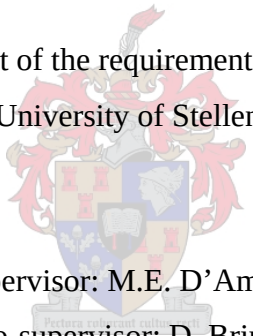
Identification of molecular markers for the diagnostic identification of the intracellular prokaryote associated with the appearance of withering syndrome in the abalone *Haliotis midae*

Candice Ockert

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch



Supervisor: M.E. D'Amato
Co-supervisor: D. Brink

April 2005

DECLARATION

I, the undersigned, hereby declare that the work contained in the thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:…….

Date:…….

ABSTRACT

Withering syndrome is a severe disease of abalone, *Haliotis* species that has been associated with mortality ranging from 99% in black, *H. cracherodii* Leach and 30% in red abalone, *H. rufescens* Swainson. The disease was first observed in California, along the west coast of North America and is an economically important disease that has led to the closure of the black abalone fishery throughout the southern California State. The causative agent of withering syndrome is a gram-negative intracellular *Rickettsiales*-like prokaryote designated *Candidatus xenohaliotis californiensis*. The geographical range of *C. xenohaliotis californiensis* is broad, besides red and black abalone it has also been reported in yellow, *H. corrugate* and blue abalone, *H. fulgens* all caught in Baja California, Mexico. In 2000 a *Rickettsiales*-like prokaryote resembling the disease-causing agent was observed in the digestive gland of the South African abalone *H. midae*. In this study we aimed to determine the relationship of the bacterium observed in the local abalone species, *H. midae* to the disease-causing agent of withering syndrome.

A specific PCR and *in situ* hybridization test using primers and probes specific for the *C. xenohaliotis californiesis* 16S rDNA gene were used to screen *H. midae* digestive gland tissues showing classical features of the *Rickettsiales*-like prokaryote. Both analyses indicated that *C. xenohaliotis californiensis* is not present in the local abalone species. We therefore aimed the identification of the organism parasiting the local abalone species by DNA sequence analysis of the 16S rDNA gene. The 16S rDNA gene was amplified by PCR, cloned and sequenced. Phylogenetic trees, constructed by maximum parsimony analysis revealed a diverse community comprised of α - *Proteobacteria*, *Mollicutes* and *Spirochaetes*. In the class α - *Proteobacteria* a novel group of sequences showing phylogenetic affinities to the order *Rickettsiales* was identified as likely candidate for forming the *Rickettsiales*-like inclusions in the digestive gland of *H. midae*. Oligonucleotide probes that bind to four variable regions of the novel group were used to confirm their presence in infected *H. midae* digestive gland tissue by *in situ* hybridization. Although these probes did not recognize the inclusions formed by the

Rickettsiales-like organisms, they revealed the presence of a group of free-living bacteria abundant in the host tissue.

We therefore conclude that (1) *C.xenohaliotis californiensis* is not present in the South- African abalone, *H. midae*; (2) the organisms isolated from the digestive gland of *H. midae* are part of the normal microflora and (3) the group of sequences showing phylogenetic affinities to the order *Rickettsiales* is not responsible for the *Rickettsiales*-like inclusions in infected digestive gland tissues but represent a novel group of organisms that are abundant in the host tissue.

O P S O M M I N G

Verwelking sindroom is ‘n siekte van perlemoen spesies wat geassosieer word met ‘n mortaliteit syfer van 99% in swart perlemoen, *H. cracherodii* en 30% in rooi perlemoen, *H. rufescens*. Die siekte is vir die eerste keer waargeneem in Kalifornië, langs die weskus van Noord-Amerika en is ‘n ekonomiese belangrike siekte wat gelei het tot die sluiting van die swart perlemoen vissery deur die hele suidelike staat van Kalifornië. Die oorsaak van verwelking sindroom is ‘n gram-negatiewe intrasellulêre *Rickettsiales*-tipe prokarioot naamlik, *Candidatus xenohaliotis californiensis*. *C. xenohaliotis californiensis* is geografies wydverspeid en buiten swart en rooi perlemoen is dit ook gerapporteer in geel, *H. corrugate* en blou perlemoen, *H. fulgens* almal vanaf Baja Kalifornië, Mexiko. In 2000 is ‘n *Rickettsiales*-tipe organisme wat ooreenkomste toon met *Candidatus xenohaliotis californiensis* waargeneem in die verteringsklier van die Suid-Afrikaanse perlemoen, *H. midae*. In hierdie studie is beoog om die verwantskap tussen hierdie *Rickettsiales*-tipe organisme en die siekte veroorsakende agent van verwelking sindrome te bepaal.

‘n Spesifieke polimerase ketting reaksie en *in situ* hibridisasie toets met inleiers en peilers spesifiek vir die *C. xenohaliotis californiensis* 16S rDNA geen is gebruik om geïnfekteerde *H. midae* verteringsklier weefsels te toets vir die teenwoordigheid van *C. xenohaliotis californiensis*. Beide analises het getoon dat *C. xenohaliotis californiensis* nie teenwoordig is in die plaaslike perlemoen spesie nie**.** Die volgende doel van die projek was dus om die *Rickettsiales*-tipe organisme wat die plaaslike perlemoen parasiteer te identifiseer deur middel van DNA volgorde analise van die 16S rDNA geen. Die 16S rDNA geen is geamplifiseer, gekloneer en die DNA volgorde bepaal**.** Filogenetiese bome, gekonstruktueer deur maksimum parsinomie analise het die teenwoordigheid van ‘n diverse groep bakterieë bestaande uit α - *Proteobacteria*, *Mollicutes* en *Spirochaetes* getoon. ‘n Nuwe groep volgordes wat ‘n filogenetiese affiniteit met die orde *Rickettsiales* toon in die klas α - *Proteobacteria* is geïdentifiseer as die waarskynlike kandidaat wat verantwoordelik is vir die *Rickettsiales*-tipe insluitsels in die verteringsklier van *H. midae*. Oligonukleotied peilers wat aan vier varieerbare streke van die nuwe groep bind is gebruik om hul teenwoordigheid deur middel van *in situ*

hibridisasie in geïnfekteerde *H. midae* weefsels aan te toon. Alhoewel die *Rickettsiales*-tipe insluitsel nie herken is deur die peilers nie, is ‘n sterk hibridisasie sein wel verkry vir ‘n groep vry-lewende bakterieë wat volop in die gasheer weefsel voorkom.

Ons kon dus tot die slotsom kom dat (1) *C. xenohaliotis californiensis* nie teenwoordig is in die Suid-Afrikaanse perlemoen *H. midae* nie, (2) die organismes geïsoleer uit geïnfekteerde verteringsklier weefsel van *H. midae* is deel van die normale mikroflora en (3) die groep volgordes wat ‘n filogenetiese affiniteit aan die orde *Rickettsiales* toon is nie verantwoordelik vir die *Rickettsiales*-tipe insluitsels in geïnfekteerde verteringsklier weefsel nie maar verteenwoordig ‘n nuwe groep organismes wat volop in die gasheer weefsel voorkom.

A C K N O W L E D G E M E N T S

I wish to express my sincere gratitude and appreciation to the following persons and institutions for their contributions to successful completion of the present study:

The Almighty for courage and faith to undertake this study;

Dr Eugenia D' Amato, my supervisor, for her encouragement, moral support and guidance.

Dr Danie Brink, my co-supervisor, for his support and guidance.

Dr Anna Mouton, for her guidance and interest in my project.

Prof Dirk Bellstedt and Benny, Department of Biochemistry, for sharing their expertise on phylogenetic analysis.

Dr Rouvay Roodt-Wilding and fellow students in lab231, for technical assistance, advice and encouragement throughout my studies.

The students and staff of the Department of Genetics, for technical assistance and advice.

Liesel and Lee-Anne, Department Medical Biochemistry, sharing their expertise on *in situ* hybridization.

National Research Foundation (NRF), for financial support.

My friend, Alicia Timm, for technical assistance, advice and encouragement throughout my studies.

To the important people in my life, my family, friends and especially Warren, for their prayers and unfailing belief in me.

LIST OF ABBREVIATIONS AND SYMBOLS

Λ	Lambda
ºC	Degrees centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
AMP	ampicillin
BCIP	5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt
BLAST	Basic local alignment search tool
bp	Base pair (s)
CaCl	Calcium chloride
CTAB	N-cetyl-N,N,N-trimethyl-ammonium bromide
ddH_2O	Double-distilled water
DIG-dUTP	Digoxigenin–labeled deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
HCL	Hydrochloric acid
ISH	*In situ* hybridization
LB	Luria Bertani
M	Molar
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium
ng	Nanogram
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

Pmol (es)	Picomole (s)
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SSU rDNA	Small subunit ribosomal deoxyribonucleic acid
TBE buffer	Tris Borate EDTA buffer
TRIS	2-amino-2 (hydroxymethyl)-1,3-propandiol
V	Volts
W	Watt

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

Emerging diseases are affecting mollusk production in several countries around the world. Given their severe impact on economic and socio-economic development, some diseases have become a primary constraint to the growth and sustainability of this sector. Currently eleven significant diseases of mollusks are listed in the *Aquatic Animal Health Code* (OIE, 2003). Withering syndrome, the most lethal disease that affects abalone, *Haliotis* spp. has also been included as one of these eleven diseases.

Withering syndrome is caused by a *Rickettsiales*-like prokaryote designated *Candidatus xenohaliotis californiensis* on the west coast of California, USA and Baja California Mexico (Haaker *et al*.1992; Steinbeck *et al*.1992; VanBlaricon *et al*.1993; Friedman *et al*.1997, 2000). The disease is most evident in black abalone, *H. cracherodii* but it also occurs in yellow, *H. corrugate*; blue, *H. fulgens* and red abalone, *H. rufescens* (Haaker *et al*. 1995; Moore *et al*. 2000; Alvarez *et al*. 2002). Withering syndrome has diminished the black abalone population by 99% and led to the closure of the black abalone fishery throughout the southern California State (Haaker *et al*. 1992; Richard & Davis, 1993). Heavy losses of up to 30% have also been reported in red abalone populations and developments in southern California suggest that the *Rickettsiales*-like prokaryote may be as devastating over time to red abalone as it has been for the black abalone (Haaker *et al*. 1995; Moore *et al*. 2000).

During a health management programme in 2000, Mouton observed a *Rickettsiales*-like organism in the digestive gland of the South African abalone, *H. midae*. Both cultured as well as wild abalone was infected with the bacterium. Transmission electron microscopy showed that the unknown bacterium was intracellular by nature and resembled the *Rickettsiales*-like prokaryote *C. xenohaliotis californiensis* considered to be the causative agent of withering syndrome. As yet none of the abalone infected with the *Rickettsiales*-like organism shows signs of withering syndrome. However, one of the characteristics of withering syndrome is that it can develop under stressed conditions, such as starvation or elevated water temperature (Steinbeck *et al*.1992; Lafferty & Kuris, 1993; Friedman *et al*. 1997; Moore *et al*. 2000). It is thus feared that changes in the abalones' normal environment, especially with environmental conditions associated with the culturing of abalone, such as handling, high stocking densities and fluctuating of water quality together with elevated temperatures may lead to the occurrence of the disease amongst South African populations.

The South African abalone, *H. midae*, or perlemoen as it is known locally, is one of the most important commercial abalone species worldwide. Of the six *Haliotis* spp. (*H. midae*, *H. parvum*, *H. spadicea*, *H. queketii*, *H. speciosa*, *H. pustulata*) endemic to the South African waters, *H. midae* is the only one that is exploited on a commercial scale (Muller, 1986; Hecht, 1994). *Haliotis midae* is highly prized in the Far East (South African abalone fetch between US$30 and US$40 per kg on the Eastern market), especially in Japan because the abalones dark, grey flesh bears a strong resemblance to Japanese abalone. At present there are approximately 15 commercial abalone farms in South Africa. Current total farm production is about 515 tons per annum live mass with a farm gate value of R125m (Anon, 2004). Because of its huge demand in the Far East, *H. midae* has suffered tremendously under the hands of poachers, which have resulted in huge financial losses to the commercial industry. The outbreak of a disease such as withering syndrome can deplete remaining abalone populations even more, leading to the loss of an important national resource. It is therefore of economic importance to determine the relationship of the unknown bacterium detected in *H. midae* to *C. xenohaliotis californiensis* before major disease outbreaks occurs.

1.1 **AIMS OF STUDY**

The intracellular *Rickettsiales*-like prokaryote infecting the South African abalone species has not been characterized before and its relationship to the disease-causing agent of withering syndrome, *C. xenohaliotis californiensis*, if any, has not been determined. In South Africa a unique opportunity therefore exists to determine the relatedness of the unknown organism to *C. xenohaliotis californiensis* by means of molecular methods before major disease outbreak occurs. Therefore, specific aims were to:

1. Determine the relationship of the intracellular *Rickettsiales*-like prokaryote infecting the South African abalone to the etiological agent of withering syndrome using *C. xenohaliotis californiensis* specific primers and probes.

2. Retrieve 16S rDNA sequences from infected tissues in order to determine the phylogenetic position of the *Rickettsiales*-like prokaryote infecting *H. midae*.

3. Confirm the presence of the *Rickettsiales*-like prokaryote residing in *H. midae* by means of *in situ* hybridization with species-specific probes designed on 16S rDNA sequences isolated from the phylogenetic study.

1.2 **REFERENCES**

Alvarez Tinajero, M.D.C., Caceres-Martinez, J. & Viles, J.G.G. 2002. Histopathological evaluation of the yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* from Baja California, Mexico. *Journal of Shellfish Research* **21**: 825-830.

Anon. 2004. Aquaculture in South Africa - the hype and the potential. *Food & Beverage Reporter*. 6-10.

Friedman, C.S., Thomson, M., Chun, J., Goodfellow, M., Gee, A. & Hedrick, R.P. 1997. Withering syndrome of black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research* **16**: 403-411.

Friedman, C.S., Andree, K.B., Beauchamp, K.A., Moore, J.D., Robbins, T.T., Shields, J.D. & Hedrick, P.P. 2000. '*Candidatus xenohaliotis californiensis'*, a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *International Journal of Systematic and Evolutionary Microbiology* **50**: 847-855.

Haaker, P.L., Richard, D.V., Friedman, C.S., Davis, G.E., Parker, D.O. & Togstad, H. 1992. Mass mortality and withering syndrome in black abalone *Haliotis cracherodii* in California. *In Abalone of the World*. Shephard, S.A., Tegner, M. J & Guzman, S. A. (eds). Blackwell Scientific Publications, Oxford, 214-224.

Haaker, P.L., Harris, S.L., Taniguchi, I.K. & Friedman, C.S. 1995. Withering Syndrome in California: does occur in subtidal populations? *Journal of Shellfish Research* **14***:267.*

Hecht, T. 1994. Behavioral thermoregulation of the abalone, *H. midae*, and applications for intensive culture. *Aquaculture* **3**: 171-181.

Lafferty, K.D. & Kuris, A.M.1993. Geographic patterns in the wasting disease of black abalone. *Bulletin of the Ecological Society of America* **73**: 240.

Moore, J.D., Robbins, T.T. & Friedman, C.S. 2000. Withering syndrome in farmed red abalone, *Haliotis rufescens*: thermal induction and association with gastrointestinal *Rickettsiales*-like prokaryote. *Journal of Aquatic Animal Health* **12**: 26-34.

Mouton, A. 2000. Health management and disease surveillance in abalone, *Haliotis midae*, in South Africa. *4th International Abalone Symposium, Cape Town, South Africa*: 526.

Muller, S. 1986. Taxonomy of the genus *Haliotis* in South Africa. *Transactions of Royal Society of South Africa* **2**: 69-77.

OIE (World Organisation for Animal health) Diagnostic manual for Aquatic Animal Diseases,, Office International Des Epizooties, Paris, France. http://www.oie.int/eng/normes/fmanual/A_summary.html

Richard, D.V. & Davis, G.E. 1993. Early warnings of modern population collapse in black abalone, *Haliotis cracherodii*, Leach 1814, at the California Channel islands. *Journal of Shellfish Research* **12**: 189-194.

Steinbeck, J. R., Groff, J.M., Friedman, C.S., McDowell, T. & Hedrick, R.P. 1992. Investigation into a coccidian-like protozoan from the California abalone, *Haliotis cracherodii*. In: *Abalone of the World*. Shephard, S.A., Tegner, M. J & Guzman, S. A. (eds). Blackwell Scientific Publications, Oxford, 203-213.

VanBlaricon, G.R., Ruediger, J.L., Friedman, C.S., Woodard, D.D. & Hedrick, R.P. 1993. Discovery of withering syndrome among black abalone *Haliotis cracherodii* Leach, 1814 Populations at San Nicolas Island, California. *Journal of Shellfish Research* **12**: 185-188.

CHAPTER 2
LITERATURE REVIEW

It is necessary to review two distinct, but pertinent, subjects to place the study in context. First, aspects of withering syndrome will be described. Next, the detection and identification of unrecognized microorganisms by molecular methods are assessed.

2.1 **WITHERING SYNDROME**

In the mid 1980's a huge decline in the Californian black abalone *H. cracherodii*, population along the Channel Island was observed. Deceased animals were severely shrunken and their empty shells were observed along the Channel Islands. After extensive studies it was shown that an infectious disease known as withering syndrome (WS) (Haaker *et al*. 1992; Steinbeck *et al*.1992; VanBlaricon *et al*. 1993; Friedman *et al*. 1997) caused the decline. The disease spread throughout the Channel Islands during the early 1990's (Lafferty & Kuris, 1993; VanBlaricon *et al*. 1993) and depleted the remaining populations, which were already greatly reduced by overfishing (Haaker *et al*. 1992; Richard & Davis, 1993). Since then, the disease has also been reported in red abalone, *H. rufescens* Swainson (Haaker *et al*. 1995; Moore *et al*. 2000), yellow abalone, *H. corrugata* and blue abalone, *H. fulgens* all caught in Baja California, Mexico (Alvarez *et al*. 2002). The geographical range of WS is suspected to be broad since infected red abalone has been transported to Chile, Japan, Israel and other countries (OIE, 2003). On the northern coast of China a similar looking disease of unknown etiology has also been reported from cultured *Haliotis discus hannai* (Guo *et al*. 1999). In 2000, Mouton *et al.* reported the presence of a *Rickettsiales*-like prokaryote in the digestive tract of the South African abalone (*H. midae*) with no associated pathology.

2.1.1 **Etiology**

Various hypotheses have been formed regarding the possible causes of WS. Pollution, a reduced food supply due to the 1983 El Niño events and an infectious agent was thought to be the main possible explanations for this phenomenon. (Haaker *et al*. 1992; Lafferty & Kuris, 1993). After extensive studies conducted on infected black abalone it was concluded that an infectious agent was responsible for the disease and that elevated temperatures, though not the sole cause, resulted in accelerated

mortalities (Lafferty & Kuris, 1993). *Pseudoklossia haliotis*, a coccid parasite that infects the kidney of the abalone (Friedman *et al*. 1995) was examined as a possible cause but no link could be made with the signs of WS (Friedman *et al*. 1993). In 1995, Gardner *et al*. reported that a *Rickettsiales*-like prokaryote was infecting the digestive tract of black abalone populations affected with WS. The prokaryote was absent in unaffected populations. Subsequent studies conducted on laboratory-held black abalone reported complex relationships between WS signs, withering syndrome-*Rickettsiales*-like prokaryote (WS-RLP) intensity, temperature and food supply, verifying that the WS-RLP presence is essential for WS expression in black abalone and suggesting its etiological role (Friedman *et al*. 1997). The WS-RLP was also found to be the causative agent of withering syndrome in red abalone (Andree *et al*. 2000; Antonio *et al*. 2000; Friedman *et al*. 2000; Moore *et al*. 2001). WS-RLP infections have also been reported in pink and green abalone (Moore *et al*. 2000).

2.1.1.1 **Phylogeny**

The etiological agent of WS was recently identified as an intracellular *Rickettsiales*-like prokaryote, designated *C. xenohaliotis californiensis* (Friedman *et al*. 2000). The classification of *C. xenohaliotis californiensis* is as follows (National Library of Medicine website[1]):

Superkingdom: Bacteria
Phylum: *Proteobacteria*
Class: *α - Proteobacteria*
Order: *Rickettsiales*
Family: *Anaplasmataceae*

C. xenohaliotis californiensis is phylogenetically placed in the order *Rickettsiales* as a member of the family *Anaplasmataceae*. The species *Wolbachia pipientis* followed by *Ehrlichia sennetsu* and *Ehrlichia risticii* are some of its closest relatives (Friedman *et al*. 2000). The term "*Candidatus*" is an indication that identification of the bacterium was based on the morphological characteristics, life history and the nucleotide sequence analysis of the 16S rDNA gene and lacks biochemical and serological characterization.

[1]http://www.ncbi.nlm.nih.gov

The order *Rickettsiales* contains a large and diverse group of obligate intracellular, gram-negative bacteria. Several species within the order cause diseases in humans and in other vertebrate or invertebrate hosts and have a worldwide distribution. The order consists of two families, *Rickettsiaceae* and *Anaplasmataceae*. Members of the family *Rickettsiaceae* grows freely in the cytoplasm of their eukaryotic host cells and includes etiological agents of human and animal diseases such as typhus, spotted fever and scrub typhus (Krieg & Holt, 1984; Roux & Raoult, 1995; Bouyer *et al*. 2001; Ishikura *et al*. 2002). In contrast to the family *Rickettsiaceae*, the family *Anaplasmataceae* replicates while enclosed in its eukaryotic host cell membrane-derived vacuole (Rikhisa, 1991). Most of the bacteria contained in this family are pathogenic to humans, domestic animals, cattle, sheep and goats (Krieg & Holt, 1984; Barlough *et al*. 1996; Noda *et al*. 1997; Parola *et al*. 2001; Lew *et al*. 2003). A large number of pathogens of aquatic animals, most of them poorly described, are also contained in the order *Rickettsiales* (Sparks, 1985). Examples of those that are well described include *Piscirickettsia salmonis*, the etiological agent of piscirickettsiosis in salmon (Fryer *et al*. 1994), the etiological agent of stained prawn disease (Bower *et al*. 1996) and NHP, the disease-causing agent of necrotizing hepatopancreatitis in shrimp (Loy *et al*. 1996).

Most terrestrial rickettsial diseases require an arthropod vector (e.g. mosquitoes, fleas, lice and ticks) for spread between individuals. Studies by Moore *et al.* (2001) and Friedman *et al.* (in press) of black and red abalone infected with *C. xenohaliotis californiensis* showed that no arthropod vector was required and that direct transmission occurred between individuals. Transmission without an arthropod vector was also seen in other marine *Rickettsiale*s-like prokaryotes e.g., *P. salmonis* infecting salmon (Cvitanich *et al*. 1991) and two that infect shrimp (Brock *et al*. 1986; Bower *et al*. 1996). *C. xenohaliotis californiensis* infects the gastrointestinal epithelium of the abalone and it was therefore suggested that, as with most gastrointestinal pathogens, the spread of the disease-causing agent between individuals is likely fecal–oral.

2.1.1.2 **Morphology**

Candidatus xenohaliotis californiensis is a gram negative, pleomorphic, obligate intracellular parasite that replicates within the cytoplasm vacuoles of the host's gastrointestinal epithelial cells. The vacuoles containing these bacteria are usually located apical to the host nucleus and are predominantly found in the cells of the post-esophagus transport ducts of the digestive gland and less frequently in the intestine (Friedman *et al*. 2000).

The dimorphic rod to spherical shaped bacterium are ribosome-rich and measure an average of 332 x 1550nm in the bacillus form and an average of 1405nm in the spherical form. They are surrounded by a trilaminar cell wall consisting of an electron dense inner plasma membrane and an outer membrane that are separated by a not so visible peptidoglycan layer (Friedman *et al*. 2000). *Candidatus xenohaliotis californiensis* replicates by binary fission within the intracytoplasmatic vacuoles, which are 14-56µm in diameter.

2.1.1.3 **Clinical symptoms**

WS is characterized by weight loss, atrophy of the foot muscle (Fig. 2.1), lethargy, weakness, mantle retraction, poor gonad development and ultimately death (Haarker *et al*. 1992; VanBlaricon *et al*. 1993). These symptoms are preceded by morphological changes in the digestive gland and foot muscle of the abalone. Morphological changes may include degeneration (atrophy of tubules, increase in connective tissues and inflammation) and or metaplasia of the digestive tubules. The metaplasia involves the replacement of secretory and absorptive cells in the digestive gland with connective tissue or less differentiated mucosal tissue (Kismohandaka *et al*. 1993; Gardner *et al*. 1995). This results in both the loss of digestive enzyme production and nutrient uptake. The animal therefore catabolizes its foot muscle tissue to meet its energy needs and this result in atrophy of the foot muscles (Kismohandaka *et al*. 1993).

Fig 2.1. The picture on the left shows an abalone shrunken inside its shell whereas the abalone on the right is healthy.

WS occurs at elevated temperatures (~18 °C). The duration of withering syndrome ranges from 3-7 months (Friedman *et al*. 1997). Cumulative mortality has been recorded at over 99% in black abalone and 30% in red abalone. (Haaker *et al*. 1992; Friedman *et al*.1997)

2.1.2 **Control of withering syndrome**

Experiments indicate that the *C. xenohaliotis californiensis* may be water-borne and does not require direct contact between infected and uninfected abalone (Moore *et al*. 2000). Above-normal water temperatures seem to have a synergistic effect on the disease (Martinez *et al*. 2000; Moore *et al*. 2000). Friedman *et al.* (1997) and Moore *et al.* (2000) showed that abalone held at elevated temperatures (black abalone – 20˚C, red abalone – 18.5˚C) are more susceptible to mortality and show more severe signs of WS. There is also an increase in infection with the *Rickettsiales*-like prokaryote in abalone at these elevated temperatures compared to abalone held in cooler waters (black abalone 13˚C, red abalone 14˚C degrees). Thus in affected cultured facilities, parasite transmission and disease expression may be curtailed if the water temperature is reduced. One major drawback of this method is that it is very difficult to modulate seawater temperature in aquaculture farms.

One way to overcome this obstacle was by using an alternative treatment. Oxytetracycline is an antibiotic in the tetracycline family and is approved by the Food and Drug Administration to treat bacterial infections in several aquatic organisms. This therapeutant has been shown to be effective in the control of the bacterial kidney disease in salmonids caused by *Renibacterium salmoninarum* (Brown *et al*. 1990) and *P. salmonis*, a marine rickettsial pathogen problematic in Chile (Cvitanich *et al.* 1991). In 2003, Friedman *et al*. assessed the therapeutic treatment of withering syndrome in black and red abalone by using oxytetracycline by injection and oral administration. The treatment was shown to effectively reduce the intensity of the bacterial infection and in some cases eliminated it entirely. This resulted in an increase in market size abalone and abalone treated with tetracycline was also much larger than the untreated animals (Friedman *et al*. 2003).

2.1.3 **Diagnostic techniques**

If *C. xenohaliotis californiensis* is detected outside its known range, light microscopy in combination with molecular techniques must be used to identify and distinguish the detected organism from other rickettsial bacteria.

Two molecular approaches have been developed to diagnose abalone with the disease-causing agent of WS. *C. xenohaliotis californiensis* specific PCR primers, based on the 16S rDNA gene were designed to screen animals showing gross signs of withering syndrome. In addition to histology this served as a presumptive method. Since PCR detects only the presence of the pathogen, other techniques must be used to link the isolated DNA to the bacteria residing in the tissues. These techniques include histological preparations and *in situ* hybridizations using the isolated DNA as probes (Andree *et al*. 2000)

In situ hybridization has recently been developed to detect *C. xenohaliotis californiensis* in tissue sections. Oligonucleotide probes specific for *C. xenohaliotis californiensis* were designed to hybridize to the small subunit ribosomal RNA of the bacterium (Antonio *et al*. 2000). The advantage of this approach is that it allows direct visualization of a specific probe hybridized to the target organism. *In situ* hybridization is therefore the method of choice to verify the identity of the rickettsial bacteria in abalone species previously not known to be susceptible to the bacterium or in a new geographical location to confirm the presence of the pathogen.

2.2 DETECTION AND IDENTIFICATION OF UNRECOGNIZED MICROORGANISMS

The aim of diagnostic microbiology is to rapidly and accurately identify bacteria in their natural environment. Traditional culture-based methods are labor intensive and time consuming and are often too selective, particularly for fastidious or yet to be cultured organisms (Wagner *et al.* 1993; Choi *et al.* 1994). The advent of molecular techniques such as PCR and subsequent hybridization or sequencing has revolutionized all fields of microbiology, and made sensitive detection and exact identification of bacteria possible.

2.2.1 **rRNA approach**

2.2.1.1 **16S rRNA gene**

The most widely used molecular marker for the detection of infectious agents or to study microbes in their natural environment is the 16S rRNA gene. Several features of the 16S rRNA gene make it an important phylogenetic tool and hence a useful target for bacterial identification. The first important feature is that this gene is present in all organisms and is therefore a universal target for bacterial identification. Secondly, its rate of change is constant over long periods and among diverse organisms

and therefore allows inferences of evolutionary distance among a wide range of life forms (Relman, 1998). Thirdly, it contains conserved as well as variable regions, which makes it possible to design general as well as specific primers and probes. Finally, it is an important cellular compound, which facilitates detection (Muyzer *et al*. 1998).

SSU rRNA sequences were the first to reveal a tripartite tree of life, the tree consisting of three domains bacteria viz. Eubacteria, Archaea (archaebacteria) and Eukarya (eukaryotes) (Muyzer *et al*. 1998). Phylogenetic analysis of other genes such as 23S rRNA, the elongation factor EF-Tu and ATPase subunit produced similar trees, which justifies the use of 16S rDNA sequences for phylogenetic inferences (Ludwig, 1998).

The most common route used to identify or to study microbes in their natural environment involves an approach – referred to as the "full cycle rRNA analysis". The full cycle approach can be divided into two phases, phase 1 – involves the retrieval of 16S rDNA sequences from its natural environment and phase 2 involves authenticating the origin of the 16S rDNA sequences retrieved in phase 1 by *in situ* hybridization. Fig. 2.2 shows a schematic outline of the "full cycle rRNA approach". (Amann *et al*. 1995).

2.2.1.2 **Phase 1 – Broad range PCR and sequencing**

Identification of a microorganism from its natural environment includes the following steps. First DNA is extracted from the environmental sample. After DNA isolation the target is amplified by broad range PCR and cloned into an appropriate vector. Next the colonies obtained from the cloning experiments are screened for clones containing the correct insert size and sequenced. The final step is to compare the sequence to a database and to determine its phylogenetic position by inferring phylogenetic trees (Amann *et al*.1995). The construction of phylogenetic trees is based on a sequence alignment. The alignment is a statement of homology, i.e. shared ancestry from which historical inferences are made. The alignment becomes essential to reconstruct phylogenies. There are two fundamental ways of treating data, as distance matrix methods (e.g. UPGMA, neighbor-joining), also known as clustering and algorithmic methods or as discrete characters (e.g. parsimony, maximum likelihood, Bayesian methods), also known as tree searching methods. Distance methods are relatively simple and faster to perform than discrete methods. Its main function is to calculate the distance (roughly, the percentage sequence differences) for all pairwise combinations of operational taxonomic units (OTUs) and to assemble it into a tree. In contrast to distance methods, discrete-date methods are time consuming but it provides more information than distance methods. These methods set a

hypothesis for every column in the alignment so that the evolution of a specific site in the molecule can be traced (Nei *et al*. 2000; Salemi *et al*. 2003).
To test the accuracy (reliability of groupings) of the phylogenetic trees obtained, bootstrap analysis can be performed (Felsenstein, 1985). Bootstrapping is a method of sampling with replacement of the sites in the alignment, and then trees are created based on those subsamples. The process is iterated multiple times (the preferred number is 1000, although a minimum of 100 can be used) and the results are compiled to allow an estimate of the reliability of a particular grouping (Nei *et al*. 2000; Salemi *et al*. 2003).

The used of the broad range PCR approach has expanded the ability of laboratories to partially characterize organism that has never been cultured before. This technique is already successfully applied in clinical microbiology to detect slow-growing bacteria (e.g. *Mycobacteria*) or organisms that are difficult to culture e.g. *Tropheryma whippelii* (Moter *et al*. 2000).

Although the use of broad range PCR using universal primers has been shown to be advantageous in the identification of microorganisms, the technique is subject to several drawbacks. Microbial contamination is one such obstacle. Even after rigorous technical precautions are taken to minimize contamination of PCR reactions, false - positives can occur. Another noticeable limitation of broad range PCR with examination of complex ecosystems with a broad microbial diversity is that a distinct band may sometimes contain more than one microbial population (Houpikian *et al*. 2002). The approach also bears an additional potential bias for the representative assessment of natural abundance of rRNA genes resulting from the preferential amplification of certain templates. In a worst case scenario it can also prevent the amplification of certain rRNAs (Amann *et al*. 1995)

PCR amplification and sequencing of the 16S rDNA gene yields information on the identity or relatedness of new sequences in comparison with species obtained from available databases. However, this is not proof that the retrieved sequences were from cells thriving in this habitat. They could also have originated from naked DNA present in the sample or from contamination (Amann *et al*. 1995). Therefore, in the second phase (Fig. 2.2), to authenticate the source of the retrieved sequences, sequence-specific hybridization probes have to be designed to identify and enumerate whole fixed cells in the original sample by *in situ* hybridization.

2.2.1.3 **Phase 2 – *In situ* hybridization using oligonucleotide probes**

In situ hybridization is the method of choice to study and identify microorganism in their natural environment. The technique permits visualization and identification of individual cells within their natural microhabitat or diseased tissues (Moter *et al*. 2000). *In situ* hybridization using oligonucleotide probes was first introduced in bacteriology by Giovanni *et al.* (1988). The advantage of using oligonucleotide probes is that they can be tailor-made to bind to targets with a wide range of specificities, from domain to strain (Hugenholtz *et al*. 1996). Also the small size of the oligonucleotide (generally 15-30bp) allows them to easily penetrate through the tissue, making access to their target much easier (Polak *et al*. 1998). Oligonucleotide probes are excellent tools for identifying bacteria and for describing microbial communities, but they rely on sequence data for their design. When designing new probes evaluation is essential, positive and negative controls should be included in all *ISH* experiments (Polak *et al*. 1998). It is also important to note that an oligonucleotide is only as precise as the sequence from which it was derived. It is thus wise to check the probes using databases such as GenBank and RDP (Ribosomal Database Project) before using it (Hugenholtz *et al*. 1996; Moter *et al*. 2000).

2.2.2 **Applications of the rRNA approach**

It is beyond this thesis to review all possible applications of the rRNA approach. Therefore a few applications are mentioned to highlight the versatility of the method.

2.2.2.1 **Microbial diversity in different environments**

Our knowledge of microbial biodiversity has been severely limited by relying on microorganisms that have been cultured. These represent only a tiny fraction of the microbial diversity in the environment (Hugenholtz & Pace, 1996). Molecular techniques such as the rRNA approach have side-stepped many of the stumbling blocks of cultivation and made possible ecological studies of microbial communities (Pace *et al.* 1986; Amann *et al*. 1995). It has been used worldwide to investigate microbial communities in natural environments. Table 2.1 shows a summary of the results of the rRNA approach from different studies exploring the diversity of microbial populations in various environments. These studies have major implications for understanding the structure and ecology of natural bacteria consortia and their population genetics in response to influences of nature and human activities.

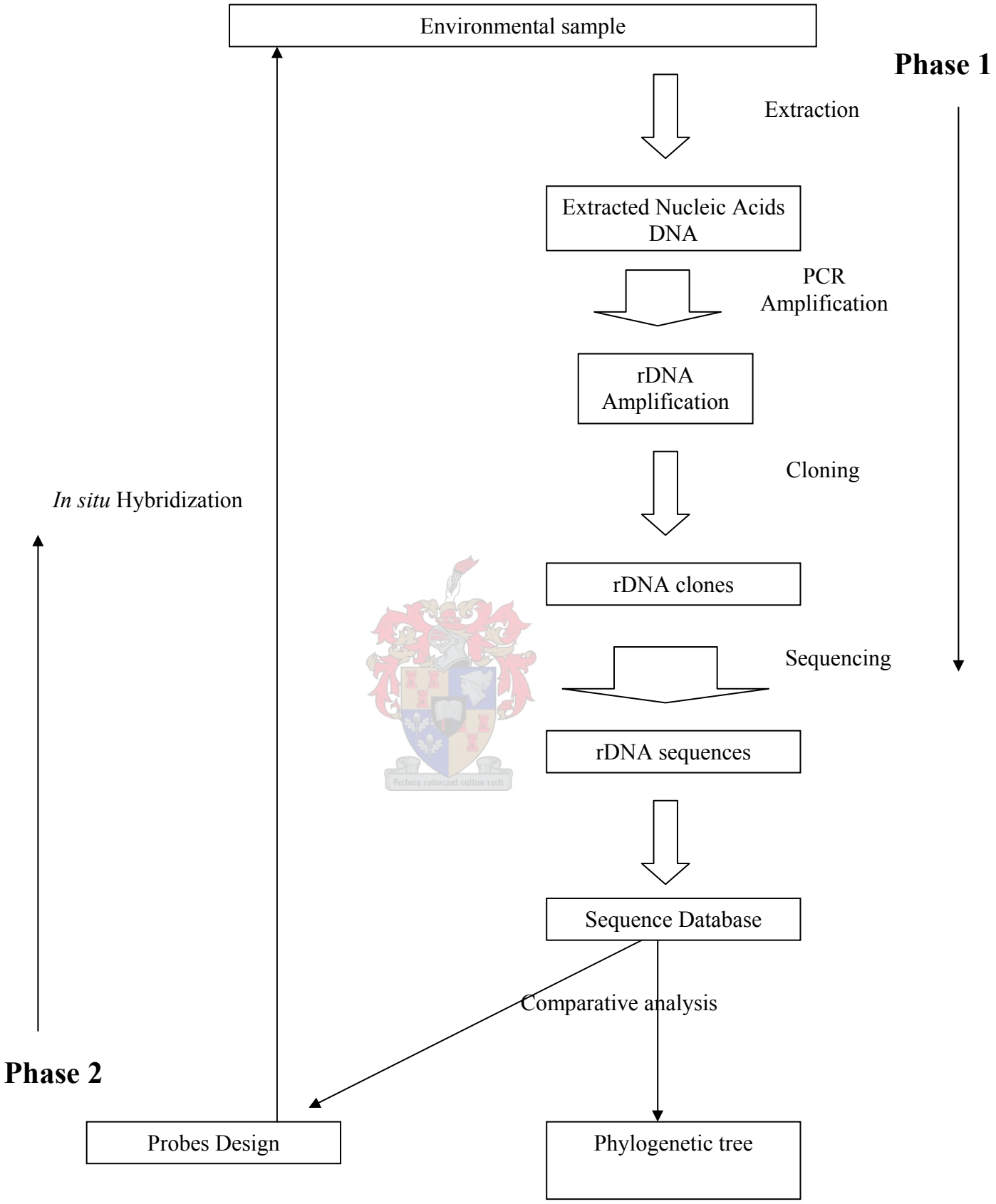


Fig 2.2. Flow chart showing the principal phases of the rRNA approach for sequencing and probing

Table 2.1: Prokaryotic diversity in different environments as revealed by the rRNA approach

Habitat	Phylogenetic affiliation of rRNA sequences	Citation
Sargasso Sea	*α –Proteobateria*, *Cyanobacteria*	Giovannoni *et al*. 1990
Agricultural soil	*Acidobacteria*, *Cytophagales*, *Chloroflexi* *γ -Proteobacteria*	Furlong *et al*. 2002
Hydrothermal vent	*γ, δ, ε – Proteobacteria*	Moyer *et al*. 1995
Great South Bay	α, β, γ, δ, ε – *Proteobacteria, Cyanobacteria* *Fusobacteria,* Low and high GC Gram-positive bacteria	Kelly *et al*. 2001
Marine sediments	γ, δ –Proteobacteria	Cifuentes *et al*. 2000
Activated sludge	α, β, γ, ε – *Proteobacteria*, *Plantomyces*, *Chamydia*	Snaidr *et al*. 1997

2.2.2.2 **Symbiotic bacteria**

Most obligate microbial symbionts are as-yet uncultured. Using the 16S rDNA approach, they can be identified and phylogenetically classified. By means of *in situ* hybridization their localization within different compartments of the host can be confirmed and at the same time their symbiotic nature proven.

2.2.2.2.1 **Bacterial symbionts of protozoa**

The 16S rDNA approach was performed for the symbiotic bacteria *Holospora obusta* that infect the macronucleus of *Paramecium caudatum* (Amann *et al*. 1991). In *P. caudatum* the situation is quite complicated. These ciliates also feed on bacteria, thus bacterial rRNA could be detected in the nuclei as well as in food particles. After amplification, cloning and sequencing of the rDNA gene fragments, *in situ* hybridization oligonucleotide probes were able to distinguish the *Holospora* species from ingested bacteria present in the food particles (Springer *et al*. 1993).

2.2.2.2.2 **Endosymbiotic bacteria in insects**

A well known example of symbiosis between bacteria and insects is the relationship between the pea aphid, *Acyrthosiphon pisum* and its primary endosymbiotic bacterium (Baumann *et al*. 1993). Phylogenetic analysis of the 16S rRNA gene placed the bacterium within the gamma subdivision of *Proteobacteria* and was subsequently named *Buchnera aphidicola*. Recently a secondary endosymbiont of the pea aphid was also identified by means of the rRNA approach. Phylogenetic analysis placed the bacterium in the order *Mollicutes* as a member of the genus *Spiroplasma*. The spiroplasma organism is said to assist the primary endosymbiont, *B. aphidicola*, in the synthesis of essential amino acids and other nutrients (Fakutsu *et al*. 2001).

2.2.2.2.3 **Identifications of pathogens**

Clinicians have long been aware of human diseases that are associated with visible but nonculturable microorganisms. The rRNA approach has been widely used to identify causative agents in humans. Whipple's disease is a bacillus infection of the duodenum and small intestine that causes fever, abdominal pain and diarrhea. By means of the rRNA approach the Wipple's disease bacterium was identified as an actinomycete not related to any known genus for which the name *Tropheryma whippelii* has been proposed (Wilson *et al*. 1991; Relman *et al*. 1992).

rRNA techniques have also been shown to be effective in the identification of animal pathogens. The causative agent of proliferative enteritis in pigs was recently described as IS (Ileal symbionts) intracelluloris. Cloning and sequencing of the 16S rRNA gene showed that the gram negative, nonflagellated, curved rod clustered within the delta subclass of *Proteobacteria*, with 91% similarity to *Desulfovibrio desulfuricans* (Gebhart *et al*. 1993).

The rRNA approach has also made possible the detection of *Mycoplasma* bacteria, known to be pathogenic to humans, animals and plants. *Mycoplasmas* are gram -positive bacteria with a low G+C content that are characterized by a complete lack of cell walls (Maniloff *et al*. 1992; Rawadi & Dussurfget, 1998). Because of their nutritional requirements, cultivation of pathogenic strains is difficult. Some of the examples where the rRNA approach was utilized to aid the detection of pathogens includes the detection of *Eperythrozoon wenyonii*, a mycoplasma that infects the erythrocytes of a wide range of vertebrate animals (Neimark *et al*. 1997), and the detection of *Mycoplasma pneumoniae*, which is the most common pathogen in lung infections (Shaw, 1989).

Diagnostic techniques of molluscan disease agents are limited and most examinations are based on histological and ultrastructural methods. Classical serological methods cannot be used for diagnostic purposes because mollusks do not produce antibodies (OIE, 2003). In recent years, several studies have used the rRNA approach to identify causative agents. One example is the work on *Haplosporiduim nelsoni*, an oyster pathogen that was responsible for periodic, large-scale oyster mortalities in estuaries of the middle Atlantic coast of the United States. After the

16S rDNA was sequenced (Fong *et al*. 1993), a 21 base oligonucleotide probe unique to *H. nelsoni* was used to establish that the retrieved sequenced originated in *H. nelsoni* cells (Stokes & Burreson, 1995).

Other studies also encompassed the identification of microorganisms in mollusks. Necroting hepatopancreatitis (NHP) is a severe disease of farmed-raised *Penaeus vannamei* that has been associated with huge mortality losses ranging from 20-95%. The NHP bacterium, which remains uncultured in part because of the absence of established shrimp cell lines, has been examined using the rRNA approach (Loy *et al*. 1996). The 16S rDNA sequence analysis revealed a close relationship to bacterial endosymbionts of the protozoa *Caedibacter caryophila* and *Holospora obtuse*. Fluorescent labeled oligonucleotide DNA probes were used to detect the bacterium in infected shrimp by *in situ* hybridization. The technique provided direct visual evidence that the 16S rDNA sequences retrieved from infected tissues were derived from the intracellular bacterium that infects farmed raised *P. vannamei* shrimp.

2.2.2.3 **Gastro-intestinal flora**

To understand the role that individual microbes play in the gastro-intestinal tract, it is important to investigate the numbers of bacterial groups in the intestine. To date studies of the microbial community in the gastro-intestinal tract have employed culture-based methods. However, numerous studies suggest that only a small fraction of the total microbial community present can be captured using culture-based techniques (Amann *et al*. 1995; Ohkuma *et al*. 1996; Felske *et al*. 1998). Culture-independent studies are therefore needed to study the remaining microbes that were not detected by the culture-based methods. Over the past years the rRNA approach has been successfully applied to determine the bacterial diversity in the gastro-intestinal tract of various organisms.

The microbial ecology of the digestive tract of fish has been investigated by many researchers (Murno *et al*. 1994; Ringo *et al*.1995; Spanggaard *et al*. 2000). This is due to its assumed importance in digestion and disease control (Austin *et al*. 1995; Bly *et al*. 1997). It has been

shown mainly from studies of intestinal flora of warm blooded animals, that the composition of the microbial community of the intestine influences the establishment of pathogenic microorganisms in the intestinal tract (Van der Waaij, 1989; Singermand & Nash, 2000). Therefore, as part of a larger study in which the potential disease-preventive effect of bacterial strains isolated from fish was evaluated (Gram *et al*. 1999; Spanggaard *et al*. 2000), the microbial community of rainbrow trout (*Oncorhynchus mykiss*, Walbaum) was investigated. Members of the gamma subclass of *Proteobacteria* dominated the bacterial population structure. *Acinetobacter*, *Pseudomonas*, *Shewanella*, *Pleisiomonas* and *Proteus* were also identified together with isolated belonging to the Beta subclass of *Proteobacteria* and Gram-positive bacteria (Huber *et al*. 2004). In another study the phylogenetic analysis of the intestinal microflora of farmed and wild salmon indicated that the predominating bacterial populations detected were *Acinetobacter junii* and a novel *Mycoplasma* phylotype (Holben *et al*. 2002). This *Mycoplasma* phylotype comprised 96% of the total microbes in wild salmon. The unusual nutritional requirements of *Mycoplasma* species for cholesterol or sterols and other serum factors (Razin, 1978; Holt *et al*. 1994) could explain why they went unnoticed in prior studies where culture-based surveys alone were employed. The novel phylotype seems to be a normal inhabitant of the gastrointestinal tract of salmon playing an as yet unknown role in the health and physiology of these animals (Holben *et al*. 2002).

Over the past years there has also been an increase in studying the diversity of bacteria residing in the gut of abalone. In a previous study by Erasmus *et al.* (1997), it was showed that bacteria isolated from the gut of the South African abalone, *H. midae* (Erasmus*,* 1996*)* produce enzymes that assist the host in breaking down seaweed. The role that these bacteria play can eventually improve the growth rate of the host (El-Shansshoury *et al*. 1994). In a similar study Tanaka *et al.* (2004) reported the first qualitative community analysis of the major bacterial groups in the gut microflora of the abalone, *H. discus hannai*, by using culture independent methods. Sequencing of cloned 16S rDNA amplicons revealed a wide range of bacteria comprised of *Alpha*-, *Gamma*, *Epsilonproteobacteria*, *Mollicutes* and *Fusobacteria*. Microscopic analysis of the same samples by means of FISH showed the predominance of *Vibrios* and *Gammaproteobacteria.*

2.3 **REFERENCES**

Alvarez Tinajero, M.D.C., Caceres-Martinez, J. & A Viles, J.G.G. 2002. Histopathological evaluation of the yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* from Baja California, Mexico. *Journal of Shellfish Research* **21**: 825-830.

Amann, R., Springer, N.W., Ludwig, W., Gortz, H.D. & Scheifer, K.H. 1991. Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. *Nature* **351**: 161-164.

Amann, R.I., Ludwig, W. & Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbial Reviews* **59**. 143-169.

Andree, K.B., Friedman, C.S., Moore, J.D. & Hendrick, R.P. 2000. A polymerase chain reaction assay for the detection of genomic DNA of a *Rickettsiales*-like prokaryote associated with withering syndrome in California abalone. *Journal of Shellfish Research* **19**: 213-218.

Antonio, D.B., Andree, K.B., Moore, J.D., Friedman, C.S. & Hedrick, R.P. 2000. Detection of *Rickettsiales*-like prokaryotes by *in situ* hybridization in black abalone, *Haliotis cracherodii*, with withering syndrome. *Journal of Invertebrate Pathology* **75**: 180-182.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effend, I. & Griffith, D.R.W. 1995. A probiotic strain of *Vibrio alginolyticus* effective in diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases* **18**: 93-167.

Barlough, J.E., Rikihisa, Y. & Madigan, J.E. 1997. Nested polymerase chain reaction for detection of *Ehrlichia risticii* genomic DNA in infected horses. *Veterinary Parisitology* **68**: 367-373.

Baumann, P., Munson, M.A., Lai, C.Y., Clark, M.A., Baumann, L., Moran, N.A. & Campbell, B.C. 1993. Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. *ASM News* **59**: 21-24.

Baumann, P., Baumann, L., Lai, C.Y., Rouhbakhsh, D., Moran, N.A. & Clark, M.A. 1995. Genetics, physiology and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annual Review of Microbiology* **49**: 55-94.

Bly, J.E., Quiniou, S.M.A, Lawson, L.A. & Clem, L.W. 1997. Inhibition of *Saprolegnia* pathogenic for fish by P*seudomonas* fluorescence. *Journal of Fish Diseases* **20**: 25-40.

Bower, S.M., Meyer, G.R., & Boutillier, J.A. 1996. Stained prawn disease (SPD) of *Pandalus platyceros* in British Columbia, Canada, caused by a rickettsial infection. *Diseases of Aquatic Organisms* **24**: 41-54.

Bouyer, D.H., Stenos, J., Crocquet-Valdes, P., Moron, C.G., Popov, V.L., Zavala-Velazquez, J. E., Foil, L.D., Stothard, D.R., Azad, A.F. & Walker, D.H. 2001. Molecular characterization of a new member of the spotted fever group, *Rickettsia felis*. *International Journal of Systematic and Evolutionary Microbiology* **52**: 339-347.

Brock, J.A., Nakagawa, L.K., Hayashi, T., Teruya, S. & Van Campen, H. 1986. Hepatopancreatic rickettsial infection of penaeid shrimp, *Penaeus marginatus* (Randall), from Hawaii. *Journal of Fish Diseases* **9**: 73-77.

Brown, L.L., Albright, L.J. & Evelyn, T.P.T. 1990. Control of vertical transmission of *Renibacterium salmoninarum* by injection of antibiotics into maturing female coho salmon *Oncorhynchus kisutch*. *Diseases of Aquatic Organism* **9**: 127-131.

Choi, B.K., Paster, B.J., Dewhirst, F.E. & Gobel, U.B. 1994. Diversity of cultivable and uncultivable oral spirochaetes from a patient with severe destructive periodontitis. *Infection Immunity* **62**: 1889-1895.

Cifuentes, A., Anton, J., Benloch, S., Donnelly, A., Herbert, R.A. & Rodriguez-Valera, F. 2000. Prokaryotic diversity in *Zostera noltii*-colinized marine sediments. *Applied and Environmental Microbiology* **66**: 1715-1719.

Cvitanich, J.D., Garate, N.O. & Smith, C.E. 1991. The isolation of a *Rickettsiales*-like organism causing disease and mortality in Chilean salmonids and its conformation by Koch's postulates. *Journal of Fish Diseases* **14**:121-145.

Douglas, A.E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* **43**: 17-37.

El-Shanshoury, A.L., Mona, M.H., Shoukr, F.A. & El-Bossery, A.M. 1994. The enumeration and characterization of bacteria and fungi associated with marine wood-boring isopods, and the ability of these microorganisms to digest cellulose and wood. *Marine Biology* **119**: 321-326.

Erasmus, J.H. 1996. The role of enteric bacteria in the abalone, *Haliotis midae*. Unpublished M.Sc. thesis, University of Cape Town.

Erasmus, J.H., Cook, P.A. & Coyne, V.E. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture* **155**: 377-386.

Fakatsu, F., Tsuchida, T., Nikoh, M. & Koga, R. 2001. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). *Applied and Environmental Microbiology* **67**: 1284-1291.

Felske, A., Akkerman, A.D.L. & De Vos, W.M. 1998. *In situ* detection of an uncultured predominant *Bacillus* in Dutch grassland soils. *Applied and Environmental Microbiology* **64**: 4588-4590.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Fong, D., Rodriquez, R., Koo, K. Sun, J., Sogin, M.L., Bushek, D., Littlewood, D.T.J. & Ford, S.E. 1993. Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus. Molecular Marine Biology and Biotechnology* **2**: 346-350.

Friedman, C.S., Roberts, W., Kismohandaka, G. & Hedrick, R.P. 1993. Transmissibility of a coccidian parasite of abalone, *Haliotis* spp. *Journal of Shellfish Research* **12**: 201-205.

Friedman, C.S., Gardner, G.R., Hedrick, R.P., Stephenson, M., Cawthorn, R.J. & Upton, S.J. 1995. *Pseudoklossia haliotis* sp.n. (Apicomplexa) from the kidney of California abalone, *Haliotis* spp. (Mollusca). *Journal Invertebrate Pathology* **66**: 33-38.

Friedman, C.S., Thomson, M., Chun, C., Haaker, P.L. & Hedrick, P.L. 1997. Withering Syndrome of the black abalone *Haliotis chacherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research* **16**: 403-411.

Friedman, C.S., Andree, K.B., Beauchamp, K.A., Moore, J.D., Robbins, T.T., Shields, J.D. & Hedrick, R.P. 2000. *Candidatus xenohaliotis californiensis*, a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *International Journal of Systematic and Evolutionary Microbiology* **50**: 847-855.

Friendman, C.S., Trevelyn, G., Robbins, T.T., Mulder, E.P. & Fields, R. 2003. Development of an oral administration of oxytetracycline to control losses due to withering syndrome in cultured red abalone *Haliotis rufescens*. *Aquacultue* **224**: 1-23.

Fryer, J.L. & Lannan, C.N. 1994. Rickettsial and chlamydial infections of freshwater and marine fishes, bivalves, and crustaceans. *Zoological Studies* **33**: 95-107.

Fryer, J.L.; Lannan, C.N.; Giovannoni, S.J. & Wood, N.D. 1992. *Piscirickettsia salmonis* gen.nov., sp.nov., the causative agent of an epizootic disease in salmonid fishes. *International Journal of Systematic Microbiology* **42**: 120-126.

Furlong, M.A., Singleton, D.R., Coleman, D.C. & Whitman, W.B. 2002. Molecular and culture based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Applied and Environmental Microbiology* **63**: 1265-1279.

Gardner, G.R., Harshbarger, J.C., Lake, J., Sawyer, T.K., Price, K.L., Stephenson, M.D., Haaker, P.L. & Togstad, H.A. 1995. Association of prokaryotes with symptomatic appearance of withering syndrome in black abalone *Haliotis cracherodii*. *Journal of Invertebrate Pathology* **66**: 111-120.

Gebhart, C.J. Barns, M.S., McOrist, S., Lin, G.F. & Lawson, G.H.K. 1993. Ileal symbiont intracellularis, an obligate intracellular bacterium of porcine intestines showing a relationship to *Desulfovibrio* species. *International Journal of Bacteriology* **43**: 533-538.

Giovannoni, S.J., Britschgi, T.B., Moyer, C.L. & Field, K.G. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**: 61-62.

Giovannoni, S.J., Delong, E.F., Olsen, G.J. & Pace, N.R. 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. *Journal of Bacteriology* **170**: 720-726.

Gouin, E., Gantelet, H., Egile, C., Lasa, I., Ohayon, H., Villiers, V., Gounon, P., Sansonetti, P.J. & Cossart, P. 1999. A comparative study of the actin based motilities of the pathogenic bacteria Listeria monocytogenes, *Shigella flexneri* and *Rickettsia conorii*. *Journal of Cell Science* **112**:1697-1708.

Gulka, G., Chang, P.W. & Marti, K.A. 1983. Prokaryotic infection associated with a mass mortality of the sea scallop, *Plactopecten magellanicus*. *Journal of Fish Diseases* **6**:355-364.

Guo, X., Ford, S.E. & Zhang, F. 1999. Molluscan aquaculture in China. *Journal of Shellfish Research* **18**:19-31.

Haaker, P.L., Richard, D.V., Friedman, C.S., Davis, G.E., Parker, D.O. & Togstad, H. 1992. Mass mortality and withering syndrome in black abalone *Haliotis cracherodii* in California. In *Abalone of the World*. Shephard, S.A., Tegner, M. J & Guzman, S. A. (eds). Blackwell Scientific Publications, Oxford. Pp. 214-224.

Haaker, P.L., Harris, S.L., Taniguchi, I.K. & Friedman, C.S. 1995. Withering syndrome in California: does it occur in subtidal populations? *Journal of Shellfish Research* **14**: 267.

Heinzen, R.A., Grieshaber, S.S., Van Kirk, L.S. & Devin C.J. 1999. Dynamics of actin-based movement by *Rickettsia rickettsii* in vero cells. *Infectious Immunity* **67**: 1926-1935.

Holben, W.E., Williams, P., Saarinen, M., Sarkilahti, L.K. & Apajalahti, J.H.A. 2002. Phylogenetic analysis of intestinal microflora indicates a novel Mycoplasma phylotype in farmed and wild salmon. *Microbial Ecology* **44**: 175-185.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams S.T. 1994. *Bergey's Manual of Determinative Bacteriology*. Williams and Wilkens, Baltimore\London

Houpikian, P. & Raoult, D. 2002. Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. *Emerging Infectious Diseases* **8**: 122-131.

Huber, I., Spanggaard, B., Appel, K.F., Rossen, L. Nielsen, T. & Gram, L. 2004. Phylogenetic analysis and *in situ* identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of Applied Microbiology* **96**: 117-132.

Hugenholtz, P. & Norman, R.P. 1996. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Reviews* **14**: 190-197.

Ishikura, M., Fujita, H., Ando, S., Matsuura, K. & Watanabe, M. 2002. Phylogenetic analysis of spotted fever group *Rickettsiae* isolated from ticks in Japan. *Microbiology Immunology* **46**: 241-247.

Kelly, K. & Chistoserdov, A.Y. 2001. Phylogenetic analysis of the succession of bacterial communities in the Great South Bay (Long Island). *FEMS Microbiology Ecology* **35**: 85-95.

Kismohandaka, G., Friedman, C.S., Roberts, W., Hedrick, R.P. & Crosby, M.P. 1993. Investigation of physiological parameters of black abalone with withering syndrome. *Journal of Shellfish Research* **12**:131-132.

Krieg, N.R. & Holt, J.G. 1984. *Bergey's Manual of Systematic Bacteriology*, vol 1. Williams and Wilkens, Baltimore\London

Lafferty, K.D. & Kuris, A.K. 1993. Geographic patterns in the wasting disease of black abalone. *Bulletin of the Ecological Society of America* **73**: 240.

Le Gall, G. & Mialhe, E. 1991. Epizootiological study of rickettsiosis of the Saint-Jacques scallop *Pecten maximus*. *Diseases of Aquatic Organisms* **10:**139-145.

Lew, A.E., Gale, K.R, Minchin, V.S. & de Waal, D.T. 2003. Phylogenetic analysis of the erythrocytic *Anaplasma* species on 16S rDNA and GroEl (HSP60) sequences of *A. marginale*, *A. centrale* and *A. ovis* and the specific detection of *A. centrale* vaccine strain. *Veterinary Microbiology* **92**: 145-160.

Loy, J.K., Dewhirst, F.E., Weber, W., Frelier, P.F., Garbar, T.L., Tasca, S.I. & Templeton, J.W. 1996. Molecular phylogeny and *in situ* detection of the etiological agent of Necrotizing Hepatopancreatitis in shrimp. *Applied and Environmental Microbiology* **62**: 3439-3445.

Ludwig, W., Struck, O., Klugbauer, S., Klugbauer, N., Weinzenegger,M., Neumair, J., Bachleitner, M. & Schleifer,K.H. 1998. Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**: 554-568.

Maniloff, J., Mcelhaney, R.N., Finch, L.R. & Baseman, J.B. 1992. *Mycoplasmas: Molecular Biology and Pathogenesis*. ASM Press, New York

Martinez, J.C., Tinajero, C.A., Renteria, Y.G. & Aviles, J.G.G. 2000. *Rickettsiales*-like prokaryotes in cultured and natural populations of the red abalone *Haliotis rufescens*, blue abalone, *Haliotis fulgans*, and the yellow abalone *Haliotis corrugata* from Baja California, Mexico. *Journal of Shellfish Research* **19**: 503.

Moore, J.D., Robbins, T.T. & Friedman, C.S. 2000. Withering syndrome in farmed red abalone, *Haliotis rufescens*: thermal induction and association with gastrointestinal *Rickettsiales*-like prokaryote. *Journal of Aquatic Animal Health* **12**: 26-34.

Moore, J.D., Robbins, T.T. & Friedman, C.S. 2001. The role of a *Rickettsiales*-like prokaryote in withering syndrome in California red abalone. *Journal of Shellfish Research* **19**: 525-526.

Moter, A. & Gobel, U.B. 2000. Fluorescence *in situ* hybridization for direct visualization of microorganisms. *Journal of Microbial Methods* **41**: 85-112.

Moyer, C.I., Dobbs, F.C. & Karl, D.M. 1995. Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Applied and Environmental Microbiology* **61**: 1555-1562.

Munson, M.A., Baumann, P. & Kinsey, M.G. 1991. *Buchnera* gen.nov. and *Buchnera aphisicola* sp. nov., a taxon consisting of mycetocyte-associated, primary endosymbionts of aphids. *International Journal of Systematic Bacteriology* **41**: 566-568.

Murno, P.D., Barbour, A. & Birkbeck, T.H. 1994. Comparison of the gut microflora of start-feeding larval turbot under different conditions. *Journal of Applied Microbiology* **77**: 560-565.

Murray, R.G.E., Brenner, D.J., Bryant, M.P., Holt, J.G., Krieg, N.R., Moulder, J.W., Pfenning, N.,Sneath, P.H.A., Staley, J.T., eds. 1984. Section 9 The Rickettsiales and Chlamydias. *Bergey's Manual of Systematic Bacteriology* vol 1: Williams and Wilkens. Baltimore 678-688.

Murray, R.G.E. & Stackebrandt, E. 1995. Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described prokaryotes. *International Journal of Systematic Bacteriology* **45**: 186-187.

Muyzer, G. 1998. Structure, function and dynamics of microbial communities: the molecular biological approach. *Advances in Molecular Ecology*: 87-117.

Nei, M. & Kumar, S. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York

Neimark, H. & Kocan, K.M. 1997. The cell wall-less rickettsia *Eperythrozoon wenyonii* is a *Mycoplasma*. *FEMS Microbiology Letters* **156**: 287-291.

Noda, H., Munderloh, U.G. & Kurtti, T.J. 1997. Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. *Applied and Environmental Microbiology* **63**: 3926-3932.

OIE (World Organization for Animal health) Diagnostic manual for Aquatic Animal Diseases. , Office International Des Epizooties, Paris, France. http://www.oie.int/eng/normes/fmanual/A_summary.html

Ohkuma, M. & Kudo, T. 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Applied and Environmental Microbiology* **62**: 461-468.

Pace, N.R., Stahl, D.A., Lane, D.J. & Olsen, G.J. 1986. The analysis of natural microbial populations by ribosomal RNA sequences. *Advanced Microbiology Ecology* **9**: 1-55.

Parola, P., Inokuma, H., Camicas, J.L., Brouqui, P. & Raoult, D. 2001. Detection and identification of spotted fever group *Rickettsiae* and *Ehrichiae* in African ticks. *Emerging Infectious Diseases* **7**: 1-5.

Polak, J.M. & McGee, J.O.D. (EDS). 1998. *In situ hybridization, principles and practice*. Oxford University press.

Rawadi, G. & Dussurget, O. 1998. Genotypic methods for diagnosis of mycoplasmal infections in humans, animals, plants and cell cultures. *Biotechnology and Genetic Engineering Reviews* **15**: 51-78.

Razin, S. 1978. The mycoplasma. *Microbial Reviews* **42**: 414-470.

Relman, D.A., Schmidt, T.M., MacDermott, R.P. & Falkow, S. 1992. Identification of the uncultured bacillus of Whipple's disease. *New England Journal of Medicine* **327**: 293-301.

Richard, D.V. & Davis, G.E. 1993. Early warnings of modern population collapse in black abalone, *Haliotis cracherodii*, Leach 1814, at the California Channel islands. *Journal of Shellfish Research* **12**: 189-194.

Ringo, E., Strom, E. & Tabachek, J.A. 1995. Intestinal flora of salmonids. A review. *Aquaculture Review* **26**: 773-789.

Roux, V. & Raoult, D. 1995. Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. *Research in Microbiology* **146**: 385-396.

Salemi, M. & Vandamme, A.M. 2003. *The Phylogenetic Handbook*. Cambridge University Press, New York.

Satoh, H., Motoi, Y., Camer, G.A., Inokuma, H., Izawa, M., Kiyuuna, T., Kumazawa, N., Muramatsu, Y., Ueno, H. & Morita, C. 2002. Characterization of spotted fever group *Rickettsiae* detected in dogs and ticks in Okinawa, Japan. *Microbiology Immunology* **46**: 263-267.

Shaw, S.B. 1989. DNA probes for the detection of Legiolella species, *Mycoplasma pneumoniae*, the members of the *Mycobacterium tuberculosis* complex. In *DNA Probes for Infection Diseases*, ED. F.C. Tenover. 101-117. CRC Press, Boca Raton.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. & Schleifer, K.H. 1997. Phylogenetic and *in situ* detection of bacteria in activated sludge. *Applied and Environmental Microbiology* **63**: 2884-2896.

Spangaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. & Gram, L. 2000. The microflora of rainbow trout: a comparison of traditional and molecular identification. *Aquaculture* **182**: 1-15.

Sparks, A.K. 1985. *Synopsis of incertebrate pathology exclusive of insects*. Amsterdam: Elsevier

Springer, N.W., Ludwif, W., Amann, R., Schnidt, H.J., Gortz, H.D. & Scheifer, K.H. 1993. Occurrence of fragmented 16SrRNA in an obligate bacterial endosymbiont of *Paramecium caudatum*. *Proceeding of the National Academy of Sciences,* USA **90**: 9892-9895.

Steinbeck, J. R., Groff, J.M., Friedman, C.S., McDowell, T. & Hedrick, R.P. 1992. Investigation into a coccidian-like protozoan from the California abalone, *Haliotis cracherodii*. In *Abalone of the World*. Shephard, S.A., Tegner, M.J. & Guzman, S.A. (eds). Blackwell Scientific Publications, Oxford, 203-213.

Stokes, N.A. & Burreson, E.M. 1995. A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni*. *Journal of Eukaryotic Microbiology* **42:** 350-357.

Szwerinksi, H., Gaiser, S. & Bardtke, D., 1985. Immunofluorescence for the quantitative determination of nitrifying bacteria: interference of the test in biofilms reactors. *Applied Microbiology Biotechnology* **21:**125-128.

Tanaka,R., Ootsubo, M., Sawabe, T., Ezur, Y. & Tajima, K. 2004. Biodiversity and *in situ* abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture* **241**: 453-463.

Unterman, B.M., Baumann, P. & Mclean, D.L. 1989. Pea aphids symbiont relationship established by analysis of the 16S rRNAs. *Journal of Bacteriology* **171**: 2970-2974.

VanBlaricon, G.R., Ruediger, J.L., Friedman, C.S., Woodard, D.D. & Hedrick, R.P. 1993. Discovery of withering syndrome among black abalone *Haliotis cracherodii Leach*, 1814 Populations at San Nicolas Island, California. *Journal of Shellfish Research* **12**: 185-188.

Van der Waaij, D. 1989. The ecology of the human intestine and its consequences to overgrowth pathogens such as *Clostridium difficile*. *Annual Review of Microbiology* **43**: 67-87.

Wagner, M., Amann, R., Lemmer, H. & Schleifer, K.H. 1993. Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependant methods for describing microbial community structure. *Applied and Environmental Microbiology* **59**: 1520-1525.

Walker, T.S. & Winkler H.H. 1987. Penetration of cultured mouse fibroblast (L cells) by *Rickettsia prowazakii*. *Infection Immunity* **22**: 200-208.

Weis, E. & Moulder, JW. 1984. Order 1. Rickettsiales Gieszczkiewicz 1939, 25 AL. In *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkens, Baltimore/London: pp 687-729.

Wilson. K.H., Blitchington, R., Frothingham, R. & Wilson, J.A. 1991. Phylogeny of the Whipple's-disease associated bacterium. *Lancet* **338**: 474-475.

Winkler, H.H. 1976. Rickettsial permeability; an ADP-ATP transport system. *Journal of Biology and Chemistry* **25**: 389-396.

Winkler, H.H., Daugherty, H.R. & Hu, F. 1999. *Rickettsia prowazekii* transport UMP and GMP, but not CMP, as building blocks for RNA synthesis. *Journal of Bacteriology* **181**: 3238-3241.

CHAPTER 3

MATERIALS AND METHODS

3.1. SCREENING OF INFECTED *H.MIDAE* FOR THE PRESENCE OF *C.XENOHALIOTIS CALIFORNIENSISS*

Two diagnostic methods were used to screen *Haliotis midae* digestive gland with classical histological features of the intracellular *Rickettsiales*-like prokaryote for the presence of *Candidatus xenohaliotis californiensis*. They include 1. a presumptive method (diagnostic PCR assay) (Andree *et al*. 2000) and 2. a confirmatory method (*In situ* hybridization) (Antonio *et al*. 2000). PCR and *ISH* are the methods of choice for screening possible host for the presence of *C. xenohaliotis californiensis.*

3.1.1 **Diagnostic PCR assay**

3.1.1.1 **Sampling**

Abalone was collected from the Irvin and Johnson (I&J) Abalone Hatchery at Danger Point, South Africa during the course of 2001. The digestive gland of the abalone showing histological features of the intracellular prokaryote was removed and stored in 100% ethanol (EtOH) at -20°C until further use.

3.1.1.2 **DNA extraction**

Fixed tissues were removed from the ethanol and dried on paper. DNA was extracted using the N-cetyl-N,N,-trimethyl-ammonium bromide (CTAB) isolation protocol adapted from Saghai-Maroof *et al.* (1984) and Doyle *et al*. (1987). Approximately 10 – 20 mg of digestive gland tissue was dissected and coarsely diced with a scalpel blade, after which it was homogenized in 500 µl of extraction buffer. RNase (4 µg/ml) (Promega) was added to each sample and left at

37°C for 2h. Proteinase K (100 µg/ml) (Roche) was added to the mixture after 2h and mixed by inversion. The samples were left in a 65°C water bath overnight. After overnight lyses of samples, an equal volume of chloroform: isoamyl alcohol (24:1) (v/v) was added and mixed thoroughly by inversion. Samples were centrifuged at 10 000 rpm for 10 min. The top aqueous phase was removed and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)(v/v) was added. Thereafter the samples were centrifuged at 10 000 rpm for 10 min. The top aqueous phase was removed and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The samples were centrifuged at 10 000 rpm for 10 min, and the supernatant transferred to a new tube. Ice-cold isopropanol [330 µl (2/3 the volume of the supernatant] (Saarchem) was added to the supernatant and slowly inverted repeatedly. The samples were left at -20°C for 3 h and were then centrifuged at 12 000 rpm for 10 min, after which the supernatant was removed. The pellet was washed once in 70% ethanol and centrifuged at 13 000 rpm for 10 min and the supernatant removed. This step was repeated. The pellets were left to dry at 55°C for 5-10 min, and resuspended in 70 µl dH_2O. Samples were then stored at -20°C.

3.1.1.3 **Standardization of samples**

DNA extractions were examined for quality and quantity on a 2 % (w/v) agarose gel (WhiteSci). Ethidium bromide (EtBr) was added for visualization of the DNA. Each sample to be loaded was prepared by adding 2 µl of 6X loading buffer (Promega) to 8 µl of the DNA sample. Lambda (λ) (Promega) standards of 0.1, 0.3 and 0.5 µg/µl were also prepared and loaded. Gels were electrophoresed at 120 V for 60 min after which it was visualized with a Geldoc™ 1000 system (BioRad).

3.1.1.4 Primer selection and synthesis

Two primer combinations were used to screen infected *H. midae* for the presence of *C. xenohaliotis californiensis*. The primer combinations and the size fragments that they produce are shown in Table 3.1.

Table 3.1 *C. xenohaliotis californiensis* specific primers

Primer combination	Primer sequence	Size (bp)	Ref.
Combination 1			
RA5-1 forward	5' – GTT GAA CGT GCC TTC AGT TTA C – 3'	160bp	Andree *et al*. (2000)
RA3-6 reverse	5' – ACT TGG ACT CAT TCA AAA GCG GA – 3'		
Combination 2			
RA5-1 forward	5' – GTT GAA CGT GCC TTC AGT TTA C – 3'	940bp	Andree *et al*. (2000)
CA1 reverse	5' – GAA GAA CCC AGC CAA ACTGA – 3'		This study

The primer CA1 was designed with the programme Oligo (dt) version 4.1 and was based on a sequence alignment with 16S rDNA sequences from *C. xenohaliotis californiensis, Ehrlichia bovis, Piscirickettsia salmonis***,** *Escherichia coli* and other sequences from the order *Rickettsiales* (See Table 3.4 for accession numbers of 16S rDNA sequences obtained from GenBank). The variable regions of the alignment were examined for regions suited to design *C. xenohaliotis californiensis* species-specific primers.

3.1.1.5 **PCR amplification**

All amplifications were performed in standard 25 µl reactions containing 1 µl 10X reaction buffer (Bioline, BIOTAQ ^{TM} DNA Polymerase), 2 mM Magnesium chloride ($MgCl_2$), 0.5 µM of each primer, 200 µM of each dinucleotide triphosphate (dNTP) and 0.975 U of *Taq* polymerase (Bioline BIOTAQ ^{TM} DNA Polymerase,). PCR was performed in 0.2 ml thin-walled tubes using

the Applied Biosystems GeneAmp® PCR System 2700. The following cycle program was used: 94°C for 3 min followed by 37 cycles with the cycle profile 40 s at 93°C, 50 s at 55°C and 1 min at 72°C. The reaction was completed with a final elongation step of 72 °C for 10 min.

3.1.1.6 **Gel electrophoresis**

To confirm amplification, 5 µl of the PCR products and 1 µl of 6X loading buffer (Promega) were examined for size and quality on a 2 % (w/v) agarose gel (WhiteSci) in 1X Tris-Borate-EDTA buffer (TBE) [100 mM 2-amino-2 (hydroxymethyl)-1,3-propandiol (Tris), 100 mM Boric acid, 2 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.3]. EtBr was added for visualization of the gel. A Promega 100 bp molecular weight ladder was used as a size marker. The gel was run at 120 V for 60 min, after which it was visualized with a Geldoc™ 1000 system.

3.1.1.7 **Sequencing of PCR products**

Verification that the positive products were from the 16S rDNA gene C. *xenohaliotis californiensis* was determined by nucleotide sequencing. The PCR products were sequenced in both the forward and reverse direction using the primer combination 1 and 2 (Table 3.1) respectively. The ABI Prism cycle sequencing kit (Big Dye Terminator cycle Sequencing kit v3.1) was used for sequencing. Sequencing reactions were performed in a total volume of 10 µl containing: 1 µM vector primers, 4 µl of PCR product, 2 µl Big Dye Terminator Ready Reaction mix and 3 µl sterile water. Cycle sequencing was performed with an Applied Biosystems GeneAmp® PCR System 2700 with 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s and extension at 60 °C for 4 min. Sequencing reaction were run on an ABI PRISM 377 DNA sequencer (Perkin Elmer, U.S.A.) at the DNA sequencing facility at Stellenbosch University.

3.1.2 *In situ* Hybridization

3.1.2.1 **Tissue fixation and histological preparations**

Microscopic slides containing infected and uninfected *Haliotis midae* digestive gland were kindly prepared at the Western Cape Provincial Veterinary Laboratory, Stellenbosch and stored in slide boxes at 4°C until used for *in situ* hybridization (*ISH*).

Microscopic slides containing black (*Haliotis cracherodii*) and red abalone (*Haliotis rufescens*) tissues infected with *C. xenohaliotis californiensis* were also prepared and served as positive controls in the *ISH* trials.

3.1.2.2 ***C.xenohaliotis californiensis* probes**

A cocktail of 4 probes viz RA5-1, CA1, RA5-6 and RA3-6 was used to hybridize to homologous sequences of the small subunit ribosomal deoxyribonucleic acid (SSU rDNA) sequences of *C. xenohaliotis californiensis* (Antonio *et al*. 2000). The sequences of the probes are given below in the 5' – 3' direction:

Table 3.2 Sequence of oligonucleotide probes*

Probe name	Probe sequence	Location
RA 5-1	5'– GTTGAACGTGCCTTCAGTTTAC – 3'	54-75bp
CA 1	5' – GAAGAACCCAGCCAAACTGA – 3'	976-994bp
RA 5-6	5' – GAAGCAATATTGTGAGATAAAGCA – 3'	1210-1233bp
RA 3-6	5' – ACTTGGACTCATTCAAAAGCGGA – 3'	189-211bp

* The approximate location of each probe (in 16S rDNA gene of *C.xenohaliotis californiensis*) is given.

3.1.2.3 **Probe labeling**

Oligonucleotides were labeled by tailing the 3' end with digoxigenin–labeled deoxyuridine triphosphate (DIG–dUTP) by using the DIG Oligonucleotide 3' – End Labeling Kit, 2nd Generation (Roche). The probes were labeled following the manufacturer's protocol.

3.1.2.4 ***In situ*** **hybridization (*ISH*)**

The *ISH* protocol used in our trials was based on the work of Friedman *et al.* (unpublished method).

Tissue deparaffinization

Tissue sections were deparaffinized by immersion in three changes of xylene (Saarchem) for 10 min each, followed by immersion in two successive absolute ethanol baths for 10 min each. Sections were then rehydrated in an ethanol series (95, 80, 70, 50 %) for 3 min each and rinsed in sterile distilled water. Tissue sections were finally equilibrated in Tris buffer (0.2 mM Tris–HCL, 2.0 mM CaCl, pH 7.2) for 5 min prior to treatment with proteinase K to permeabilize the tissues.

Permeabilization of tissues

For optimum probe penetration to target tissues, sections were treated with proteinase K (50 µg/ml) in Tris buffer, at 37ºC for 45 min. The reaction was stopped by washing the sections in three changes of 1X phosphate buffered saline (PBS) for 10 min each prior to prehybridization.

Prehybridization

Prehybridization was performed to reduce background in tissue sections. The prehybridization solution contained in 1.0 ml solution: 0.51 ml deionized formamide (Sigma), 0.20 ml 20X standard saline citrate (SSC), 0.05 ml heat-denatured sperm DNA (10 mg/ml) (Invitrogen, Life Technologies), 0.20 ml 50 % dextran sulfate (Sigma) that was preheated at 40 ºC, and 0.02 ml 50 X Denhardt's (Sigma) solution. The tissue sections were covered with prehybridization solution and incubated for 2 h without cover slips at room temperature in a humid chamber.

Hybridization

After 2 h, the prehybridization solution was discarded, briefly rinsed in buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) and then replaced with the hybridization solution. The hybridization solution contained the same components as the prehybridization solution except that the probe was added at 1:1000 (10 fmol/ul) (v:v). The tissue sections and the hybridization solution were simultaneously denatured on a heating block for 10 min at 99ºC. The slides were cooled at room temperature for 1 min, covered with DNase-free cover slips, and then hybridized overnight at 40ºC in a humid chamber.

Stringency washes

Unbound probe was removed by washing the sections twice for 10 min in 2X SSC at 40ºC, twice for 10 min in 1X SSC at 40ºC, and once for 10 min in 0.5X SSC at room temperature. The sections are then equilibrated in buffer 1 for 10 min at room temperature. To block non-specific binding areas, the sections were placed in buffer 1 supplemented with 0.3 % Triton X-100 (Sigma) and 2 % sheep serum (Sigma) for 1 h at room temperature with mild agitation.

Detection of hybridization signals

Anti-digoxigenin alkaline phosphatase antibody conjugate (Boehringer Mannheim) was diluted 1:1000 in buffer 1 supplemented with 0.3 % Triton X-100 and 1 % sheep serum. The conjugate solution (0.5 - 1.0 ml) was carefully added to a dry section and incubated without cover slip at room temperature for 2 h in a humid chamber. After 2 h the slides were rinsed twice with buffer 1 for 5 min each and twice in buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 min each. The slides were then incubated with color development solution [45 µl nitroblue tetrazolium (NBT), 35 µl 5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (Boehringer Mannheim) in 10 ml of buffer 2] for 2 h in the dark in a humid chamber. The reaction was stopped by three changes with distilled water of 3 min each. To enhance the tissue morphology against the signal, sections were counter stained with 0.05 % aqueous Bismarck Brown Y (Sigma) for 3 min in the dark. The slides were then rinsed in distilled water followed by a brief dehydration in 70 % and 100 % ethanol, air dried and finally mounted with a cover slip by using DPX mountant mounting medium (Saarchem (Pty) Ltd). Hybridization signals were

evaluated by light microscopy for areas showing a purple precipitate where the probe had bound to *C. xenohaliotis californiensis* DNA.

3.2. **ISOLATION OF 16S RDNA SEQUENCES FROM THE DIGESTIVE GLAND OF *H. MIDAE* INFECTED WITH THE *RICKETTSIALES*-LIKE PROKARYOTE**

3.2.1 **DNA isolation**

DNA was extracted from digestive gland tissues showing histological features of the *Rickettsiales*-like prokaryote. Uninfected tissues that include gill, mantle and gonad were also dissected from the abalone and stored in 100 % EtOH at -20°C until further use. These tissues served as positive controls. The CTAB isolation procedure described earlier (3.1.1.2) was use to extract DNA. The DNA was separated on a 2 % agarose gel, stained with EtBr and electrophoresed at 120 V in 1X TBE. The gel was visualized with a Geldoc™ 1000 system (Biorad).

3.2.2 **16S rDNA PCR amplification**

PCR amplification of the 16S rDNA gene was carried out to amplify a fragment of ~ 1500 bp using bacterial universal primers, E94 (5' – GAG AGT TTG ATY MTG GCT CAG – 3') and D88 (5' – GAA GGA GGT GWT CCA RCC GCA – 3') (Paster *et al*. 2001). PCR was performed using a 25 µl mixture containing: 1µl 10X reaction buffer (Bioline, BIOTAQ™ DNA Polymerase), 2 mM MgCl, 0.5 µM of each primer, 200 µM of each dNTP and 0.975 U of *Taq* polymerase (Bioline BIOTAQ™ DNA Polymerase). Thermal cycles were performed in the GeneAmp® PCR System 2700 DNA thermocycler (Applied Biosystems). The amplification profile consisted of denaturation at 94°C for 3 min followed by amplification under the following conditions: 93°C for 40 s, annealing at 59°C for 50 s, and elongation at 72°C for 1

min. A total of 37 cycles were performed, which were followed by a final elongation step of 72°C for 10 min. Successful amplification was determined by agarose gel electrophoresis of 5 µl of the amplification product.

3.2.3 **Cleaning of PCR products**

The desired PCR fragment were excised from the gel and extracted using the NucleospinR extract kit (Macherey-Nagel). The products were eluted in 25 – 50 µl elution buffer NE and separated on a 2 % agarose gel to confirm whether the products were still present.

3.2.4 **16S rRNA cloning**

Four microlitre of the purified PCR products were cloned in a pCR 4 – TOPO vector and transformed in *E. coli* strain TOP 10 ^{r} competent cells using the "TOPO TA cloning^{r} kit" (Invirogen ^{TM}, Life Technologies). Clones were plated on Luria Bertani (LB) (1,0% Tryptone, 0.5% Yeast extract, 1,0% NaCl) plates [supplemented with 15 g/l Agar Bacto (SIGMA)] containing 100 µg/ml of ampicillin (amp) (USB^{TM}). The plates were left at 37°C for overnight growth.

3.2.5 **Colony PCR**

After overnight growth at 37°C, PCR reactions were performed directly on *E. coli* cells to determine the correct size of the inserts. Each clone was inoculated in PCR buffer [containing 2.5 µl 10X reaction buffer (Promega^{TM} DNA Polymerase), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM of each dNTP and 0.5 U of *Taq* polymerase (Promega^{TM} DNA Polymerase)]. The size of the inserts (approximately 1500 bp) was determined by using flanking vector primers designated: M13F (5' – GTAAAACGACGGCCAG – 3') and M13R (5' – CAGGAAACAGCTATGAC – 3') included in "TOPO TA cloning^{r} kit".

The PCR reaction was incubated at 94°C for 10 min. Amplification was performed for 25 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min). This was followed by a final extension at 72°C for 10 min. The results of the colony PCR were checked for size on a 2 % (w/v) agarose gels.

3.2.6 **Isolation of plasmids**

The positive clones were cultivated in 5 ml LB medium containing 100 μg/ml of ampicillin and grown overnight at 37°C with vigorous shaking. Plasmids were isolated from the overnight cultures using the Wirzard[r] Plus SV Minipreps DNA purification systems (Promega). The plasmids were electrophoresed on a 2 % agarose/TBE gel.

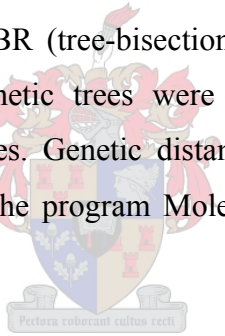
3.2.7 **Sequencing of 16S rDNA inserts**

The clones were sequenced in both the forward and reverse direction using the vector primers M13F and M13R (-20) The ABI Prism cycle sequencing kit (Big Dye Terminator cycle Sequencing kit v3.1) was used for sequencing. Sequencing reactions were performed in a total volume of 10 μl containing: 1 μM vector primers, 4 μl of PCR product, 2 μl Big Dye Terminator Ready Reaction mix and 3 μl sterile water. Cycle sequencing was performed with an Applied Biosystems GeneAmp® PCR System 2700 with 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 10 s and extension at 60°C for 4 min. Sequencing reaction were run on an ABI PRISM 377 DNA sequencer (Perkin Elmer, U.S.A.) at the DNA sequencing facility at Stellenbosch University.

3.2.8 **Phylogenetic analysis of 16S rDNA sequences**

The sequences obtained were compared to 16S rRNA of organisms deposited in GenBank (See Table 3.4 for accession numbers of 16S rDNA sequences obtained from GenBank) using the BLAST[1] algorithm to find homologous or similar sequences (Altshul *et al*. 1990). Sequences obtained from the study were then aligned with closely related sequences identified from the BLAST search using ClustalW (Thomson *et al*. 1994) as implemented in the program BioEdit (Hall 1999).

Maximum parsimony (MP) trees were reconstructed using the heuristic search option in the program PAUP, version 4.0b10 (Swofford 2002). Maximum parsimony (MP) analyses were performed using equal weights for all characters with gaps treated as missing data. Analysis was conducted with 1000 repeated tree searches in which the starting tree was constructed by random taxa addition and swapped with the TBR (tree-bisection-reconnection) algorithm. Confidence values for the nodes of the phylogenetic trees were calculated by the bootstrap method (Felsenstein 1985) using 1000 replicates. Genetic distances were obtained using Kimura's 2 parameter model (Kimura 1980) with the program Molecular Evolutionary Genetics Analysis version 2 (MEGA) (Kumar *et al*. 2001)



[1] http://www.ncbi.nlm.nih.gov/BLAST/

3.3 *IN SITU* HYBRIDIZATION WITH SPECIES-SPECIFIC PROBES

3.3.1 **Design of species-specific oligonucleotide probes**

Using the information obtained from the phylogenetic analysis, species-specific probes were designed for the newly isolated sequences obtained from the abalone digestive gland showing phylogenetic affinities to the order *Rickettsiales*. The sequences of the probes are given below in the 5' – 3' direction:

Table 3.3 Sequence of oligonucleotide probes

Probe name	Probe sequence
RI 96	5'– ATACGCATTTATGTGTAGAGTACAGC– 3'
RI 630	5' – ATTTTGGAGAGGATAGTGGAATTTA– 3'
RI 1076	5' – GCCATTATTTGCCATCATTAAGTT – 3'
RI 1275	5' – TTAATCTTACCGTGGTTAGCTGC – 3'

Potential target sites for the probes consisted of regions of 20-26 nucleotides that perfectly matched corresponding sites for all the target organisms. Selection of the potential "best" probes was made according to the following criteria: (1) target sites located in the more conserved region of the specific group and (2) target sites with a maximum number of mismatches with non-target organisms (variable region of alignment).

3.3.2 ***In situ*** **Hybridization**

The *in situ* hybridization procedure described earlier (3.1.2) was use to hybridize the species-specific oligonucleotide probes designed in section 3.3.1 on histological sections of *H. midae* containing the *Rickettsiales*-like prokaryote. Uninfected *H. midae* and black and red abalone infected with *C. xenohaliotis californiensis* served as controls in these *in situ* hybridization trials.

Table 3.4 Accession numbers of 16S rDNA sequences obtained from GenBank

Class	Species	Accession number
Aquificae		
	Aquifex aquanticus	AJ309733
α – Proteobacteria		
	Ehrlichia sp. EH1087	AY309971
	Ehrlichia sp. Ehf669	AY309969
	Ehrlichia canis	M733221
	Cowdria ruminantium	X6659
	Candidatus neoehrlichia mikurensis	AB084582
	Ehrlichia phagocytophila	M73224
	Ehrlichia belluno	AY098730
	Ehrlichia bovis	U03775
	Anaplasma marginale	M60313
	Candidatus xenohaliotis californiensis	AF133090
	Caedibacter caryophila	X71837
	Wolbachia sp. 16S rDNA	U83097
	Wolbachia pipientis	X61768
	Wolbachia sp. from *Haliotis danicus*	AJ001604
	Wobachia endosymbiont of *Brugia malayi*	AJ010275
	Wolbachia endosymbiont of *Dirofilaria immitis*	AF487892
	Ehrlichia sennetsu	M73225
	Ehrlichia risticii	M21290
	Neorickettsia helminthoeca	U12457
	Rickettsia africae	L36098
	Rickettsia parkeri	L36673
	Rickettsia japonica	L36213
	Rickettsia JL-02	AY158006
	Rickettsia typhii	U12463
	Rickettsia prowazekii	M21789
	Pelibacter ubique strain	AF510192
	Bacterium UNSW4	AF425999
	Glacial ice bacterium	AF479378
	Bacterium CAGY7	AF538746
	Sphingomonas parapaucimobilis	X72721
	Spingomonas pituitosa	AJ243751
	Sphingomonas SaS2	AJ620194
	Rhizomonas sp. K6	AJ000918
	Spingomonas herbicidovorans	AB022428
	Spingomonas chlorophenocolica	X87161

Table 3.4 – (continued)

Class	Species	Accession number
γ – Proteobacteria		
	Escherichia coli	J01695
	Piscirickettsia salmonis	X60783
	Coxiella burnetii	M21291
ε – *Proteobacteria*		
	Uncultured acrobacter sp. clone 15	AY069963
	Unidentified epsilon proteobacteria strain BD1	AB015529
	Acrobacter sp. clone D1A1	AJ271654
	Uncultured eubacterium CHA3-437	AJ132728
	Uncultured eubacterium CHA3-127	AJ132726
	Candidatus Acrobacter sulfidicus	AY035822
	Acrobacter butzleri	AF314538
Mollicutes		
	Uncultured mycoplasma sp. clone A9	AY720906
	Uncultured mycoplasma sp. clone B58	AY70915
	Mycoplasma bovoculi	U44768
	Mycoplasma conjuctivae	U44770
	Mycoplasma flocculare	X62699
	Mycoplasma ovipneumoniae	U44771
	Spiroplasma sp. 16S rDNA	AJ132412
	Spiroplasma sp. Y32	M2447
	Spiroplasma symbiont of Antonina	AB030022
	Eperythrozoon wenyonii	AF016546
	Mycoplasma ovis	AF338268
	Mycoplasma suis	AY492086
	Candidatus Mycoplasma haemominutum	U88564
	Haemobartonella muris	U82963
Fusobacteria		
	Illyobacter tartaricus	AJ307982
	Propiongenium modestum	X54275
	Illyobacter insuetus	AJ307980
	Uncultured Fusobacteria	AJ575990
	Fusobacteria bacterium KO711	AF550592
	Fusobacterium gonidoformans	M58679

Table 3.4 (continued)

Class	Species	Accession number
Spirochaetes		
	Borrelia lonesteri	AY166715
	Borrelia coriaceae	AF210136
	Borrelia anserina	M64312
	Borrelia lusitaniae	AB091819
	Borrelia burgdorferi	AF091368
	Cristispira CP1	U42638
	Spirochaeta zuelzerae	M887265
	Spirochaeta stenostrepta	M88724
	Spirochaeta aurantia	M57740
	Spirochaeta MWH-HuW8	AJ565433

3.4 **REFERENCES**

Altschul, S.F., Gish, W.M., Myers, E.W. & Lipman, D.J. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403-404.

Andree, K.B., Friedman, C.S., Moore, J.D. & Hedrick, R.P. 2000. A polymerase chain reaction assay for the detection of genomic DNA of a *Rickettsiales*-like prokaryote associated with withering syndrome in California abalone. *Journal of Shellfish Research* **19**: 213-218.

Antonio, D.B., Andree, K.B., Mcdowell, T.S. & Hedrick, R.P. 1998. Detection of *Myxobolus cerebralis* in Rainbrow trout and oligochaete tissues by using nonradioactive *in situ* hybridization (*ISH*) protocol. *Journal of Aquantic Animal Health* **10**: 338-347.

Antonio, D.B., Andree, K.B., Moore, J.D., Friedman, C.S. & Hedrick, R.P. 2000. Detection of *Rickettsiales*-like prokaryotes by *in situ* hybridization in black abalone, *Haliotis cracherodii*, with withering syndrome. *Journal of Invertebrate Pathology* **75**: 180-182.

Doyle, J.J. & Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry* **19**: 11-15.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using bootstrap. *Evolution* **39**: 783-791.

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

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular evolution* **16**: 111-120.

Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis Software. *Bioinformatics* **50**: 602-612.

Moore, J.D., Robbins, T.T. & Friedman, C.S. 2000. Withering syndrome in farmed red abalone, *Haliotis rufescens*: thermal induction and association with gastrointestinal *Rickettsiales*- like prokaryote. *Journal of Aquantic Animal Health* **12**: 26-34.

Paster, B.J., Boches, S.K., Galvin, J.L., Ericson, R.E., Lau, C.N., Levanos, V.A., Dahasrabudhe, A. & Floyed, E.D. 2001. Bacterial diversity in human subgingival Plaque. *Journal of Bacteriology* **183**: 3770-3783.

Saghai-Maroof, M.A., Soliman, K.M, Jorgensen, R.A & Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* **81**: 8014 - 8019.

Swofford, D.L. 2002. PAUP*. Phylogenetic analysis using parsimony (*other methods): Version 4.0b10. Sinour associates

Thomson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673 – 4680.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 SCREENING OF INFECTED *H. MIDAE* FOR THE PRESENCE OF *C. XENOHALIOTIS CALIFORNIENSIS*

4.1.1 Diagnostic PCR assay

4.1.1.1 Primer selection

Fig. 4.1 shows the variable region (990bp – 1035bp) of the 16S rDNA sequence alignment that was chosen to design the *C. xenohaliotis californiensis* specific primer, Ca1. The primer Ca1 was chosen after alignment showed sequences obtained from GenBank, *C.burnetii*, *E.coli*, *P. salmonis* and species of the order *Rickettsiales* (Accession numbers - Table 3.4) found no homologous site for hybridization. The oligonucleotide was 20 bp in length and targeted the region 990–1035 bp of the *C. xenohaliotis californiensis* 16S rDNA gene.

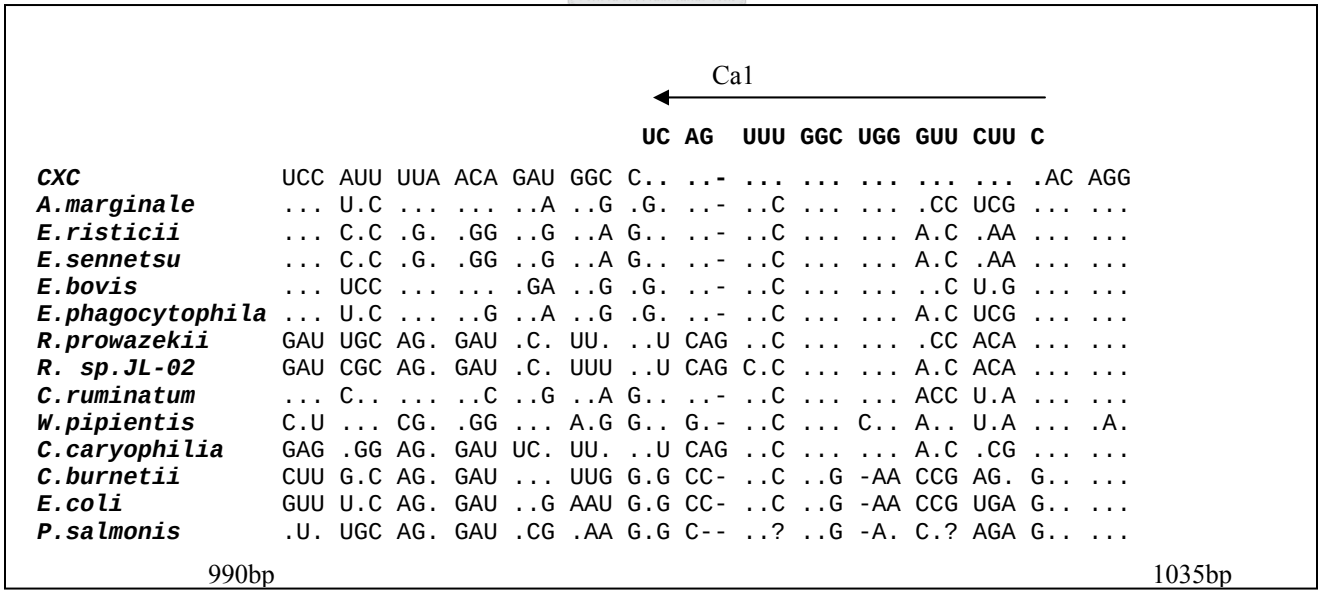


Fig 4.1. Sequence comparison between *C. xenohaliotis californiensis* specific primer (CA1) and corresponding regions in 16S rDNA of *E. coli*, *P. salmonis*, *C. burnetii* and various species from the order *Rickettsiales* (Table 3.4). Dots include nucleotides identical to *C. xenohaliotis californiensis* and a bar indicates a gap.

4.1.1.2 **PCR amplification with *C. xenohaliotis californiensis* specific primers**

The local abalone species, *H. midae*, was examined by PCR for the presence of the withering syndrome bacterium. DNA extracted from black abalone (*Haliotis cracherodii*) heavily infected with the etiological agent served as a positive control. The PCR primers amplified *C. xenohaliotis californiensis* SSU rDNA from genomic DNA of infected black abalone (Fig. 4.2: lane 3, Fig. 4.3: lane 3) but no product was obtained from genomic DNA extracted from the local abalone species (Fig. 4.2: lanes 4-9, Fig. 4.3: lanes 4-11). Verification that the positive products were from the 16S rDNA gene C. *xenohaliotis californiensis* was determined by nucleotide sequencing.

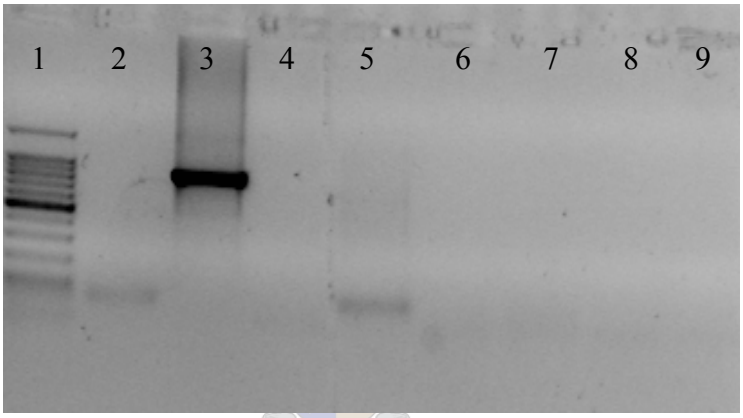


Fig. 4.2. PCR amplification with *C. xenohaliotis californiensis* primers, RA5-1 and Ca1. Lane1: 100 bp ladder molecular standard; Lane 2: negative control (PCR buffer without DNA); Lane 3: 940 bp amplification product obtained from infected DNA of black abalone; Lane 4-9: No amplification product obtained from infected *H. midae* DNA.

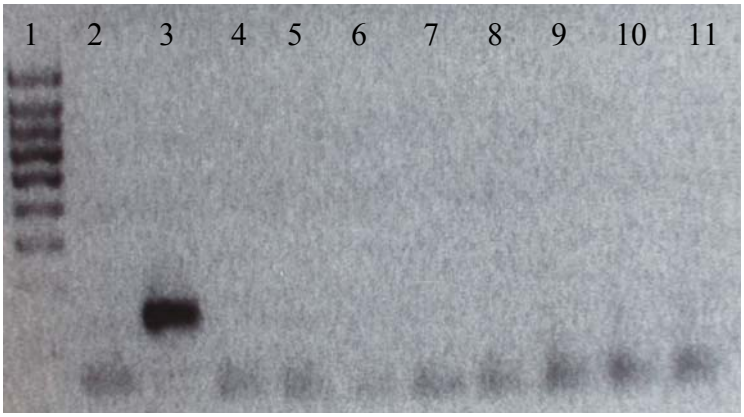


Fig. 4.3. PCR amplification with *C. xenohaliotis californiensis* primers, RA5-1 and RA3-6. Lane1: 100 bp ladder molecular standard; Lane 2: negative control (PCR buffer without DNA); Lane 3: 160 bp amplification product obtained from infected DNA of black abalone; Lane 4-11: No amplification product obtained from infected *H. midae* DNA.

4.1.2 *In situ* hybridization

4.1.2.1 Optimization of protocol

The optimum dilution of the probes was 1:1 000 (v.v; 10 fmol/µl). Increasing the concentration of the probes to 12.5 and 15 fmol/µl did not significantly increase the intensity of the hybridization signal. The main disadvantage of oligonucleotide probes is their rather low specific activity. One method that has shown success in alleviating this problem is the use of mixtures of oligonucleotides directed at different sequences along the same gene (Haper *et al*. 1997). In this study different probe combinations were also tested to determine which mixtures would demonstrate the best binding ability to the SSU rDNA of *C. xenohaliotis californiensis.* The most intense hybridization signal were obtained when the cocktail of three to four probes were used. As soon as the combinations were reduced to only two and one probe no hybridization signal was observed. Moreover, hybridization and stringency washes at 40° were effective for optimum binding of the probe and reducing backgrounds.

4.1.2.2 Detection of parasite DNA

In situ hybridization of the probes to tissue sections of red (Fig. 4.4 A) and black (Fig. 4.4 B) abalone infected with *C. xenohaliotis californiensis* yielded strong hybridization of the probes with no background hybridization to abalone tissue. Positive hybridization signals were evident in inclusion bodies located in the epithelium of the digestive gland. These inclusions were observed as purple precipitants therefore indicating the presence of the parasite.

The bacterial inclusions formed by the *Rickettsiales*-like prokaryote in *H. midae* were not recognized by the *C. xenohaliotis californiensis* specific probes (Fig 4.5). Increasing the probes concentration to 12.5 fmol/µl and 15 fmol/µl also did not yield any observable hybridization signal. Uninfected *H. midae* tissue sections that served as a negative control in the *in situ* hybridization trial were also not recognized by the *C. xenohaliotis californiensis* probes (Fig. 4.6).

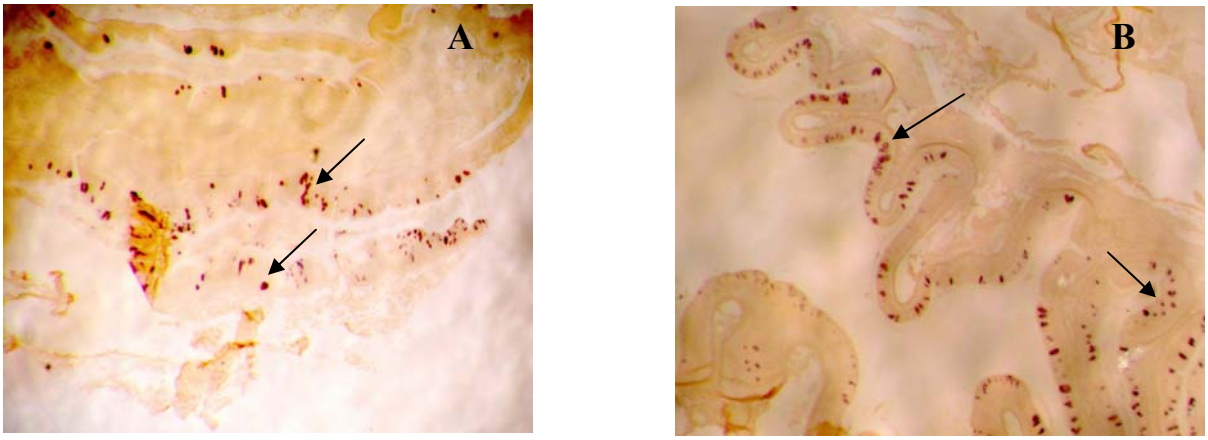


Fig 4.4. In situ hybridization with *C. xenohaliotis californiensis* oligonucleotide probes on histological sections of red (A) and black (B) abalone infected with *C. xenohaliotis californiensis*. Arrows indicate the inclusion bodies located in the epithelium of the digestive gland

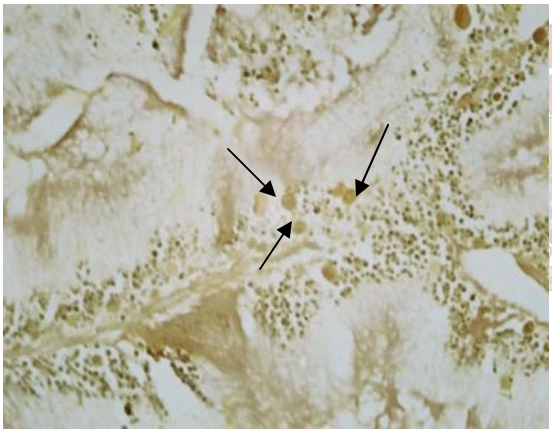


Fig 4.5. *In situ* hybridization with *C. xenohaliotis californiensis* oligonucleotide probes on histological sections of *H. midae* infected with the *Rickettsiales*-like prokaryote. Arrows indicate the non-hybridization of inclusions formed by the *Rickettsiales*-like prokaryote.

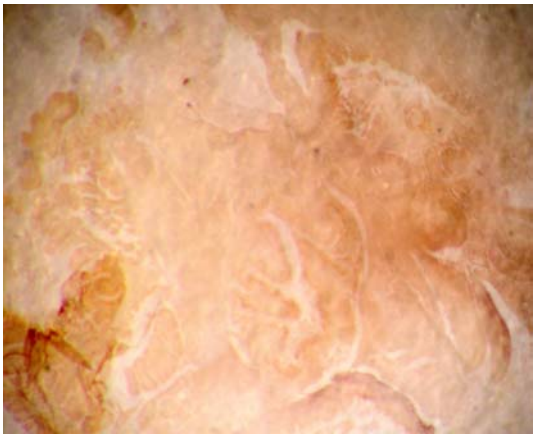


Fig 4.6. *In situ* hybridization with *C. xenohaliotis californiensis* oligonucleotide probes to uninfected *H. midae* tissue sections.

Both the PCR assay and the *in situ* hybridization test provide evidence that the etiological agent of withering syndrome is not present in the local abalone species. The fact that the *C. xenohaliotis californienis* PCR primers/probes did not recognize the *Rickettsiales*-like inclusions in *H. midae* indicated that a different bacterium is infecting the local abalone species.

4.2. ISOLATION OF 16S RDNA SEQUENCES FROM INFECTED *H. MIDAE* DIGESTIVE GLAND TISSUES

The 16S rDNA gene isolated from infected digestive gland was amplified and subsequently cloned into the pCR 4 – TOPO vector. A total of six clone libraries were obtained. Forty clones per library were screened for clones containing the correct insert size. Clones of the correct size (Table.4.1) were sequenced and subjected to phylogenetic analysis. The phylogenetic analysis indicated that the 16S rDNA sequences retrieved from infected digestive gland tissue fell into three classes, *α -Proteobacteria*, *Mollicutes* and *Spirochaetes*. The distribution of the sequences obtained from the 16S rDNA libraries within each of these classes is shown in Table 4.1. In Figs 4.9 through 4.12, the phylogenetic diversity within each class is shown and is discussed in detail below.

4.2.1 Phylogenetic analysis of the 16S rDNA sequences isolated from infected *H. midae* digestive gland tissue

4.2.1.1 *α - Proteobacteria*

The most abundant group of sequences isolated from the digestive gland of infected abalone showed phylogenetic affinities with the group *α - Proteobacteria*. A heuristic search from the aligned DNA sequence data of the SSU rDNA gene produced a phylogenetic tree (Fig. 4.8) of 2525 steps (CI = 0.505, RI = 0.803). This tree was rooted by using *E.coli* a member of the class γ

– *Proteobacteria* as an outgroup, and shows two orders of the class *α* - *Proteobacteria*: *Sphingomonadales* and *Rickettsiales***.**

Table 4.1 16S rDNA sequences identified in the clone libraries from the digestive gland microbes of the abalone *H.midae*

Name of clone library	Number of clones sequenced	Affiliation (Class, Order)
Clone library **10**	2 2	*Mollicutes, Mycoplasmatales* *Alphaproteobacteria*, *Sphingomonodales*
Clone library **14**	3	*Mollicutes, Mycoplasmatales*
Clone library **18**	2	*Spirochaetas*, *Spirochaetales*
Clone library **63**	4 5 2	*Alphaproteobacteria*, *Rickettsiales* *Alphaproteobacteria*, *Sphingomonodales* *Mollicutes, Mycoplasmatales*
Clone library **72**	2	*Alphaproteobacteria, Sphingomonodales*
Clone library **132**	2	*Mollicutes, Mycoplasmatales*

Of the thirteen sequences assigned to the group *α* - *Proteobacteria,* nine of them clustered with members of the order *Sphingomonadales* (numbers 80,97,28,22,58,18,62,76,77) in Fig 4.9). High similarities between the sequences isolated and species in this order were obtained (Table 4.1). The order *Sphingomonadales* contains aerobic bacteria and is frequent in marine environments (Takeuchi *et al*.1994; Prescott *et al*. 1999). We therefore speculate that the sequences showing similarities to the order *Sphingomonodales* might therefore have been taken in from the surrounding water during digestion of food particles or are resident in the digestive tract of the abalone.

The remaining four sequences (numbers 20,63,96,39 in Fig 4.9) of those assigned to α - *Proteobacteria* clustered together on a single branch and are basal to members of the order *Rickettsiales*. They showed a low phylogenetic affinity to *Pelagibacter ubique strain* (bootstrap value 52%), an uncultured rickettsia known to occur in bacterioplankton (Rappè *et al*. 2002), and to the families *Rickettsiaceae* and *Anaplasmataceae.*

Table 4.2 **Kimura's two-parameter genetic distances illustrating the relationship of the digestive gland bacteria and members of the order *Sphingomonodales*. A total of 1501 nucleotides of the 16S rDNA gene were included in the dataset after missing information and gaps were removed.**

Taxon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	0.22																		
3	0.22	0.01																	
4	0.21	0.03	0.03																
5	021	0.08	0.08	0.07															
6	0.20	0.06	0.07	0.05	0.05														
7	0.20	0.06	0.06	0.05	0.05	0.00													
8	0.21	0.07	0.06	0.06	0.08	0.09	0.09												
9	0.22	0.06	0.07	0.06	0.11	0.09	0.09	0.06											
10	0.22	0.06	0.06	0.06	0.11	0.09	0.09	0.06	0.02										
11	0.22	0.07	0.07	0.07	0.11	0.09	0.09	0.06	0.01	0.01									
12	0.22	0.07	0.07	0.07	0.11	0.09	0.09	0.06	0.01	0.01	0.00								
13	0.21	0.08	0.08	0.07	0.10	0.09	0.09	0.05	0.02	0.02	0.02	0.02							
14	0.21	0.08	0.08	0.07	0.10	0.09	0.09	0.05	0.02	0.02	0.02	0.02	0.00						
15	0.21	0.08	0.08	0.07	0.10	0.09	0.09	0.05	0.02	0.02	0.02	0.02	0.00	0.00					
16	0.23	0.09	0.09	0.09	0.12	0.11	0.10	0.07	0.04	0.04	0.04	0.04	0.01	0.01	0.01				
17	0.21	0.08	0.08	0.08	0.11	0.09	0.09	0.06	0.02	0.02	0.02	0.03	0.00	0.00	0.00	0.02			
18	0.25	0.10	0.11	0.10	0.13	0.12	0.12	0.08	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.04	0.03		
19	0.25	0.10	0.11	0.10	0.13	0.12	0.12	0.08	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.04	0.03	0.00	

*1.*E.coli*	2.*S.herbidovorans*	3.*S.chlorophenocolica*	4.*R.sp.K6*
5.S.SaS2	6.**76**	7.**77**	8.*S.pituitosa*
9.*S.parapaucimobilis*	10.*B.UNSW4*	11.*B.CAGY7*	12.*Glacial ice bacterium*
13.**18**	14.**62**	15.**28**	16.**58**
17.**22**	18.**80**	19.**97**	

The family *Rickettsiaceae* is composed of obligate intracellular bacteria that grow freely in the cytoplasm of eukaryotic host cells and includes etiological agents of human diseases such as typhus and spotted fever. Members of the family *Anaplasmataceace* are also obligate intracellular and replicate while enclosed in an eukaryotic host cell membrane-derived vacuole

(Rikihisa, 1991). Most of the bacteria contained in this family are pathogenic to domestic animals, cattle, sheep and goats. The disease-causing agent of withering syndrome, *C. xenohaliotis californiensis,* is also a member of this family. The genetic distances illustrating the relationship among the digestive gland bacteria and members of the order *Rickettsiales* are shown in Table 4.3.

The relationship of the clones isolated from infected *H. midae* digestive gland tissue to the order *Rickettsiales* was fairly well-supported (bootstrap value 92%) and clustering with members of this group was interesting. The *Rickettsiales*-like prokaryote residing in infected *H. midae* digestive gland tissue shares common characteristics with the order *Rickettsiales*. They are intracellular by nature and replicate in vacuoles within their eukaryotic host cell (pers. comm. A. Mouton). We therefore speculates that because of their association with the order *Rickettsiales*, based on sequencing and morphological data, these clones might be responsible for the inclusions formed in infected digestive gland tissues.

4.2.1.2 ***Mollicutes***

A maximum parsimony tree was constructed to show the placement of eight clones (numbers 92,35,21,14,27,26,75,10 in Fig 4.10) as members of the class *Mollicutes*. The heuristic search from the aligned DNA sequence data (1464 characters) of the SSU rDNA gene produced a most parsimonious tree of 2320 steps (CI = 0.715, RI = 0.807). The tree shows the relationship between sequences obtained from infested abalone digestive gland and members of the genus *Mycoplasma,* and is rooted with the branch leading to *Bacillus subtilis* a member of the order *Bacillales*.

Although most *Mollicutes* species are considered to be either pathogenic or parasites, there are examples of commensal and saprophytic *Mollicutes* (Holt *et al*. 1994). The clones isolated in this study showed phylogenetic affinities to two uncultured *Mycoplasma* species: *Uncultured mycoplasma sp. clone A9* (genetic distance ranging from 0.02-0.04, Table 4.4) and *Uncultured sp. clone B58* (genetic distance ranging from 0.06-0.08, Table 4.4), which were previously isolated from the digestive tract of the abalone *H. discus hannai* (Tanaka *et al*. 2004).

Table 4.3 **Kimura's two-parameter genetic distances illustrating the relationship of the digestive gland bacteria and members of the order *Rickettsiales*. A total of 1501 nucleotides of the 16S rDNA gene were included in the dataset after missing information and gaps were removed.**

Taxon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1																														
2	0.22																													
3	0.22	0.00																												
4	0.22	0.01	0.01																											
5	0.22	0.01	0.01	0.00																										
6	0.22	0.01	0.01	0.00	0.00																									
7	0.22	0.01	0.00	0.00	0.00	0.00																								
8	0.22	0.01	0.01	0.01	0.00	0.00	0.00																							
9	0.24	0.18	0.18	0.18	0.18	0.18	0.18	0.18																						
10	0.25	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.03																					
11	0.25	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.03	0.00																				
12	0.29	0.19	0.19	0.19	0.19	0.18	0.19	0.18	0.21	0.21	0.21																			
13	0.25	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.19																		
14	0.25	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.16	0.19	0.04																	
15	0.24	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.18	0.02	0.04																
16	0.25	0.16	0.16	0.15	0.15	0.16	0.16	0.16	0.15	0.15	0.15	0.19	0.02	0.04	0.02															
17	0.25	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.03	0.05	0.02	0.02														
18	0.25	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.03	0.05	0.02	0.02	0.01													
19	0.24	0.16	0.15	0.15	0.15	0.15	0.15	0.15	0.16	0.16	0.16	0.16	0.11	0.11	0.10	0.10	0.11	0.10												
20	0.24	0.15	0.15	0.14	0.14	0.14	0.14	0.15	0.15	0.15	0.15	0.16	0.10	0.10	0.10	0.09	0.10	0.10	0.01											
21	0.24	0.16	0.16	0.16	0.15	0.15	0.15	0.16	0.15	0.16	0.16	0.15	0.11	0.12	0.11	0.11	0.13	0.13	0.05	0.05										
22	0.25	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.15	0.15	0.16	0.15	0.12	0.12	0.12	0.12	0.13	0.13	0.07	0.06	0.02									
23	0.25	0.17	0.17	0.16	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.15	0.11	0.11	0.11	0.11	0.13	0.12	0.05	0.05	0.01	0.01								
24	0.25	0.16	0.16	0.16	0.16	0.15	0.15	0.16	0.15	0.16	0.16	0.15	0.11	0.11	0.11	0.11	0.12	0.12	0.05	0.05	0.01	0.01	0.00							
25	0.23	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.14	0.14	0.13	0.14	0.15	0.15	0.09	0.08	0.07	0.07	0.07	0.07						
26	0.29	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.26	0.26	0.25	0.25	0.23	0.23	0.23	0.23	0.24	0.24	0.24	0.24	0.25	0.25	0.24	0.24	0.25					
27	0.22	0.16	0.16	0.16	0.16	0.15	0.15	0.15	0.15	0.15	0.15	0.16	0.13	0.12	0.13	0.14	0.14	0.14	0.07	0.07	0.07	0.06	0.06	0.06	0.03	0.26				
28	0.26	0.19	0.19	0.18	0.18	0.18	0.18	0.19	0.18	0.19	0.19	0.27	0.21	0.21	0.20	0.20	0.21	0.21	0.20	0.19	0.18	0.19	0.18	0.19	0.21	0.25	0.20			
29	0.26	0.19	0.18	0.19	0.19	0.18	0.18	0.18	0.19	0.19	0.19	0.24	0.20	0.19	0.19	0.19	0.20	0.19	0.18	0.17	0.17	0.17	0.16	0.17	0.19	0.25	0.18	0.05		
30	0.26	0.19	0.18	0.19	0.19	0.18	0.18	0.18	0.19	0.19	0.19	0.24	0.20	0.19	0.19	0.19	0.20	0.19	0.18	0.17	0.17	0.17	0.16	0.17	0.19	0.26	0.18	0.05	0.00	
31	0.23	0.20	0.20	0.20	0.19	0.20	0.20	0.20	0.21	0.20	0.20	0.23	0.15	0.17	0.16	0.15	0.16	0.16	0.17	0.17	0.18	0.19	0.18	0.18	0.18	0.23	0.19	0.19	0.17	0.18

*1.*E.coli* 2.*R.typhii* 3.*R.prowazekii* 4.*R.japonica* 5.*R.JL-02* 6.*R.slovaca* 7.*R.africae* 8.*R.parkeri* 10.*E.sennetsu* 11.*E.risticii* 12.*C.xenohaliotis californiensis* 13.*W.endosymbiont of Brugia malayi* 14.*W.endosymbiont of Dirofilaria immitis* 15.*W.sp.WAU* 16.*W.sp. from H.danicus* 17.*W.sp. 16SrDNA* 18.*W.pipientis*19.*C.neoehrlichia mikurensis* 20.*E.belluno* 21.*C.ruminantium* 22.*E.canis* 23.*E.sp.EH1087* 24.*E.sp.EHF669* 25.*E.bovis* 26.*P.ubique strain* 27.*Amarginale* 28.**96** 29.**20** 30.**63** 31.**39**

These *Mycoplasmas* were found to be dominant in the digestive tract of *H. discus hannai*; therefore supporting the notion that the *Mycoplasma* species isolated in this study might represent normal inhabitants of the digestive gland of *H. midae*.

Table 4.4 **Kimura's two-parameter genetic distances illustrating the relationship of the digestive gland bacteria and members of the class *Mollicutes*. A total of 1464 nucleotides of the 16S rDNA gene were included in the dataset after missing information and gaps were removed.**

Taxon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
[1]																									
[2]	0.00																								
[3]	0.00	0.00																							
[4]	0.01	0.01	0.01																						
[5]	0.19	0.20	0.19	0.20																					
[6]	0.31	0.30	0.31	0.31	0.29																				
[7]	0.30	0.30	0.30	0.31	0.26	0.04																			
[8]	0.35	0.35	0.35	0.36	0.34	0.09	0.10																		
[9]	0.32	0.33	0.32	0.33	0.31	0.09	0.09	0.03																	
[10]	0.25	0.24	0.25	0.25	0.26	0.16	0.16	0.16	0.14																
[11]	0.24	0.23	0.24	0.24	0.24	0.15	0.13	0.17	0.15	0.04															
[12]	0.24	0.23	0.24	0.24	0.24	0.15	0.13	0.17	0.15	0.04	0.00														
[13]	0.25	0.24	0.25	0.25	0.26	0.16	0.16	0.14	0.13	0.02	0.03	0.03													
[14]	0.26	0.25	0.26	0.26	0.27	0.16	0.16	0.16	0.15	0.00	0.05	0.05	0.02												
[15]	0.25	0.24	0.25	0.25	0.26	0.16	0.16	0.14	0.13	0.02	0.03	0.03	0.00	0.02											
[16]	0.25	0.24	0.25	0.25	0.27	0.16	0.16	0.15	0.13	0.01	0.04	0.04	0.01	0.01	0.01										
[17]	0.25	0.24	0.25	0.25	0.27	0.16	0.16	0.14	0.13	0.01	0.04	0.04	0.01	0.01	0.01	0.00									
[18]	0.29	0.30	0.29	0.30	0.25	0.18	0.16	0.16	0.13	0.07	0.07	0.07	0.07	0.08	0.07	0.07	0.06								
[19]	0.25	0.25	0.25	0.25	0.26	0.15	0.15	0.14	0.13	0.03	0.04	0.04	0.02	0.03	0.02	0.03	0.03	0.08							
[20]	0.36	0.37	0.36	0.37	0.29	0.35	0.37	0.34	0.34	0.33	0.35	0.35	0.33	0.33	0.33	0.33	0.33	0.35	0.33						
[21]	0.34	0.35	0.34	0.35	0.31	0.37	0.35	0.35	0.37	0.30	0.34	0.34	0.32	0.31	0.32	0.31	0.31	0.33	0.32	0.21					
[22]	0.32	0.33	0.32	0.34	0.31	0.32	0.34	0.35	0.37	0.40	0.39	0.39	0.40	0.40	0.40	0.40	0.40	0.42	0.40	0.14	0.23				
[23]	0.34	0.35	0.34	0.36	0.30	0.34	0.34	0.33	0.34	0.35	0.38	0.38	0.35	0.36	0.35	0.35	0.34	0.36	0.36	0.10	0.18	0.08			
[24]	0.32	0.33	0.32	0.33	0.27	0.34	0.34	0.35	0.35	0.35	0.37	0.37	0.34	0.35	0.34	0.35	0.34	0.36	0.35	0.10	0.19	0.09	0.03		
[25]	1.41	1.46	1.41	1.46	1.02	1.16	1.06	1.12	1.16	1.17	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.15	1.25	1.09	1.05	1.19	1.12	1.22	

*1.*S.symbiont of Antonina*	2. *S.sp. 16SrDNA*	3.*S.sp.Y32*	4. *S.sp.YR-2*	5.*M.sp.PG50*
6.*M.bovoculi*	7.*M.conjuctiviae*	8.*M.flocculare*	9.*M.ovipneumonia Y-98*	
10.**75**	11.**27**	12.**26**	13.**21**	14.**10**
15.**14**	16.**92**	17.**35**	18.*U.mycoplasma sp.cloneB58*	
19.*U.mycoplasma sp. clone A9*	20.*C.mycoplasma haemominutum*		21.*H.muris*	22.*M.suis*
23.*E.wenyonii*	24. *M.ovis*	25.*B.subtillus*		

The function of *Mollicutes* in the digestive gland of the abalone is still unclear, but they are known to assist pea aphid endosymbiont *Buchnera* in the synthesis of essential amino acids and

outer nutrients for host (Douglas, 1998, Fukatsu *et al*. 2001). The *Mycoplasmas* isolated in this study could therefore play an important role as an amino acid synthesizer for host abalone, but this must still be investigated.

4.2.1.3 ***Spirochaetes***

The trees presented in Figs 4.11 shows the position of two distinctive sequences (72 and 18) within the class *Spirochaetes*. A single most parsimonious tree of 1296 steps (CI = 0.700, RI = 0.730) was produced. The tree was rooted with the branch leading to *Leptospira biflexa,* a member of the family *Leptospiraceae*. The two sequences formed a single clade and showed a genetic distance of 0.17 (Table 4.5) to their sister taxon *Cristipira Cp1*. The association of these two sequences with *Cristipira Cp1* is not surprising and was supported by a bootstrap value of 95%. *Cristipira Cp1* is a member of the genus *Cristipira,* which are large spirochetes found primarily in a crystalline style in the digestive tract of bivalve mollusks. The crystalline style is a long, tapered, gelatinous rod that aids in the digestion of food particles (Breznak, 1984; Margulis *et al*. 1991; Paster *et al*. 1995).

Table 4.5 **Kimura's two-parameter genetic distances illustrating the relationship of the digestive gland bacteria and members of the class *Spirochaetes*. A total of 1454 nucleotides of the 16S rDNA gene were included in the dataset after missing information and gaps were removed.**

Taxon*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.														
2.	0.01													
3.	0.17	0.17												
4.	0.19	0.19	0.22											
5.	0.20	0.20	0.23	0.03										
6.	0.25	0.25	0.22	0.19	0.18									
7.	0.24	0.24	0.23	0.15	0.15	0.10								
8.	0.20	0.20	0.21	0.18	0.19	0.22	0.21							
9.	0.19	0.19	0.20	0.19	0.19	0.22	0.21	0.03						
10.	0.19	0.19	0.20	0.19	0.19	0.21	0.21	0.03	0.00					
11.	0.20	0.20	0.21	0.18	0.19	0.22	0.21	0.02	0.04	0.03				
12.	0.21	0.21	0.21	0.18	0.18	0.22	0.20	0.02	0.03	0.03	0.02			
13.	0.26	0.26	0.24	0.18	0.18	0.10	0.12	0.22	0.22	0.22	0.22	0.22		
14.	0.30	0.29	0.32	0.23	0.23	0.28	0.27	0.26	0.27	0.27	0.26	0.26	0.30	

*1.**72** 2.**18** 3.*Cristispira CP1* 4.*S.aurantia* 5.*S.MWH-HUW8*

6.*S.zuelzerae* 7.*S.stenostrepta* 8.*B.lonesteri* 9. *B.coriaceae* 10.*B.burgdorferi*

11.*B.lusitaniae* 12.*B.anserina* 13.*T.sp.2-149* 14. *L.biflexa*

4.2.2 **Phylogenetic analysis of the organisms residing in uninfected gonad, mantle and gill tissues of *H. midae***

Phylogenies were also inferred to identify organisms residing in uninfected gonad, mantle and gill tissues. The experiment was run in parallel with the phylogenetic analysis of the bacteria residing in infected digestive gland. This served as a control to ensure that the universal primers were not amplifying bacterial contaminants from the infected digestive gland tissues.

A maximum parsimony tree of 2660 steps (CI = 0.624, RI = 0.851) was produced (Fig. 4.12). The tree showed the placement of the 16S rDNA sequences isolated from gonad, gill, and mantle in the classes *Mollicutes*, *ε-Proteobacteria* and *Fusobacteria* respectively. The tree was rooted with *Aquifex aquaticus* a member of the family *Aquificaceae*.

The 16S rDNA sequence from gonad tissue clustered within the genus *Mycoplasma* with the sequence designated 14 previously isolated from the digestive gland (Section 4.2.1.2) as its closest relative (genetic distance 0.01, Table 4.6). The association of the *Mycoplasma* sequence isolated from gonad with the sequence 14 isolated from the digestive gland is supported by the fact that these two tissues are closely associated with one another. Therefore, it is expected to observe similar microflora in these two tissues.

The sequence obtained from mantle tissue was placed in the phylum *Fusobacterium* with the closest relatives among species of the genera *Ilyobacter* and *Propionigenium* (bootstrap value of 97%, genetic distance table). The *Fusobacterium* isolated, showed sequence similarities to two uncultured *Fusobacterium* bacteria*, Fusobacterium KO711* (genetic distance 0.03)[(previously isolated from basalt and on extinct smoker pipe at Kolbeinsey Ridge (Steinsbuk *et al*. 2002, unpublished)] and *Uncultured fusobacteria bacterium* (genetic distance 0.05) [previously isolated from deep-sea hydrothermal vents (Alain *et al*. 2004)], with a bootstrap value of 99%. We suggest that the isolate may come from the surrounding water because *Fusobacteria* are frequent in marine environment (Watson *et al*. 2000; Brune *et al*. 2002).

Parsimony analysis placed the 16S rDNA sequence isolated from gill tissue in the genus *Acrobacter* in **ε-***Proteobacteria*. The sequence showed phylogenetic affinities with an uncultured *Acrobacter*, previously isolated from oyster microbiota (Romero *et al*. 2002), that was supported by a bootstrap value of 100% and a genetic distance of 0.02 (Table 4.6). Over the past years *Acrobacter* spp. have been described from a variety of habitats in marine environments, including activated sludge (Snaidr *et al*. 1997), salt marsh sediments (Nelson & Jannasch, 1983), Wadden Sea sediments (Llobet-Brossa *et al*. 1998), North Sea bacterioplankton (Eilers *et al*. 2000) and hypersaline cyanobacterial mats from Solar Lake (Teske *et al*. 1996). The *Acrobacter* clone isolated in this study probably represents bacteria from the surrounding water that was established on the outer surface of the gills.

The bacteria isolated from mantle, gill and gonad seem to be normal inhabitants of these tissues and did not appear to be related to the sequences obtained from infected digestive gland, with the exception of the sequence isolated from gonad. As stated above, the latter case is, likely, a

consequence of the proximity between these tissues. Therefore the universal primers used in this study did not amplify bacterial contaminants.

4.3 *In situ* hybridization with species – specific probes

As a result of the universal nature of the bacterial primers used in the phylogenetic study, a wide spectrum of bacteria representing normal inhabitants of the digestive gland was amplified. From all the bacteria obtained, the group of sequences (sequences marked with (●) Fig 4.8) showing phylogenetic affinities to the order *Rickettsiales* were identified as likely candidates for forming the *Rickettsiales* - like inclusions in the digestive gland of *H. midae*. To determine the origin of the sequences designated 20, 39, 63 and 96 in infected digestive gland tissues, *in situ* hybridization assays were performed using oligonucleotide probes.

The sequences 20, 39, 63 and 96 served as templates for oligonucleotide design. Four variable regions specific for all four sequences were designed and hybridized on histological section showing classical feature of the *Rickettsiales*-like prokaryote. Uninfected *H. midae* were included in the hybridization trials as negative controls. Black and red abalone tissues infected with *C. xenohaliotis californiensis* were also examined by *in situ* hybridization.

4.3.1 Optimization of the protocol

Numerous *in situ* hybridization trials were performed with the species-specific probes. To optimize the protocol the following variables were modified: (a) concentration of probe (b) stringency, by changing the temperature of washes, and (c) length of incubation of BCIP-NBT substrate. The optimum dilution of the probes was 1:1 000 (v.v; 10 fmol/µl). Increasing the concentration of the probes to 12.5 and 15 fmol/µl did not significantly increase the intensity of the hybridization signal. Post hybridization washes at 40°C resulted in decrease background compared with parallel washes conducted at room temperature. Binding with the BCIP-NBT

substrate at room temperature for 2h gave strong signal and minimal backgrounds. Incubating the substrate beyond 4h increase the signal but increased nonspecific staining.

4.3.2 **Detection of hybridization signal**

The oligonucleotide probes did not hybridized to the inclusions formed by the *Rickettsiales*-like prokaryote. A positive hybridization signal was however observed for a group of abundant extracellular bacteria residing in the digestive tract, thereby authenticating the origin of the clones 39, 96, 20 and 63. Dense colonies of these bacterial cells were observed throughout the host tissue.

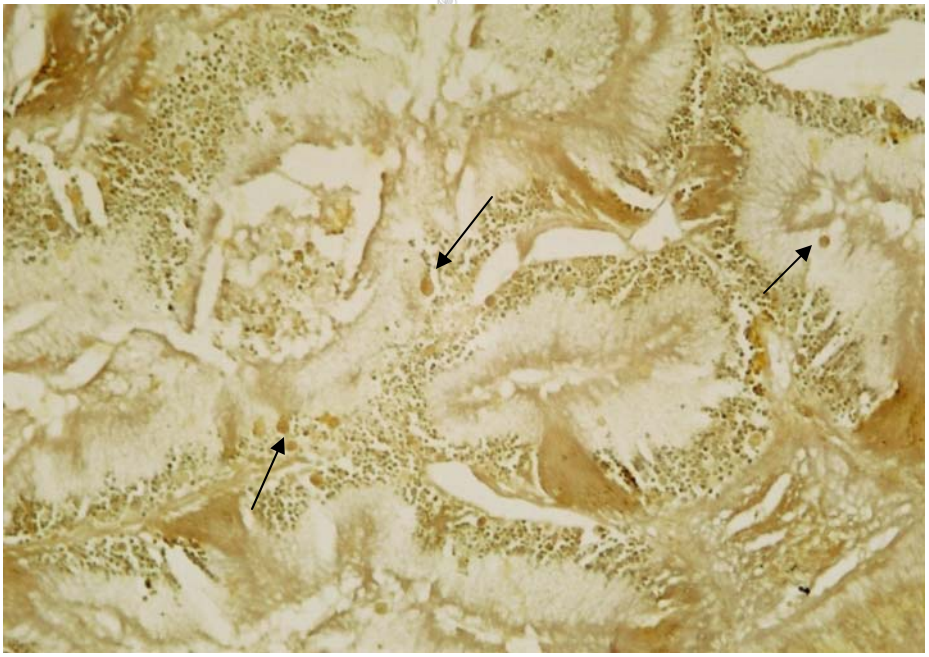


Fig 4.7. *In situ* hybridization with oligonucleotide probes specific for templates 20,39,63 and 96 on histological sections of *H. midae* infected with the *Rickettsiales*-like prokaryote. Arrows indicate non-hybridization of the inclusions formed by the *Rickettsiales*-like prokaryote.

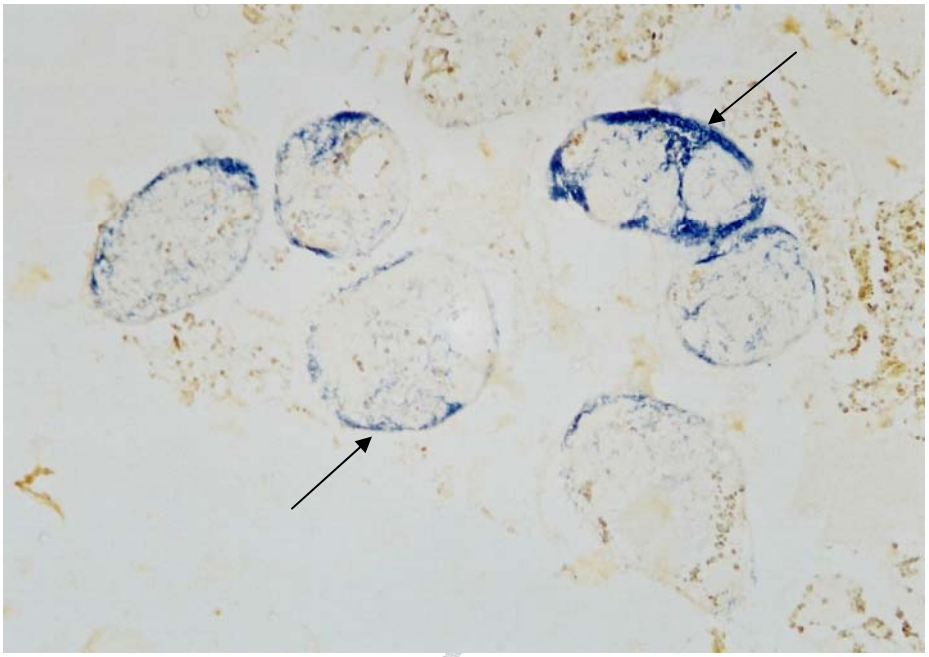


Fig 4.8. *In situ* hybridization with oligonucleotide probes 20,39,63,96 on histological sections of *H. midae* infected with the *Rickettsiales*-like prokaryote. A positive hybridization signal was observed for a group of abundant extracellular bacteria residing in the digestive tract. Arrows indicate dense colonies of bacterial cells.

Based on these results, the identity of the *Rickettsiales*-like prokaryote residing in infected digestive gland tissues could not be confirmed. All bacteria isolated in this study seemed to be normal microflora of the digestive tract. Various reasons could explain these results. Firstly, because of the universal nature of the primers, the normal microflora of the digestive tract is competing for hybridization to the primers and secondly the unknown organism may be under represented in the samples used.

Finally, based on the phylogenetic analysis (section 4.2.1.1) the group of sequences that gave hybridization signals is clearly members of the class α-*Proteobacteria* and is distinct but related to members of the order *Rickettsiales* (Fig 4.8A & B). Their closest relative, *P. ubique* strain is a free-living rickettsia common in bacterioplankton. They might therefore represent a novel group of rickettsias residing in the gut of the abalone. Both the phylogenetic and *in situ* hybridization

analysis indicates that this group of organisms represents a novel α-*Proteobacteria*, which appears to be abundant in the digestive tract of the abalone, *H. midae*.

Table 4.6 **Kimura two-parameter genetic distances illustrating the relationship of the gonad, mantle and gill to members of the classes *Mollicutes*, *Fusobacteria* and *ε-Proteobacteria* respectively. A total of 1537 nucleotides of the 16S rDNA gene were included in the dataset after missing information and gaps were removed.**

Taxon *	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1																																					
2	0.01																																				
3	0.05	0.06																																			
4	0.09	0.11	0.11																																		
5	0.05	0.07	0.03	0.09																																	
6	0.06	0.07	0.04	0.06	0.02																																
7	0.10	0.11	0.10	0.08	0.09	0.09																															
8	0.06	0.07	0.04	0.06	0.02	0.00	0.09																														
9	0.38	0.39	0.38	0.40	0.37	0.38	0.37	0.38																													
10	0.36	0.36	0.36	0.38	0.34	0.36	0.35	0.36	0.03																												
11	0.37	0.37	0.36	0.38	0.35	0.36	0.35	0.36	0.02	0.03																											
12	0.34	0.34	0.34	0.36	0.33	0.33	0.33	0.33	0.05	0.03	0.04																										
13	0.38	0.38	0.39	0.41	0.38	0.40	0.38	0.40	0.04	0.05	0.05	0.07																									
14	0.40	0.40	0.39	0.41	0.39	0.39	0.36	0.39	0.04	0.04	0.04	0.05	0.04																								
15	0.34	0.34	0.34	0.38	0.33	0.35	0.33	0.35	0.08	0.08	0.09	0.10	0.09	0.09																							
16	0.37	0.37	0.37	0.39	0.36	0.36	0.34	0.36	0.05	0.05	0.05	0.04	0.05	0.03	0.09																						
17	0.33	0.33	0.33	0.32	0.36	0.35	0.28	0.35	0.32	0.33	0.32	0.32	0.33	0.32	0.33	0.29																					
18	0.34	0.34	0.34	0.32	0.37	0.35	0.29	0.35	0.33	0.33	0.33	0.33	0.33	0.32	0.33	0.29	0.02																				
19	0.30	0.30	0.31	0.31	0.33	0.31	0.27	0.31	0.35	0.36	0.36	0.35	0.35	0.34	0.33	0.31	0.04	0.04																			
20	0.33	0.33	0.33	0.32	0.36	0.35	0.28	0.35	0.32	0.33	0.32	0.32	0.33	0.32	0.33	0.29	0.01	0.01	0.04																		
21	0.32	0.33	0.33	0.31	0.35	0.34	0.27	0.34	0.32	0.33	0.32	0.32	0.32	0.31	0.32	0.29	0.02	0.01	0.04	0.01																	
22	0.30	0.30	0.31	0.31	0.33	0.31	0.27	0.31	0.35	0.36	0.36	0.35	0.35	0.34	0.33	0.31	0.04	0.04	0.00	0.04	0.04																
23	0.33	0.33	0.34	0.32	0.36	0.35	0.28	0.35	0.32	0.32	0.33	0.32	0.32	0.30	0.32	0.29	0.03	0.04	0.05	0.04	0.03	0.05															
24	0.35	0.35	0.35	0.34	0.38	0.36	0.30	0.36	0.34	0.35	0.34	0.34	0.35	0.33	0.35	0.31	0.02	0.03	0.05	0.02	0.02	0.05	0.05														
25	0.34	0.35	0.35	0.33	0.37	0.36	0.29	0.36	0.33	0.33	0.34	0.33	0.33	0.32	0.33	0.30	0.04	0.05	0.06	0.05	0.04	0.06	0.02	0.03													
26	0.32	0.32	0.33	0.31	0.35	0.33	0.26	0.33	0.32	0.32	0.32	0.31	0.32	0.30	0.32	0.29	0.03	0.03	0.04	0.02	0.02	0.04	0.03	0.04	0.04												
27	0.33	0.33	0.35	0.32	0.36	0.34	0.30	0.34	0.34	0.35	0.34	0.35	0.37	0.35	0.34	0.35	0.09	0.10	0.09	0.09	0.09	0.09	0.09	0.09	0.10	0.10											
28	0.40	0.40	0.40	0.38	0.41	0.39	0.38	0.39	0.37	0.34	0.37	0.35	0.38	0.36	0.36	0.36	0.15	0.16	0.16	0.15	0.15	0.16	0.17	0.16	0.18	0.15	0.15										
29	0.36	0.36	0.39	0.40	0.39	0.38	0.38	0.38	0.43	0.42	0.42	0.41	0.40	0.41	0.37	0.39	0.17	0.17	0.14	0.17	0.16	0.14	0.17	0.18	0.18	0.15	0.17	0.11									
30	0.42	0.44	0.43	0.41	0.43	0.41	0.39	0.41	0.39	0.36	0.39	0.37	0.40	0.38	0.38	0.37	0.16	0.16	0.17	0.15	0.16	0.17	0.18	0.17	0.19	0.16	0.18	0.03	0.11								
31	0.38	0.38	0.39	0.42	0.41	0.39	0.37	0.39	0.41	0.40	0.41	0.40	0.41	0.39	0.34	0.38	0.15	0.16	0.13	0.15	0.15	0.13	0.17	0.17	0.18	0.14	0.19	0.11	0.06	0.10							
32	0.37	0.37	0.37	0.39	0.36	0.38	0.38	0.38	0.30	0.31	0.30	0.32	0.29	0.31	0.32	0.30	0.25	0.25	0.25	0.25	0.24	0.25	0.26	0.27	0.27	0.24	0.30	0.31	0.27	0.31	0.29						
33	0.38	0.38	0.36	0.40	0.37	0.39	0.37	0.39	0.30	0.32	0.30	0.33	0.30	0.30	0.33	0.30	0.25	0.24	0.24	0.25	0.24	0.24	0.25	0.27	0.26	0.24	0.31	0.32	0.28	0.32	0.28	0.00					
34	0.37	0.37	0.37	0.39	0.36	0.38	0.38	0.38	0.30	0.31	0.30	0.32	0.29	0.31	0.32	0.30	0.25	0.25	0.25	0.25	0.24	0.25	0.26	0.27	0.27	0.24	0.30	0.31	0.27	0.31	0.29	0.00	0.00				
35	0.38	0.38	0.36	0.40	0.37	0.39	0.37	0.39	0.31	0.32	0.31	0.33	0.31	0.31	0.33	0.31	0.25	0.25	0.25	0.25	0.24	0.25	0.26	0.27	0.27	0.24	0.31	0.32	0.28	0.32	0.28	0.01	0.00	0.01			
36	0.33	0.33	0.32	0.38	0.32	0.34	0.32	0.34	0.33	0.33	0.33	0.35	0.34	0.35	0.29	0.35	0.30	0.30	0.27	0.30	0.29	0.27	0.29	0.31	0.29	0.28	0.29	0.31	0.29	0.33	0.29	0.17	0.17	0.17	0.18		
37	0.32	0.32	0.29	0.32	0.29	0.31	0.31	0.31	0.28	0.31	0.30	0.32	0.31	0.30	0.31	0.32	0.37	0.37	0.36	0.36	0.36	0.36	0.37	0.38	0.37	0.36	0.38	0.38	0.43	0.41	0.39	0.30	0.30	0.30	0.30	0.27	

*1.*U.eubacteria CHA3-437* 2.*U.eubacteriaCHA3-127* 3.*U.epsilon proteobacterium* 4.*A.sulfidicus* 5.*A.sp. 16Sr DNA* 6. *U.acrobacter sp.clone15* 7.*A.butzeleri* 8.**Gill** 9.*P.modestum* 10. *I.insuetus* 11.*I.tartarticus* 12.*P.maris* 13.*U.fusobacteria bacterium* 14. *F.bacterium KO711* 15.*F.gonidoformans* 16.**Mantle**17.92 18.**Gonad** 19.27 20.21 21.14 22.26 23.10 24.35 25.75 26.*U.mycplasma sp.clone A9* 27. *U.mycoplasma sp.clone B58* 28.*M.ovipneumonia Y-98* 29.*M.conjuctiviae* 30. *M.flocculare* 31.*M.bovoculi* 32.*S.sp.Y32* 33.*S.sp.16SrDNA* 34.*S.symbiont of Antonina* 35.*S.sp.YR-2* 36.*M.sp. PG50* 37.*A.aquanticus*

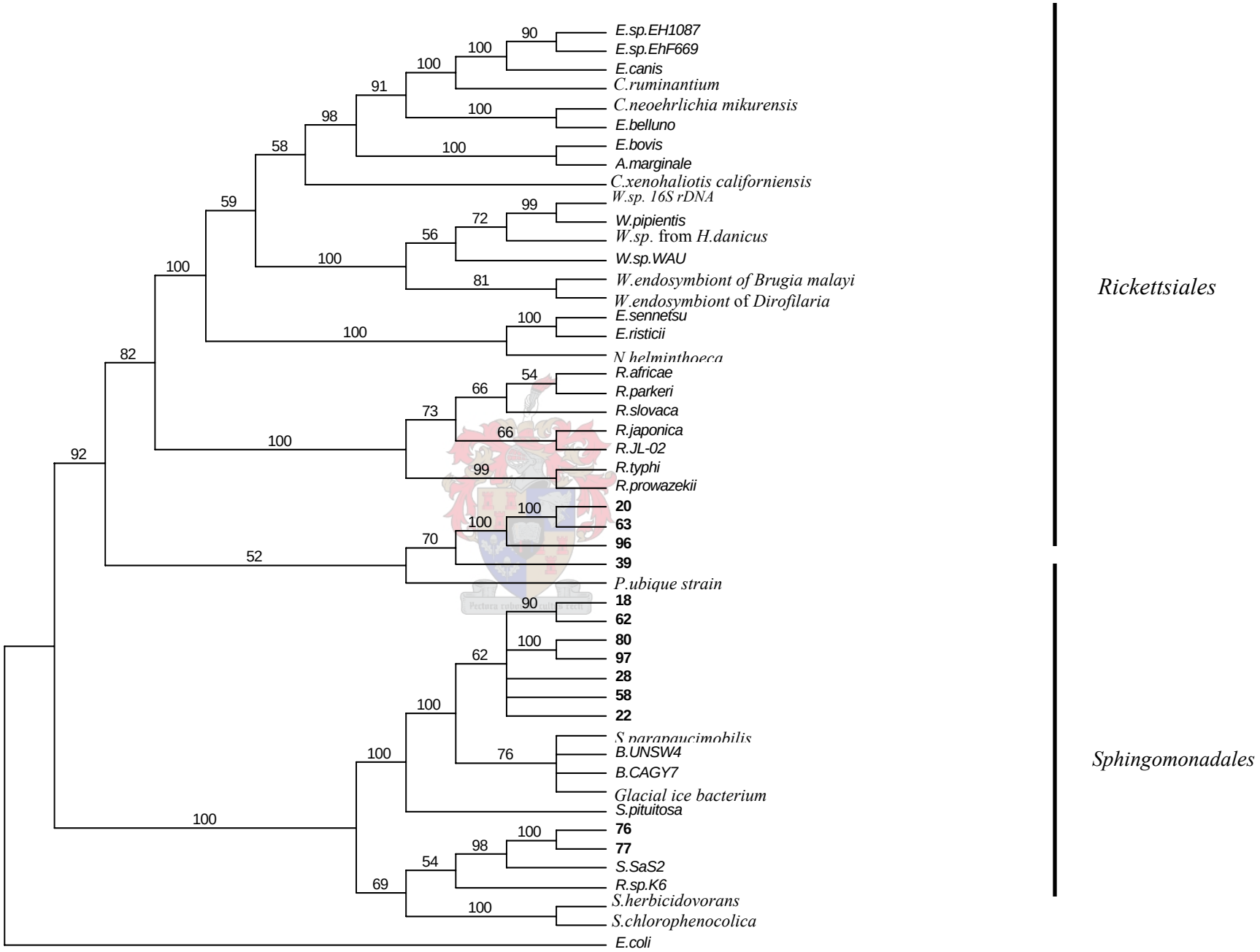


Fig 4.9: Maximum parsimony tree comparing the digestive gland bacteria (Sequences indicated in numbers) isolated from infected *H. midae* tissues with various members of the α - *Proteobacteria*. The number at the top of the branch depicts the percent occurrence of a given branch during 1000 replicates of the bootstrap analysis. *E.coli* was used as an outgroup.

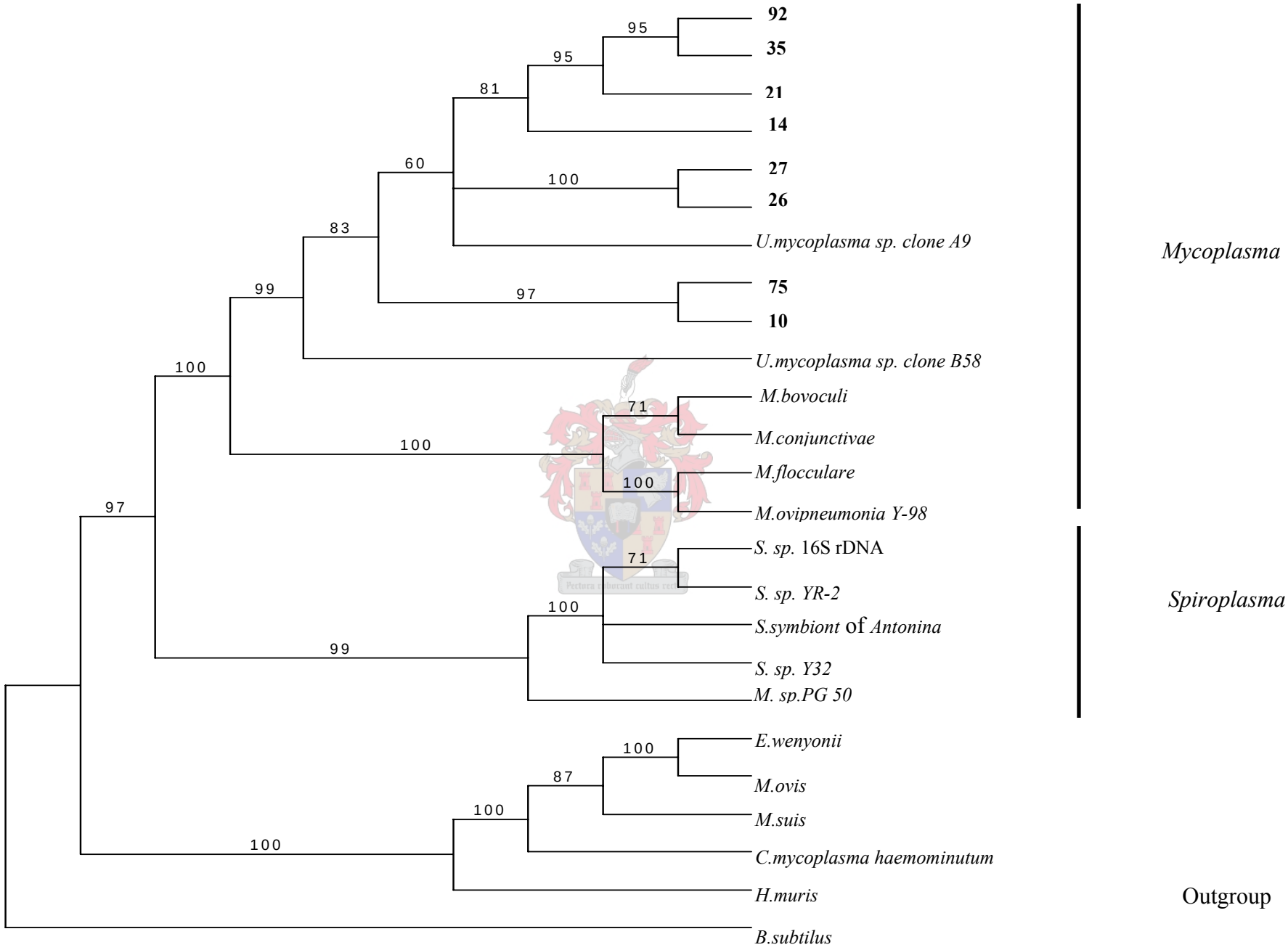


Fig 4.10: Maximum parsimony phylogenetic tree comparing the digestive gland bacteria (Sequences indicated in numbers) with various members of the class *Mollicute*s. The number at the top of the branch depicts the percent occurrence of a given branch during 1000 replicates of the bootstrap analysis. *B.subtilis* was used as an outgroup

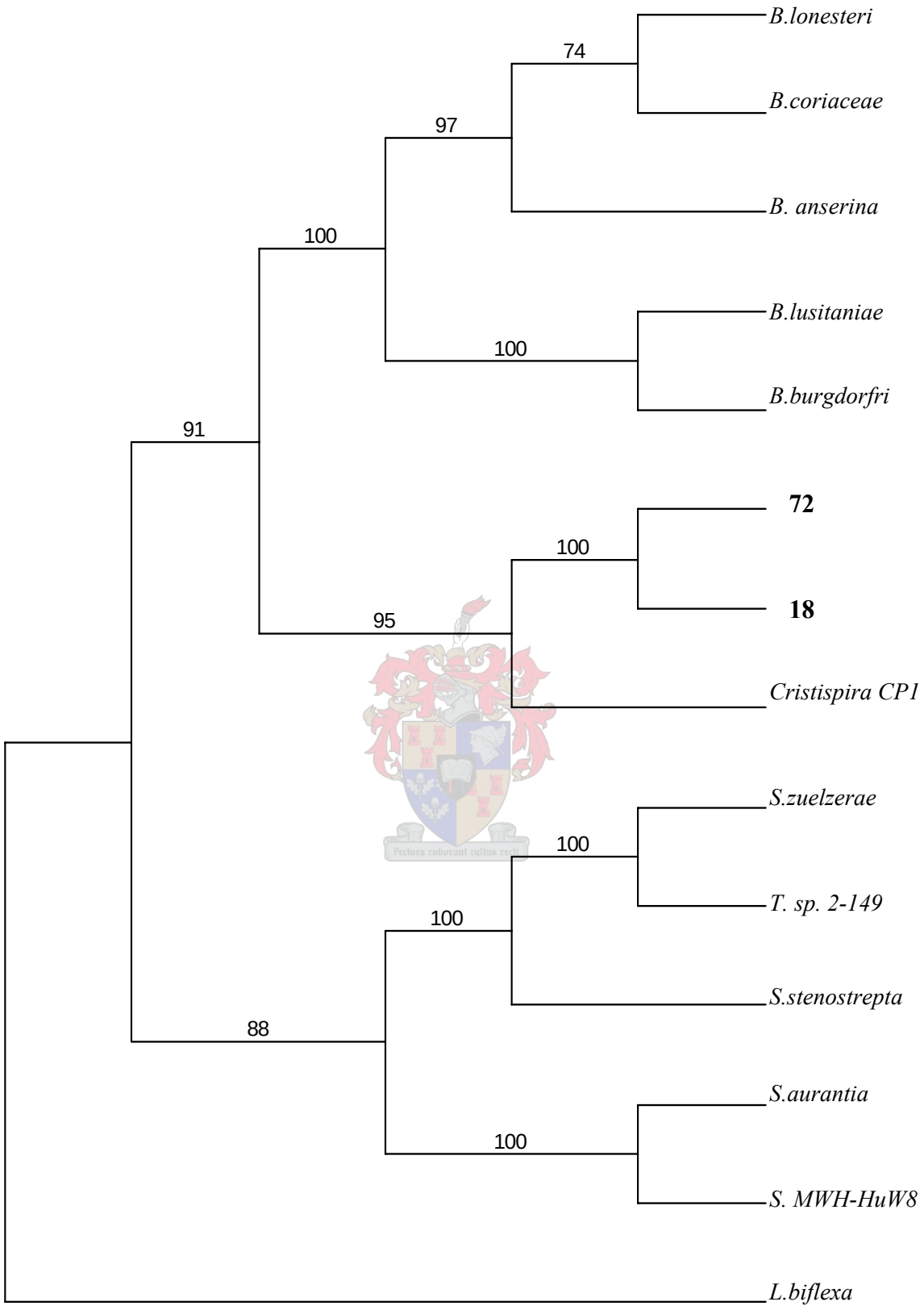


Fig 4.11: Maximum parsimony phylogenetic tree comparing the digestive gland bacteria (Sequences indicated in numbers) isolated from infected digestive gland with various members of class *Spirochaetes*. The number at the top of the branch depicts the percent occurrence of a given branch during 1000 replicates of the bootstrap analysis. *L. biflexa* was used as an outgroup.

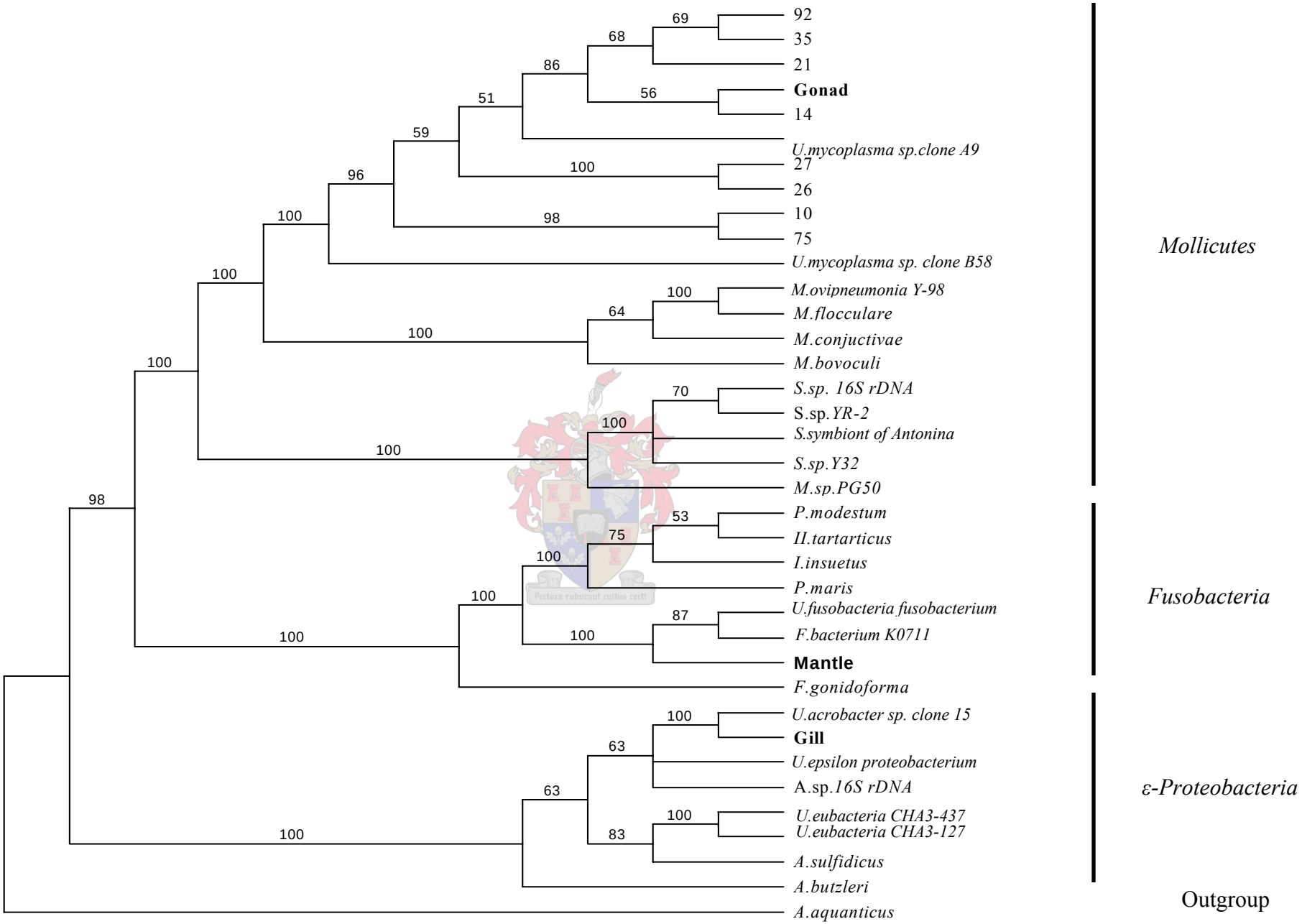


Fig 4.12: Maximum parsimony phylogenetic tree comparing the 16S rDNA sequences isolated from gonad, mantle and gill with the classes *Mollicutes, Fusobacteria and ε-Proteobacteria r*espectively. Numbers indicates – Sequences isolated from digestive gland. The number at the top of the branch depicts the percent occurrence of a given branch during 1000 replicates of the bootstrap analysis. *A.aquanticus* was used as an outgroup.

4.4 **R E F E R E N C E S**

Alain, K., Zbindin, M., Le Bris, N., Lesongeur, F., Querellou, J., Gaill, F. & Cambon-bonavita, M.A. 2004. Early steps in microbial colonization process at deep-sea hydrothermal vents. *Environmental Microbiology* **6**: 227-241.

Breznak, J.A. 1984. Genus II. *Cristispira* Gross 1910, 44^{AL}. *In* N.R. Krieg and J.G. Holt (ed), *Bergey's manual of systematic bacteriology* 1: 46-49. Williams & Wilkens, Baltimore.

Brune, A., Evens, S., Kaim, G., Ludwig, W. & Shink, B. 2002. Ilyobacter insuetus sp. nov., a fermentative bacterium specialized in the degradation of hydroaromatic compounds. *International Journal of Systematic and Evolutionary Microbiology* **52**: 429-432.

Douglas, A.E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* **43**: 17-37.

Eilers, H., Pernthaler, J., Glöckner, F.O. & Amann, R. 2000. Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology* **66**: 3044-3051.

Fukatsu, T., Tsuchida, T., Nikoh, N. & Koga, R. 2001. Spiroplasma symbiots of the pea aphid. *Acyrthosiphon pisum*. *Applied and Environmental Microbiology* **67**: 1284-1291.

Haper, S.J., Bailey, E., Mckeen, C.M., Stewart, A.S., Pringle, J.H., Freehally, J. & Brown, T. 1997. A comparative study of digoxigenin, 2.4-dinitrophenel, and alkaline phosphatase as deoxyoligonucleotide labels in non – radioisotopic in situ hybridization. *Journal of Clinical Pathology* 50: 686-690

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. 1994. *Bergey's Manual of Determinative Bacteriology*. Williams and Wilkins, Baltimore.

Llobet-Brossa, E., Rosselló-Mora, R. & Amann, R. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Applied and Environmental Microbiology* **64**: 2691-2696.

Nelson, D.C. & Jannasch, H.W. 1983. Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. *Archeology Microbiology* **136**: 262-269.

Margulis, L., Nault, L. & Sieburth, J. 1991. *Cristispira* from oyster styles: complex morphology of large symbiotic spirochetes. *Symbiosis* **11**: 1-19.

Paster, B.J., Pelletier, D.A. Dewhirst, F.E., Weisburg, W.G., Fussing, V., Poulsen, K.L., Dannenberg, S. & Schroeder, I. 1995. Phylogenetic position of the Spirochetal genus Cristipira. *Applied and Environmental Microbiology* **62**. 942-946.

Prescott, L., Harley, J.P. & Klein, D.A. 1999. *Microbiology* 4th edition. WCB McGraw – Hill.

Rappè, M.S., Connon, S.A., Vergin, K.L. & Giovannoni S.J. 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**: 630-633.

Romero, J.,Garcia-Varela, M., Lacette,J.P. & Espejo, R.T. 2002. Bacterial 16S rRNA gene analysis revealed that bacteria related to *Acrobacter* spp. constitute an abundant and common component of the oyster microbiota (*Triostrea chilensis*). *Microbial Ecology* **44**: 365-371.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. & Schleifer, K. 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Applied and Environmental Microbiology* **63**: 2884-2896.

Takeuchi, M., Sawada, H., Oyaizu, H. & Yokota, A. 1994. Phylogenetic evidence for *Sphingomonas* and *Rhizomonas* as nonphotosynthetic members of the alpha – 4 subclass of the *Proteobacteria*. *International Journal of Systematic Bacteriology* **44**: 308-314.

Tanaka, R., Ootsubo, M., Sawabe, T., Ezur, Y. & Tajima, K. 2004. Biodiversity and *in situ* abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques**.** *Aquaculture* **241**: 453-463.

Teske, A., Sigalevich, P. Cohen, Y. & Muyzer, G. 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology* **62**: 4210-215.

Watson, J., Matsui, G.Y., Leaphart, A., Wiegel, J., Rainey, F.A. & Lovell, C.R. 2000. Reductively debrominating strains of Propiongenium maris from burrows of bromophenol-producing marine infauna. *International Journal of Systematics and Evolutionary Microbiology* **50**: 1035-1042.

CHAPTER 5
CONCLUSION

Withering syndrome is one of the most destructive abalone diseases in the world. This study attempted to evaluate the presence of the disease-causing organism in the South African species *H. midae* and examine the nature of the *Rickettsiales*-like prokaryote residing in infected digestive gland tissues of this species. Significant advances were made in this regard and during this process interesting results were obtained regarding the diversity of the microflora in *H. midae*.

The main objective of this study was to determine the relationship of *C. xenohaliotis californiens* to the *Rickettsiales*-like prokaryote residing in infected digestive gland tissues of *H.midae*. A specific PCR test and *ISH* designed to detect the 16S rDNA gene of *C. xenohaliotis californiensis* was used to screen *H. midae* tissues infected with the *Rickettsiales*-like prokaryote. Both techniques confirm that the disease-causing agent of Withering syndrome is not present in *H. midae*. The absence of this bacterium in local abalone species has significant positive implications for the South African abalone industry.

Since there was no relationship between the *Rickettsiales*-like organism and *C.xenohaliotis californiensis*, the next objective was to retrieve 16S rDNA sequences from infected digestive gland tissues in order to determine the taxonomic position of the *Rickettsiales*-like prokaryote residing in *H. midae*. The universal nature of the primers used and the competition of the normal gut flora for hybridization to the primers resulted in phylogenetic analysis revealing a diverse community comprised of α-*Proteobacteria*, *Mollicutes* and *Spirochaetas.* The class α-*Proteobacteria* contained a novel group of bacteria showing phylogenetic affinities to the order *Rickettsiales*. The novel group was identified as likely candidate for forming the *Rickettsiales*-like inclusions in the digestive gland of *H. midae***.** *In situ* hybridization with species-specific probes designed for the novel group revealed the presence of extracellular bacteria that were abundant in the host tissue and seems to be normal inhabitants of the abalones digestive gland.

We therefore conclude that all the bacteria isolated in this study represents normal microflora of the abalone digestive tract and that none of them are responsible for the inclusions formed by the *Rickettsiales*-like organism. Future work to determine the identity of the *Rickettsiales*-like organisms should consider a more careful physical isolation of the *Rickettsiales* inclusions before DNA extractions. The use of density gradient centrifigation has shown to be a valuable method to purify *Rickettsiales* – like organisms in mollusk. Investigation of this method in the future could aid in the identification of the *Rickettsiales* - like prokaryote infecting *H.midae.* Finally, a more careful dissection of the digestive gland should also be consider in the future in order to prevent PCR amplification of microflora of the digestive tract.