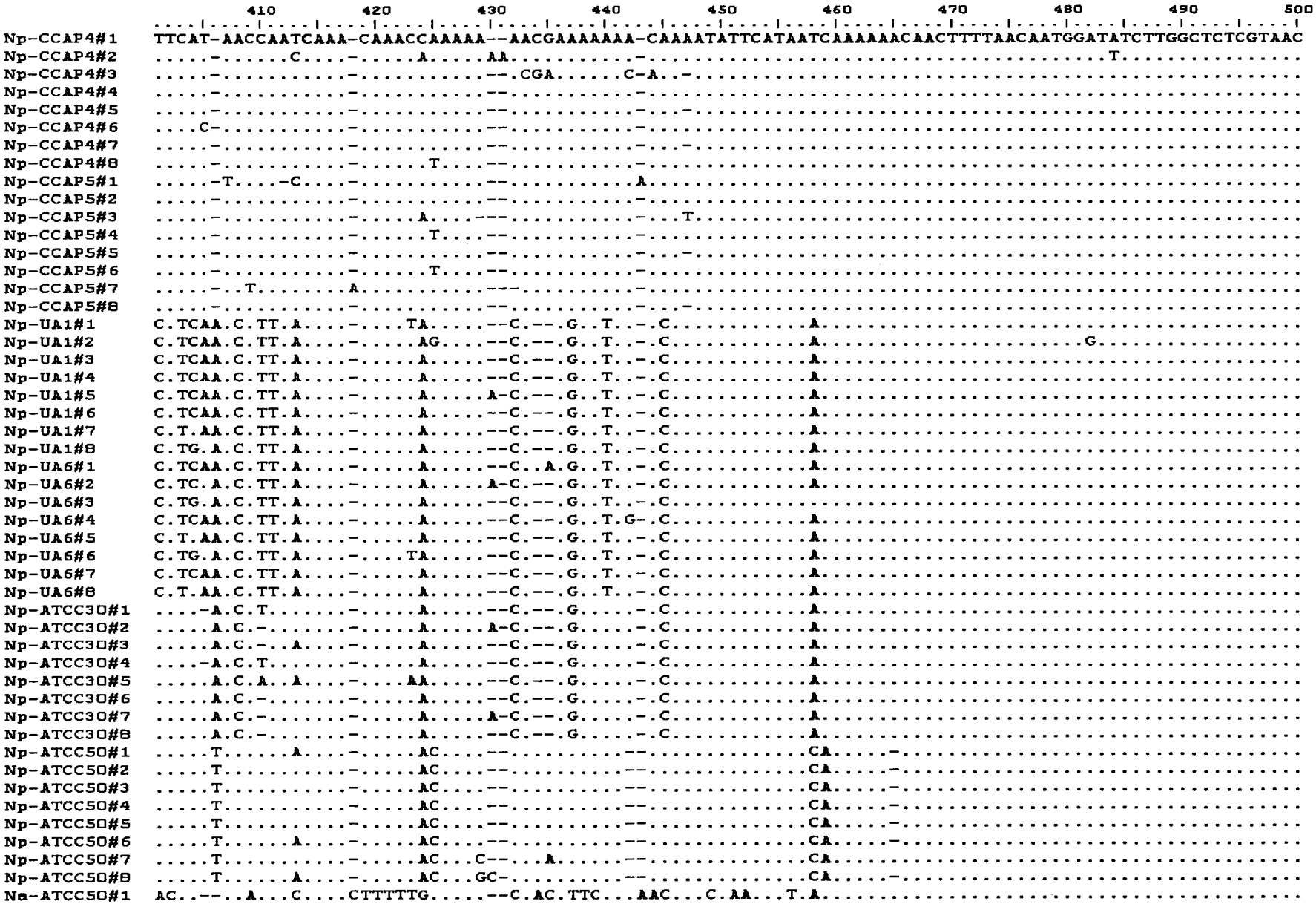
(ClustalW mutiple alignment performed with BioEdit software version 7.0.4.1)

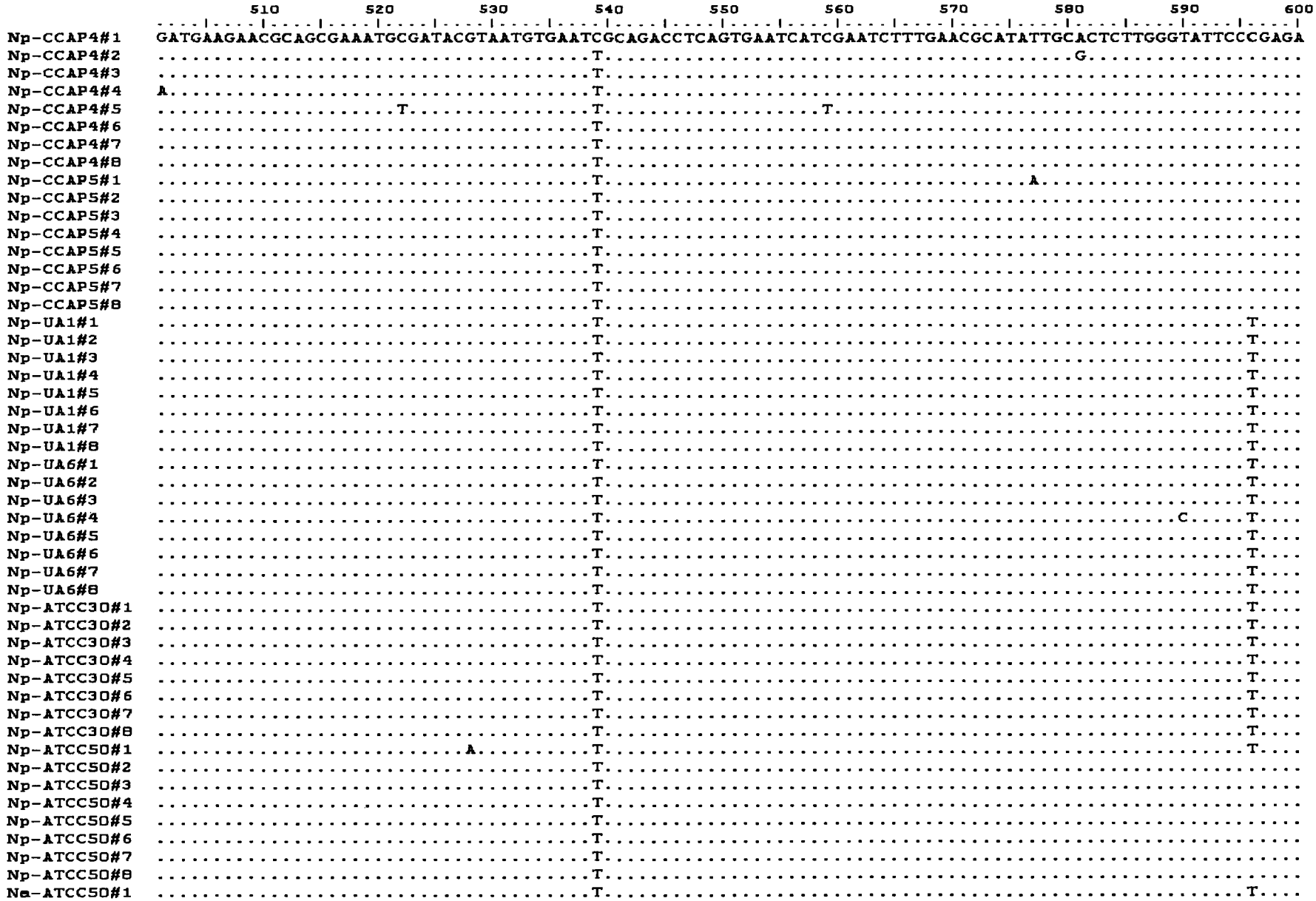


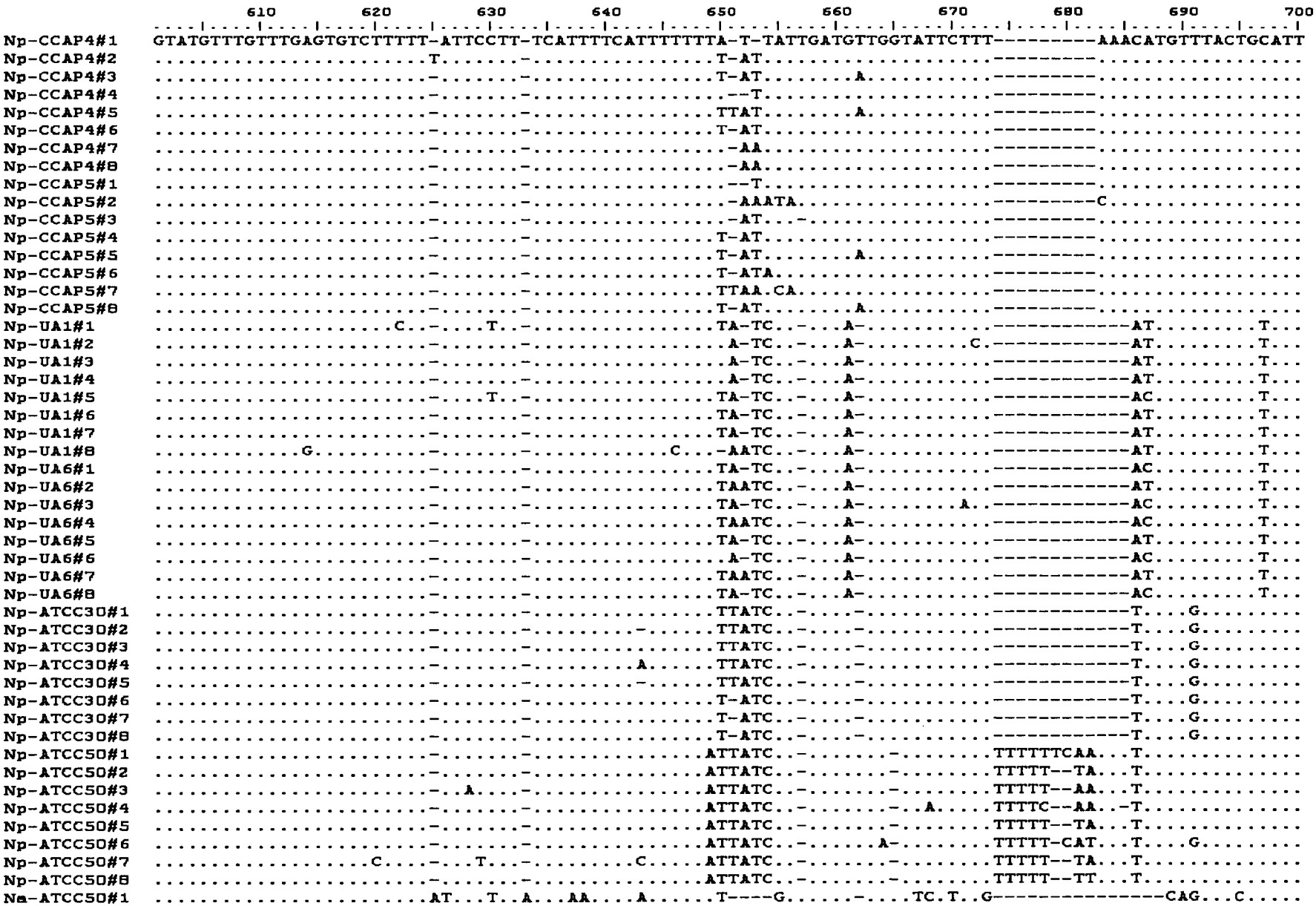


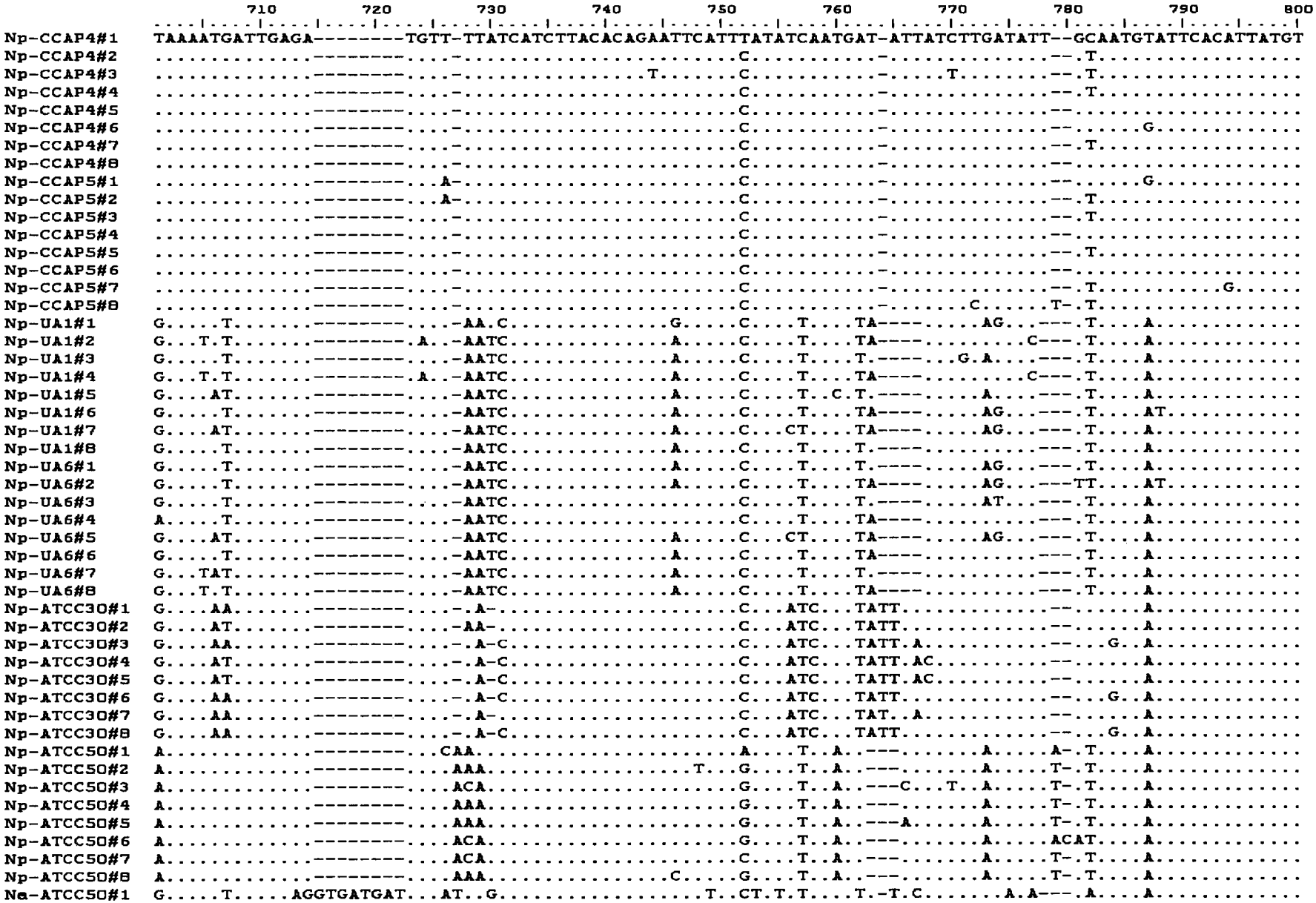


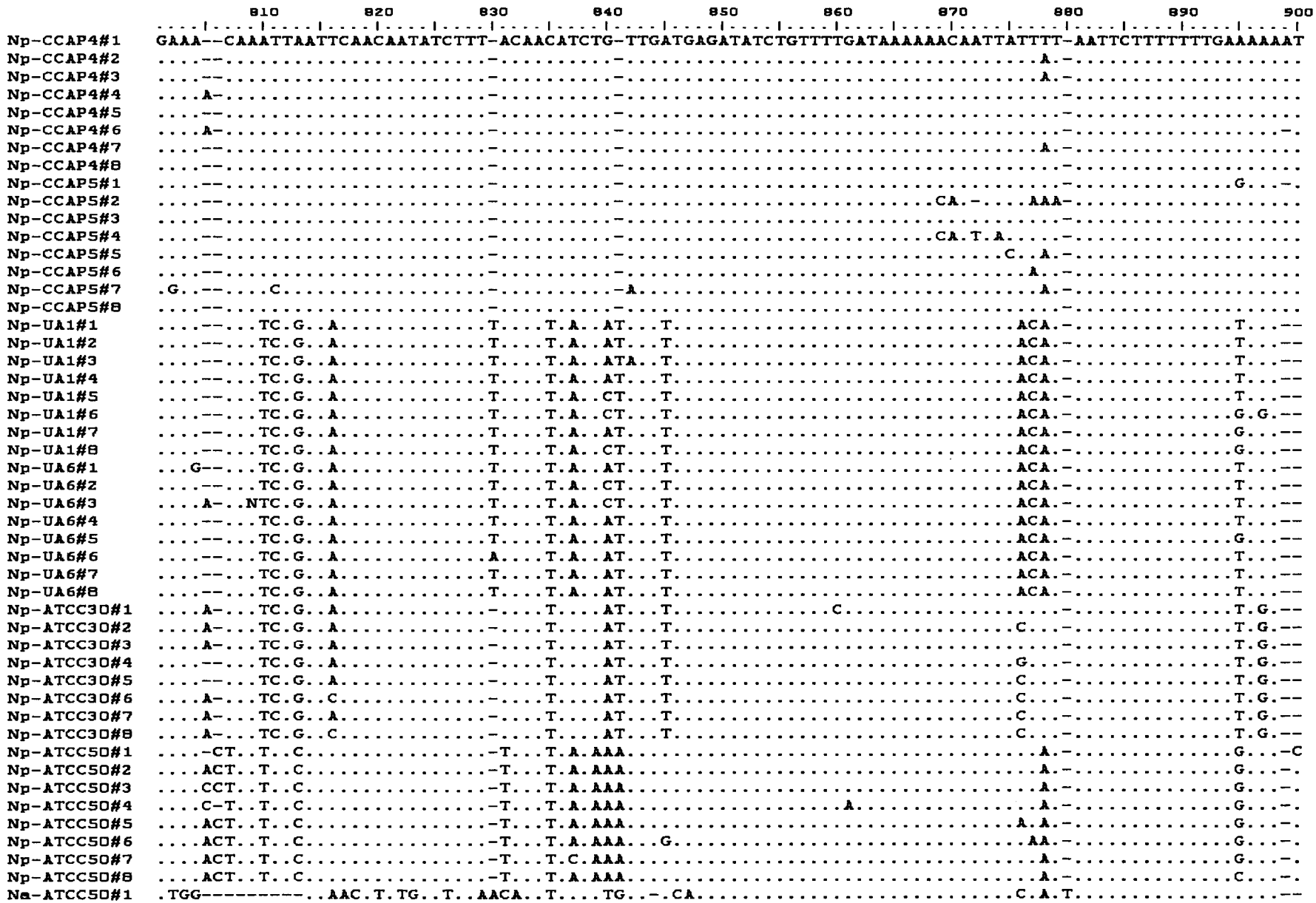


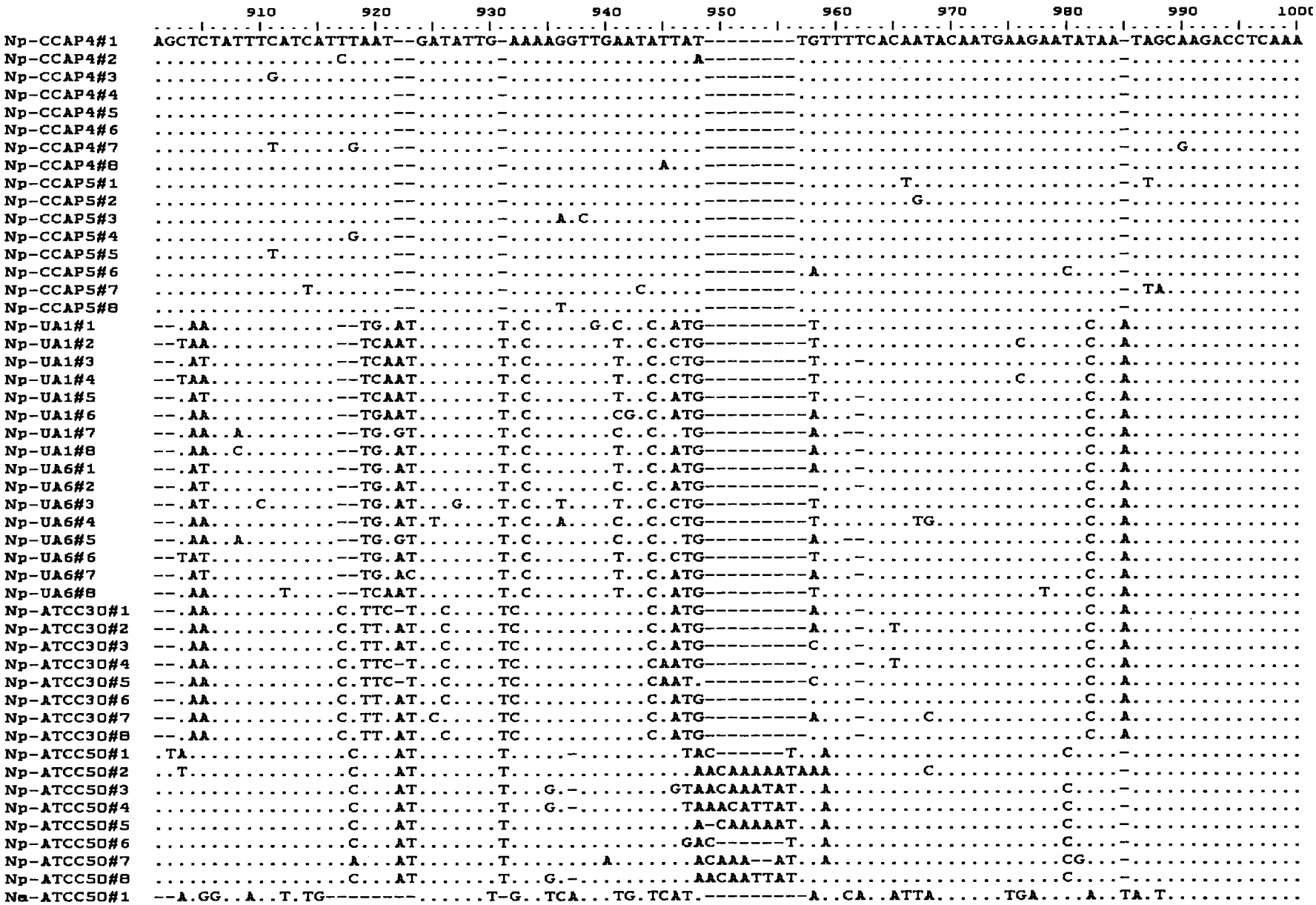


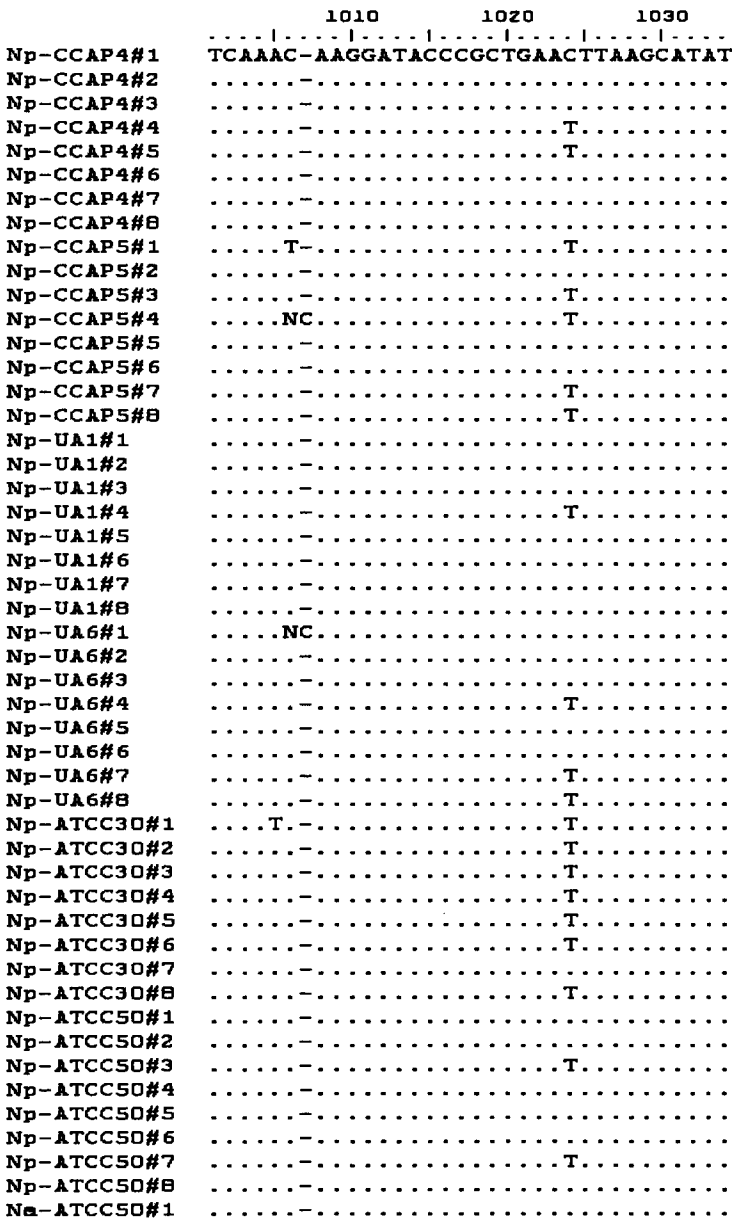










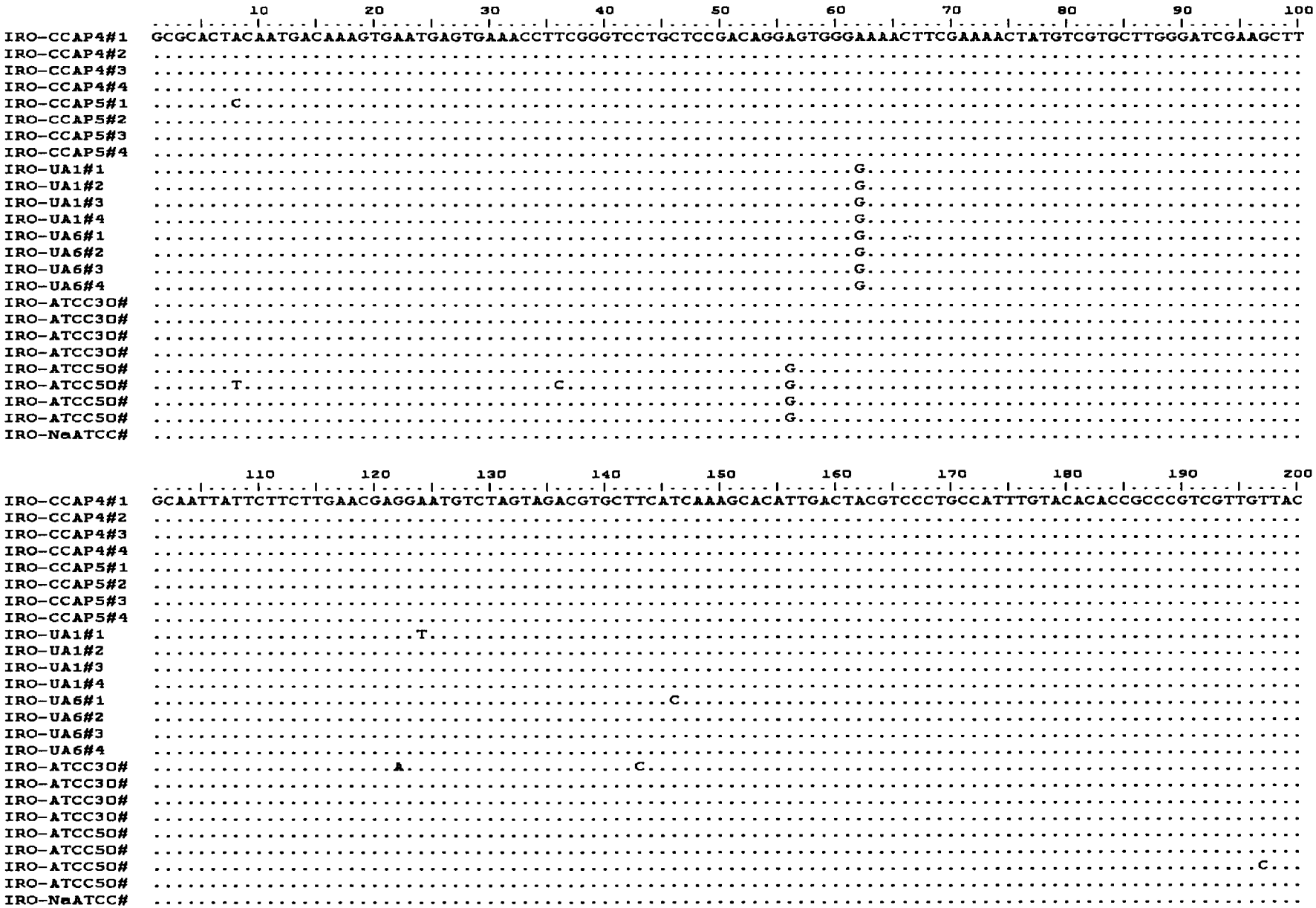


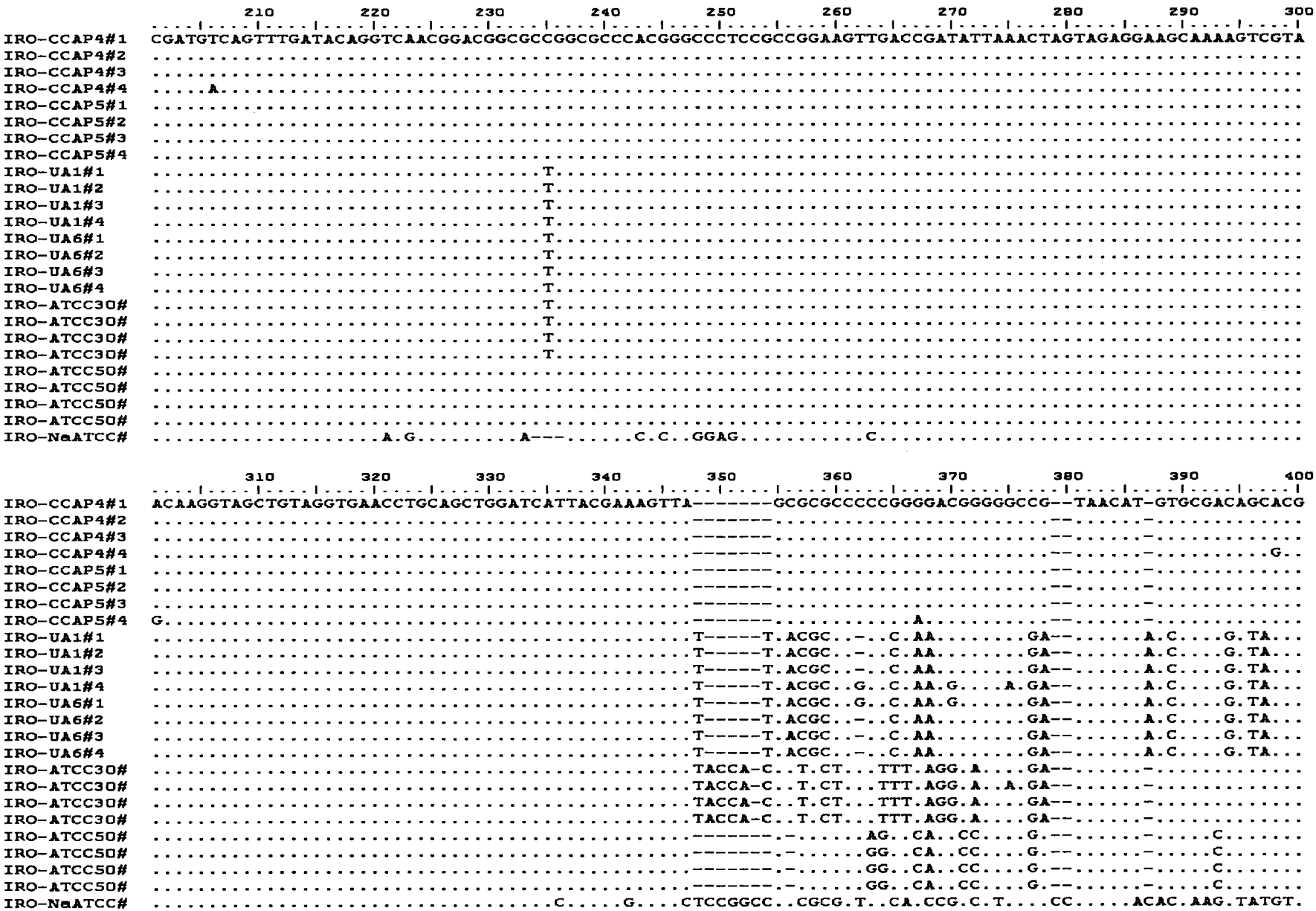
APPENDIX 17

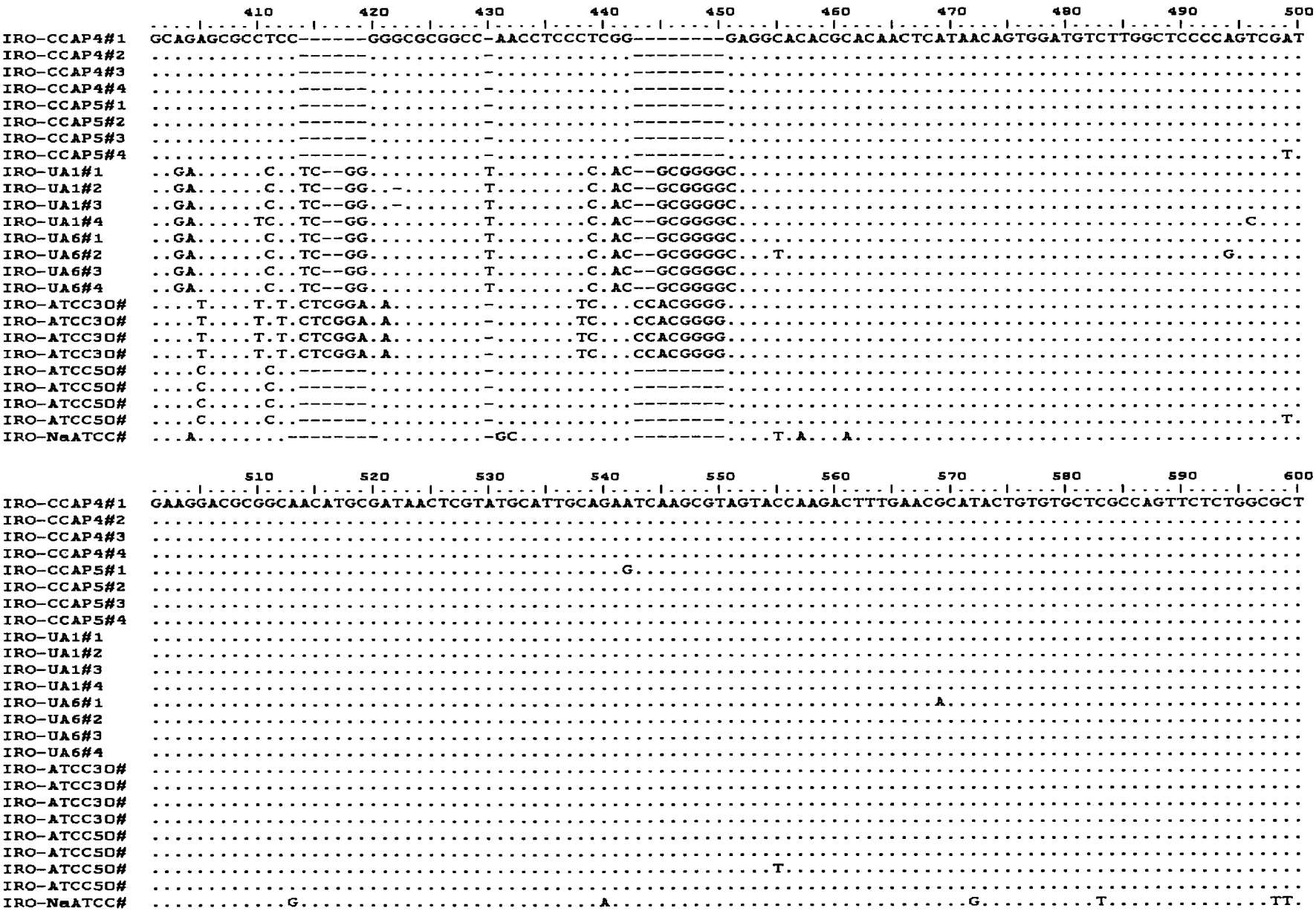
Ichthyobodo necator Related Organism ITS region alignment

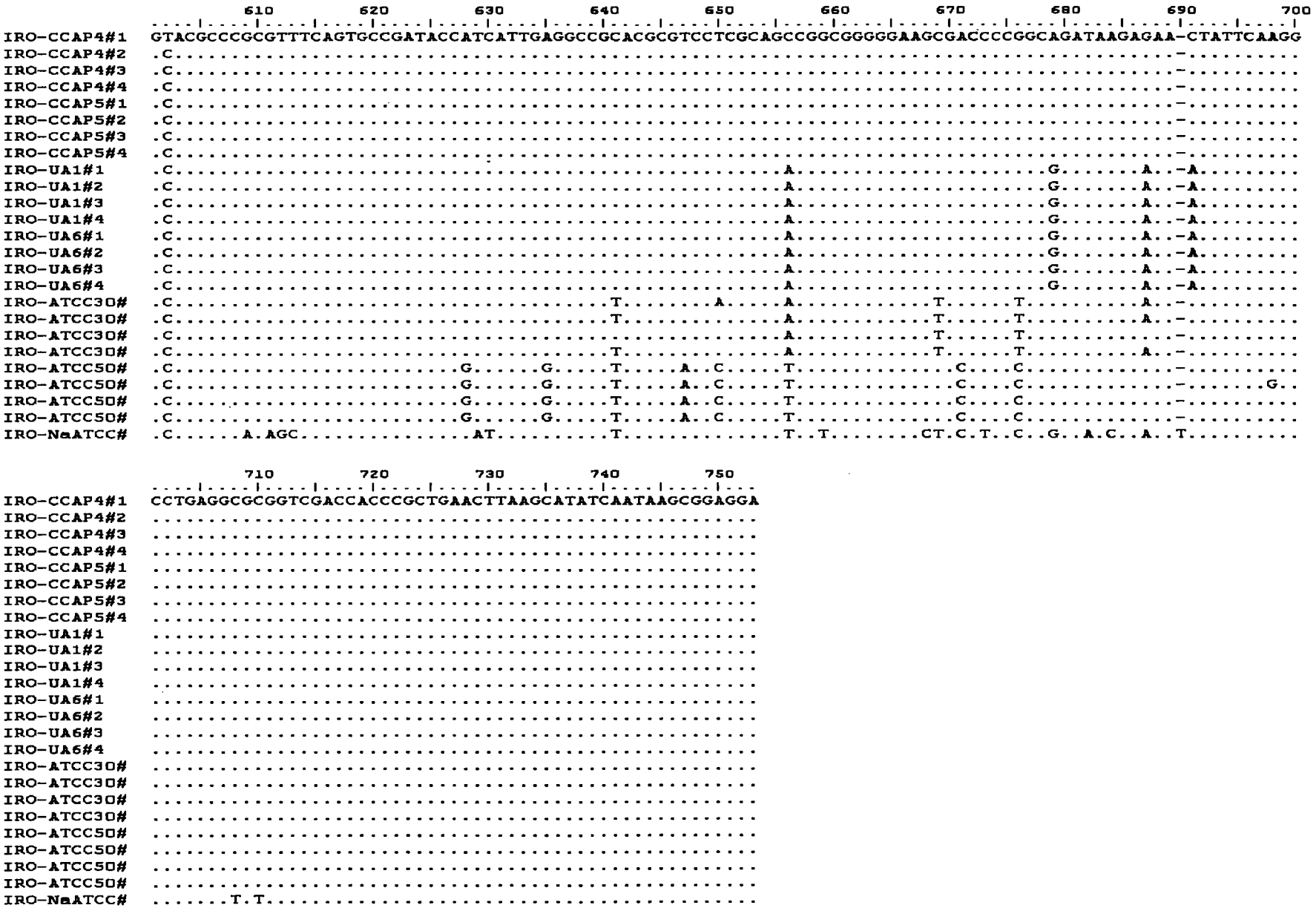
A total of 25 sequences including
four cloned sequences for each IRO-*Neoparamoeba pemaquidensis* isolates
and one cloned sequence for the IRO-*Neoparamoeba aestuarina* isolate (outgroup)

(ClustalW mutiple alignment performed with BioEdit software version 7.0.4.1)



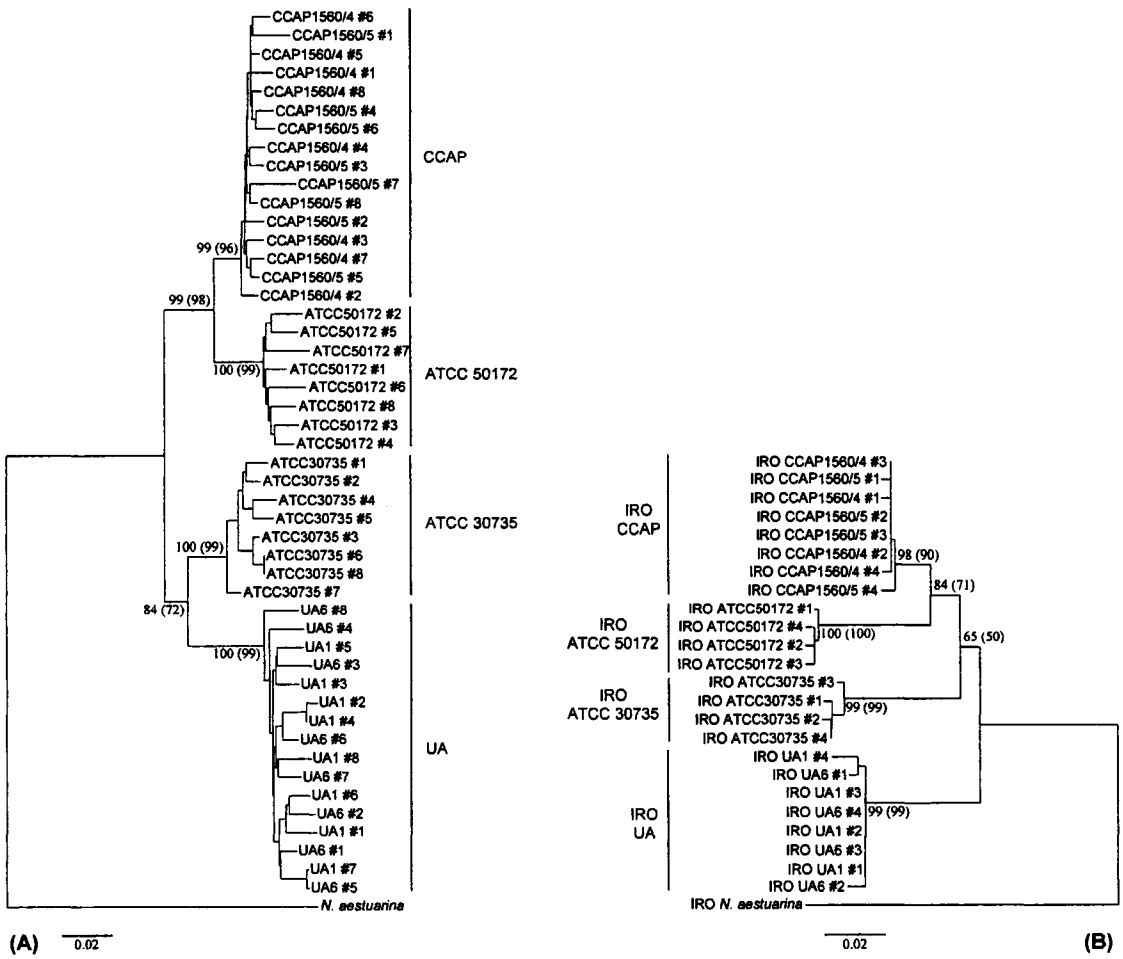






APPENDIX 18

Original phylogenetic analysis of *Neoparamoeba pemaquidensis* and respective endosymbiont *Ichthyobodo necator* Related Organism based on ITS sequences



(A) *Neoparamoeba pemaquidensis* Neighbor-joining phylogram based on the alignment of 49 sequences of 828 nucleotides long. (B) *Ichthyobodo necator* Related Organism Neighbor-joining phylogram based on the alignment of 25 sequences of 383 nucleotides long. Values at nodes represent the percentages of bootstrap replications: Neighbor-joining and Maximum Parsimony (within brackets), only values equal to or greater than 50 % are shown.