AN ABSTRACT OF THE THESIS OF

Michael J. Mauel for the degree of Doctor of Philosophy in Microbiology presented on September 10, 1996. Title: Evidence of Genetic Diversity in Piscirickettsia salmonis.

Abstract approved: Redacted for Privacy John L. Fryer

Piscirickettsia salmonis is the etiologic agent of piscirickettsiosis, a systemic disease of salmonids. A nested polymerase chain reaction (PCR) was developed to detect genomic DNA of P. salmonis. The nested PCR assay detected less than one P. salmonis tissue culture infectious dose 50 (TCID50). Using the P. salmonis-specific primers in a single amplification allowed detection of 60 TCID50. The specificity of the PCR was assessed with a panel of four salmonid and 15 bacterial genomic DNA preparations. Products from amplification were observed only from P. salmonis DNA.

Restriction fragment length polymorphism (RFLP) analysis of the 16S products from 6 isolates of P. salmonis demonstrated that one isolate, EM-90, was unique. Two additional primers were developed to differentiate EM-90 from the five other P. salmonis isolates. To assess the genetic variability in this species or species-complex, the 16S ribosomal DNA of five isolates from three different geographical locations were amplified using PCR. The PCR products were sequenced and compared with other previously published bacterial small subunit rRNA sequences. The results of this analysis confirm that P. salmonis is a member of the gamma subunit of the proteobacteria and show that the five isolates form a tight monophyletic cluster.

Three groups of sequences were identified. Two of the isolates, LF-89 and SLGO-94 were closely related (>99.7% similarity) and were isolated from the same geographic location but from different host species. ATL-4-91 and NOR-92, both isolated from Atlantic salmon but from different geographic areas form another group (99.7% similarity). EM-90 was unique with similarity values ranging from 98.5 -
98.9% when compared to the other four isolates. To further clarify the genetic variability in this genus, the internal transcribed spacer (ITS) and 23S rDNA of six isolates were amplified, sequenced and analyzed. The analysis again indicated that the six isolates form a tight monophyletic cluster. One spacer sequence was identified per isolate and the ITS region did not contain a tRNA gene. The ITS sequences were 311-bp in length and varied among the isolates (95.2-99.7% similarity). Approximately 1900-bp of the 23S rDNA gene were amplified for the six isolates and similarities ranged from 97.9 to 99.8% between the isolates. Phylogenetic trees were constructed with the 16S, ITS and 23S rDNA data and were of similar topography, a finding that suggested that the three regions provided similar phylogenetic information. Within *P. salmonis* three groups of ITS and 23S rDNA sequences were identified. Three isolates, LF-89, C1-95, and SLGO-94 are closely related (99.1% - 99.7% ITS and 99.3 - 99.8% 23S rDNA similarities) and have similar geographic but different host species origins. Another group is composed of ATL-4-91 and NOR-92. These organisms were both obtained from Atlantic salmon but from different geographic areas in the northern hemisphere and show sequence similarity values of 99.4% ITS and 98.7% 23S rDNA.

The sequence of isolate EM-90 was unique with similarities ranging from 95.2 - 96.9% ITS and 97.6 - 98.5% 23S rDNA when compared to the other five isolates. The *P. salmonis* ITS region has diverged on average 3.15 times faster than the 16 S rDNA gene, while the 23S rDNA gene has diverged 1.6 times faster than the 16S rDNA gene.
Evidence for Molecular Diversity of *Piscirickettsia salmonis*

by

Michael J. Mauel

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SEPTEMBER 10, 1996

APPROVED:

Redacted for Privacy
Major Professor, representing the Department of Microbiology

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Chair of the Department of Microbiology

Redacted for Privacy
Dean of Graduate School

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Michael J. Mauel, Author
CONTRIBUTION OF AUTHORS

I wish to thank and acknowledge the contributions of Dr. J. L. Fryer and Dr. S. J. Giovannoni. While I have been responsible for the laboratory work their contributions of guidance, expertise, advice, interest and patience in assisting me with the development of the experimental methods, analysis of the data and editing of the manuscripts have been invaluable in completing this work.
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DEDICATION

I dedicate this manuscript to my parents, Robert and Marjorie and to my wife Lynne. My parents gave me a great start on life by teaching me the value of reading and learning and by always encouraging my curiosity. They have been supportive of my life choices and have always been there for me when I needed help or to celebrate my accomplishments. I wish Mom was still here to see the final draft but I thank God that Dad is here for both of them.

Lynne has arrived in my life only recently but she has made my life complete as it has never been before. I thank her for her encouragement, understanding, patience and love. May I never be without her.

I also wish to thank my teachers, professors, friends and fellow graduate students for their encouragement and enthusiasm that has helped guide me to and keep me on this path.

Look Mom, its finally done!
EVIDENCE FOR GENETIC DIVERSITY IN *Piscirickettsia salmonis*

CHAPTER 1

**INTRODUCTION and LITERATURE REVIEW**

It is now recognized that rickettsia are serious pathogens of fish and can have a major economic impact on the mariculture industry and perhaps on the survival of feral fish. Prior to 1989 the role of rickettsia-like organisms as etiologic agents of fish disease was unrecognized. In fact, scientists argued against the possibility of a rickettsial pathogen for fish; stating that since all known terrestrial rickettsia-like organisms require an insect vector and since there were few if any, parasitic insects in aquatic environments there should be no aquatic rickettsia (Wolf 1981).

**Rickettsia-like organisms infecting invertebrates**

There have been many reports of rickettsia-like organisms infecting aquatic invertebrates (Table 1). These organisms have been observed mainly in hosts from the marine environment but there are also reports of infections in fresh water (mollusc; Harshbarger et al. 1977, Otto et al. 1977, Comps et al. 1977a,b, Buchanan 1978, Comps and Deltreil 1979, Meyers 1981, Leibovitz et al. 1984, Couch 1985, Comps 1985; crustacean: Federici et al 1974, Pappalardo and Bonami 1980, Larsson 1982, Johnson 1984, Brock et al. 1986, Anderson et al. 1987, Brock 1988). The organisms observed in mollusc have been reported as rickettsia-like but their taxonomy is unclear. They are pleomorphic, coccoid to ovoid, rod shaped and often benign. However case histories describing extensive mortality have been noted. These observations have been limited to light and electron microscopy of sectioned tissue showing an ultrastructure of an infectious agent similar to rickettsia (Anderson et al. 1965).
Table 1.1 Selected reports of Rickettsia-like infections in molluscs and crustaceans

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<th>Host species</th>
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<td>Argopecten irradians</td>
<td>Prince Edward Island, Canada</td>
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<td>Bay scallop</td>
<td>NE Atlantic Coast, USA</td>
<td>Leibovitz et al. (1984)</td>
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<tr>
<td>Chlamys varia</td>
<td>not reported</td>
<td>Legall et al. (1991)</td>
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<td>scallop</td>
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<td></td>
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<tr>
<td>Crassostrea gigas</td>
<td>France</td>
<td>Comps et al. (1977a,b)</td>
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<tr>
<td>Pacific oyster</td>
<td>Alaska, USA</td>
<td>Meyers et al. (1990)</td>
</tr>
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<td></td>
<td>Atlantic Coast, Spain</td>
<td>Azevedo and Villalba (1991)</td>
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<tr>
<td>Crassostrea virginica</td>
<td>New York, USA</td>
<td>Meyers (1981)</td>
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<td>Eastern oyster</td>
<td>Gulf Coast, USA</td>
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<tr>
<td>Donax trunculus</td>
<td>Mediterranean Coast, France</td>
<td>Comps (1985)</td>
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<td>Wedge shell</td>
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<td>Mercenaria merceneria</td>
<td>Delaware, USA</td>
<td>Fries and Grant (1991, 1992)</td>
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<td>Northern guahog</td>
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<td>Mya arenaria</td>
<td>Chesapeake Bay, USA</td>
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<td>Softshell clam</td>
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<td>Mytilus edulis</td>
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<td>Blue mussel</td>
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<td>Pacific razor clam</td>
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<td><strong>Crustaceans</strong></td>
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<td>Ripulogammarus pulex</td>
<td>Sweden</td>
<td>Larsson (1982)</td>
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<td>freshwater amphipod</td>
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<td>Carcinus mediterraneus</td>
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<td>Paralithodes platypus</td>
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<td>Blue king crab</td>
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<td>Panaeus marginatus</td>
<td>Hawaii, USA</td>
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<td>Panaeus merguiensis</td>
<td>Singapore, Malaysia</td>
<td>Brock (1988)</td>
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<td>Panaeus monodon</td>
<td>Singapore</td>
<td>Anderson et al. (1987)</td>
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<td>Malaysia</td>
<td>Brock (1988)</td>
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Modified from Fryer and Lannan 1994
The reports of rickettsia-like organisms in crustacea have been frequently linked to mortality (Federici et al. 1974, Leibovitz 1988, Krol et al. 1991). The hepatopancreas is the typical target organ but infections in the epidermis, digestive tract (Federici et al. 1974), fat cells and connective tissue occur (Larsson 1982). They exhibit pleomorphic developmental stages similar to the Chlamydia or members of the genus Rickettsiella (Leibovitz 1988).

Only one of the organisms infecting aquatic invertebrates has been characterized sufficiently for taxonomic placement. This agent infecting the fresh water amphipod, *Crangonyx floridanus*, has been placed, on the basis of morphological and developmental similarities, in the species *Rickettsia grylli*, a pathogen of insects (Weiss and Moulder 1984). The other agents have been designated rickettsia-like based on light and electron microscopy.

**Early reports of rickettsia-like organisms in fish**

Prior to 1981 there had only been two reports of rickettsia-like organisms in fish (Table 2). The was reported by Mohamed (1939) in a diseased puffer-like fish called a fokaka, *Tetrodon fahaka*. This was an unconfirmed case report and limited to observation by light microscope. Mohamed observed small coccoid forms in the monocytes and plasma of a dead fish brought to the laboratory for examination. The organisms stained pink with Giemsa and did not grow on artificial media.

A rickettsia-like organism was observed in RTG-2 fish cells (Wolf and Quimby, 1962) that were inoculated with material from rainbow trout (*Oncorhynchus mykiss*) infected with viral hemorrhagic septicemia (VHS) virus (Ozel and Schwanz-Pfitzer 1975). Examination of the Rickettsia-like organism was by light and electron
Table 1.2 Reported Rickettsial and Rickettsia-like infections of fish

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<td><em>Callionymus lyra</em> (Dragonet)</td>
<td>Cardigan Bay Wales</td>
<td>Davies (1986)</td>
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<td>Taiwan</td>
<td>Chern and Chao (1994)</td>
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<td><em>Oreochromis aureus</em> (Blue tilapia)</td>
<td>Taiwan</td>
<td>Chern and Chao (1994)</td>
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<td><em>Oreochromis zilii</em> (Redbelly tilapia)</td>
<td>Taiwan</td>
<td>Chern and Chao (1994)</td>
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<td><em>Oreochromis hornorum</em> (Wami tilapia)</td>
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<td>Chern and Chao (1994)</td>
</tr>
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<td><em>Oreochromis niloticus</em> (Nile tilapia)</td>
<td>Taiwan</td>
<td>Chern and Chao (1994)</td>
</tr>
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<td><em>Oreochromis unisexual broods</em></td>
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<td>Chen et al. (1994)</td>
</tr>
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<td><em>Panaque suttoni</em> (Blue-eyed plecostomus)</td>
<td>Colombia</td>
<td>Khoo et al. (1995)</td>
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<td><em>Dicentrarchus labrax</em> (Sea bass)</td>
<td>France</td>
<td>Comps et al. (1996)</td>
</tr>
<tr>
<td><em>Oncorhynchus kisutch</em> (Coho salmon)</td>
<td>Southern Coast Chile</td>
<td>Fryer et al. (1990, 1992)</td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em> (Rainbow trout)</td>
<td>Germany Southern Coast Chile</td>
<td>Ozel &amp; Schwanz-Pfitzner (1975)</td>
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<tr>
<td><em>Oncorhynchus mykiss</em> (Rainbow trout)</td>
<td>Germany Southern Coast Chile</td>
<td>Fryer et al. (1992)</td>
</tr>
<tr>
<td><em>Oncorhynchus tshawytscha</em> (Chinook salmon)</td>
<td>Southern Coast Chile</td>
<td>Cvitanich et al. (1991)</td>
</tr>
<tr>
<td><em>Oncorhynchus gorbushcha</em> (Pink salmon)</td>
<td>Pacific Coast Canada</td>
<td>Fryer et al. (1992)</td>
</tr>
<tr>
<td><em>Salmo salar</em> (Atlantic salmon)</td>
<td>Southern Coast Chile</td>
<td>Evelyn (1992)</td>
</tr>
<tr>
<td><em>Salmo salar</em> (Atlantic salmon)</td>
<td>Pacific Coast Canada</td>
<td>Evelyn (1992)</td>
</tr>
<tr>
<td><em>Tetrodon fahaka</em></td>
<td>Egypt</td>
<td>Mohamed (1939)</td>
</tr>
</tbody>
</table>

Modified from Fryer and Lannan 1994
microscopy only and the organism was not maintained. While the rainbow trout population did have mortalities, the cause was not determined and no attempt was made to ascertain the pathogenicity of the rickettsia-like organism. In 1986 another microscopic observation of a rickettsia-like organism was reported in a dragonet (Callionymus lyra) collected off the coast of Wales (Davies 1986). No indication of pathogenesis was reported and the organism was not cultured (Table 2).

**New salmonid disease signs in Chile**

During the 1980’s salmonid mariculture experienced a rapid expansion in southern Chile that centered around Chiloe Island near Puerto Montt. While salmonid fish are not native to South America there are numerous locations suitable for the netpen culture of salmon. Large numbers of salmonid eggs were imported from the Pacific North West of North America to maintain the growing industry. This importation of eggs meant that naive fish were repeatedly introduced into the region. These fish had no experience with the pathogens in Chile and would not have developed resistant mechanisms to the native pathogens. By the mid 1980’s the Chilean salmon industry experienced recurring epizootics with losses reaching 90% in coho salmon (Oncorhynchus kisutch) at some locations 6-12 weeks after transfer from fresh water to the marine net pens (Bravo and Campos 1989, Fryer et al. 1990, Cvitanich et al. 1990, 1991, Branson and Diaz-Munoz 1991, Fryer et al. 1992). The epizootics would peak in the fall and rise again the following spring (Cvitanich et al. 1991). Initially losses were only noted in coho even though other species of salmonids were being reared in the same area. Later losses also occurred in chinook salmon (Oncorhynchus tshawytscha), Atlantic salmon (Salmo salar) and rainbow trout reared in net pens in southern Chile (Garces et al. 1991).
Isolation of pathogen

Bravo and Compos (1989) observed an unidentified parasite in the blood and internal organs of infected fish by light and electron microscopy. Initial attempts made to culture the pathogen using more than 35 different types of artificial media met with no success.

That same year kidney tissue from infected moribund coho salmon was inoculated onto the cell line CHSE-214 from chinook salmon embryo cells. A previously unknown rickettsia-like bacterium was isolated in the CHSE-214 cells (Fryer et al. 1990). Supernant from the infected CHSE-214 culture was inoculated into a number of other cell lines; CSE-119 from coho salmon; CHH-1 (ATCC CRL 1680) from chum salmon (*Oncorhynchus keta*) (Lannan et al. 1984); RTG-2 (ATCC CCL 55) from rainbow trout (Wolf and Quimby, 1962); and four cell lines derived from warm water fish: EPC from the common carp (*Cyprinus carpio*) (Fijan et al. 1983); FHM (ATCC CCL 42) originating from the fathead minnow (*Pimephales promelas*) (Gravell and Malsberger 1965); BB (ATCC CCL 59) from the brown bullhead (*Ictalurus nebulosus*) (Wolf and Quimby, 1962); and BF-2 (ATCC CCL 91) derived from the bluegill (*Lepomis macrochirus*) (Wolf et al. 1966). CPE was observed in the CSE-119, CHH-1, RTG-2, EPC and FHM cell lines. Growth was best in the salmonid cell lines and no CPE was observed in the BF-2 or BB cell lines.

The suspected pathogenic bacterium grown in CHSE-214 cells was passed three times and then injected into previously unexposed coho salmon in both fresh and sea water aquaria to fulfill Koch’s postulates. All injected fish died from symptoms identical to the signs observed in coho salmon. The rickettsia-like organisms were reisolated from the artificially infected fish thereby fulfilling Koch’s postulates and establishing the organism isolated as the etiologic agent (Cvitanich, et al. 1990, Garces et al. 1991, Fryer et al. 1990).
Pathology

Fish infected with this organism often show skin lesions that range from raised areas of scales of less than 0.5 cm in diameter to hemorrhagic ulcers 2 cm in diameter (Branson and Neito Diaz-Munoz 1991). Many of the infected fish are dark and lethargic with some ascities, while others appear completely normal externally. Infected fish aggregate in the corners of the net pens near the surface, and are anorexic. Internally the fish organs will have a pale color (anemia), slightly enlarged spleens, grey swollen kidneys and livers with pale small lesions. Hematocrits fall to 2-20% compared to the normal 40-45%. The disease and death start to occur 6-12 weeks after the smolts are placed in saltwater net pens. Ring shaped lesions present in the liver were found to be diagnostic (Cvitanich, et al. 1990, Schafer et al. 1990, Garces et al. 1991, Lannan and Fryer 1993).

Histopathology

Three papers (Branson and Nieto Diaz-Munoz 1991, Cvitanich et al. 1990, Garces et al. 1991) have described the histopathology of piscirickettsiosis. There are extensive pathological changes in many tissues of infected fish. The gills show multifocal epithelial hyperplasia, with some severe consolidation of secondary lamellae and necrosis in the hyperplasia tissue. Basophilic granules are present in the gill lesions. Gut tissue is severely damaged, with necrosis and sloughing of the epithelial cells. Skin lesions have necrosis of the dermis and epidermis and some degeneration of the subdermal musculature.

There is invasion by inflammatory cells in the kidney which shows generalized necrosis of haemopoietic tissue with oedema with some fibrosis. Tubular cells are generally unaffected except in the most severe cases. Necrotic thrombi are present in some smaller blood vessels often with necrotic changes to the vessel endothelium.
Enlarged macrophages containing basophilic granules and tissue debris are common. The haemopoietic cells of the spleen displayed the same pathology as those in the kidney. Multi-focal to general necrosis with invasion by inflammatory cells, and an increase level of eosinophilic ground substance was present in the liver. Basophilic granules were present within macrophages, hepatocytes and free in the tissue. Macrophages in the liver are enlarged and contain tissue debris and basophilic granules. The basophilic granules described in the tissues are the pathogenic bacterium which have invaded the tissue.

**Infectivity**

In studies designed to test the infectivity of the rickettsia, groups of 40 juvenile coho salmon and groups of 30 juvenile Atlantic salmon were injected with ten-fold dilutions of spent medium from a LF-89-infected cell culture. Over the 42 days of the experiment, mortality ranged from 88 to 100%. LF-89 was reisolated from moribund fish in each group and while the lethal dose 50% (LD₅₀) was not determined there was a clear dose-response pattern. Mortality was the only gross sign of disease in Atlantic salmon, while in the coho salmon all the typical signs of the disease were present (Garces et al. 1991).

**Taxonomic characteristics**

The organism has been placed in the Rickettsaeae because it is an obligate intracellular parasite, it replicates in membrane-bound cytoplasmic vacuoles, displays a rippled gram negative cell wall morphology and electron-lucent spherical structures described for certain rickettsial species (Anderson et al. 1965). In addition, although pleomorphic it does not have the alternating forms characteristic of the *Chlamydia* and it does not react with monoclonal antibody against the chlamydial genus-specific LPS antigen. Host specificity and reactions to serological testing suggested that the bacterium
did not fit in any of the established rickettsial genera. The organism has a lower optimum growth temperature 15-18°C, than other characterized rickettsia (Fryer et al 1992).

The organism is seen either singly or in pairs within membrane-bound cytoplasmic vacuoles. Three layers enclose the organisms, a wavy outer membrane, a gram negative cell wall, and a closely opposed inner membrane. An area with DNA-like material was located near the center and an electron-dense region containing ribosome-like structures were concentrated near the cell membrane. Many organisms were observed apparently undergoing binary fission (Cvitanich et al. 1990, Garces et al. 1991).

**Development of diagnostic indirect fluorescent antibody test**

Lannan et al. (1991) developed an indirect fluorescent antibody test (IFAT) for detection of the rickettsia-like agent causing disease in Chilean salmon. Procedures included the purification of the organism from the CHSE-214 cell culture debris with a series of filtration and centrifugation steps. Polyclonal antiserum was produced against the purified bacterium in New Zealand white rabbits. The polyclonal antiserum was used in IFAT and was effective when applied to blood smears and tissue sections. Unlike the Giemsa stain the IFAT is specific and able to differentiate the pathogen from other bacteria. The antisera did not react with four other bacterial pathogens associated with disease in salmonids. There is no requirement for aseptic technique with the IFAT, it is simple to perform and the results are rapidly available. Culturing the organism is difficult as conditions are seldom ideal when collecting tissues from infected fish which increases the opportunity for contamination. Approximately 14-21 days are required before CPE is detected, unlike the IFAT where results are available in hours. The development of the IFAT provided a rapid, sensitive and simple technique for diagnosis of the disease.
Development of immunohistological diagnostic test

Alday-Sanz et al. (1994) developed an immunohistological diagnostic test for piscirickettsiosis as an alternative to the IFAT. The test used the same antisera described by Lannan et al. (1991) but instead of using fluorescent label secondary antibody they used an antirabbit-horseradish peroxidase labeled secondary antibody. Color was then developed using 1% hydrogen peroxidase in 1.5 mg 3,3-diaminobenzidine tetrahydrochloride in TBS. Tissue from Chile and Ireland displayed distinct dark-brown coloration in infected cells and organisms within cells in the kidney of experimentally infected fish. The color also appeared in the spleen, liver, heart, kidney, and pancreas of naturally infected fish from Ireland. No reaction was evident when the procedure was tried with fish tissues infected with Aeromonas salmonicida, Vibrio anguillarum or Renibacterium salmoninarum.

Neorickettsia helminthoeca

Salmon in the Pacific Northwest can be infected with the metacercaria of the trematode Nanophyetus salmincola. This trematode has a complex life cycle involving passage through a snail host to a fish host and then a mammal or a bird host. The trematode is itself infected with a rickettsial agent, Neorickettsia helminthoeca, which causes the disease referred to as salmon poisoning, in canines. Canines become infected by the ingestion of salmonid fish tissue that is parasitized with the trematode containing the rickettsia. In an aquatic system where N. helminthoeca is present all of the trematodes and 90+ % of the fish hosts are infected (A. Amandi, Oregon Department of Fisheries and Wildlife, personal communication). The rickettsia lives in the trematode and does not infect the fish (Milleman and Knapp 1970). Attempts to culture N. helminthoeca demonstrated it would grow in canine and mouse cell cultures but did not
grow in the fish cell cultures tested (Noonan 1973). The rickettsia Neorickettsia helminthoeca is a member of the tribe Ehrlichieae.

To determine if there are common antigens between the coho salmon rickettsia (P. salmonis) and the Ehrlichieae, sera from animals infected with Ehrlichial agents were tested with the salmonid rickettsia by IFAT. Both acute and recovered sera from dogs infected with N. helminthoeca and sera from dogs infected with Ehrlichia canis were tested. There was no reaction from the anti-Neorickettsia serum but there was a low-level reaction observed with the anti-E. canis serum suggesting the presence of a small number of shared antigens. (Lannan et al. 1991).

16S rDNA Taxonomy

The bacterium’s 16S rRNA gene was sequenced and its phylogenetic relationship mapped. The organism is a gamma proteobacterium distantly related to the genera Coxiella and Wolbachia. Based upon the unique 16S sequence, the organism’s temperature requirements, host range and serologic characteristics Fryer et al (1992) determined that the bacterium belonged to a novel genus and species and proposed the name Piscirickettsia salmonis which translated means “fish rickettsia from salmon” (Fryer et al. 1992). Piscirickettsia salmonis is taxonomically placed in the order Rickettsiales, the family Rickettsiaceae. It has been deposited with the American Type Culture Collection as Piscirickettsia salmonis gen nova, sp. nova ATCC VR 1361 and the type strain is LF-89.
Description of *Piscirickettsia salmonis*

*Piscirickettsia salmonis* is described as a pleomorphic predominately coccoid, gram negative, nonmotile bacterium, about 0.5 to 1.5 μm in diameter, and occurs as pairs of curved rods and occasionally in ring forms. Replication is by binary fission in membrane bound cytoplasmic vacuoles of host cells. In thin section, the bacterium displays a typical gram negative cell wall and the protoplasmic structure of a prokaryote. *Piscirickettsia salmonis* is Gimenez-negative (Gimenez 1964), stain dark blue with Giemsa and retains basic fuchsin when stained by Pinkerton’s method for rickettsia and chlamydia (Simmons and Gentzkow 1944). The organism does not react with monoclonal antibody made against the group-specific Chlamydial LPS antigen (Fryer et al. 1990). Four species of salmonids (coho salmon *O. kisutch*, chinook salmon *O. tshawytscha*, rainbow trout *O. mykiss*, and Atlantic salmon *S. salar*) have had epizootics with this organism and two (coho and Atlantic salmon) have been artificially infected (Fryer et al. 1992).

The organism can be cultured in fish cell lines but does not grow on artificial media. Cytopathic effect (CPE), in the form of clusters of rounded cells appearing ten days after inoculation, is present on the cell monolayer (Fryer et al. 1990). The optimum growth temperature is between 15 to 18°C, growth is slowed below 10°C and above 20°C. No growth occurs at 25°C or above. Titers of $10^6$ to $10^7$ TCID$_{50}$/ml are reached in the infected fish cell cultures. After one cycle of freeze thaw at -70°C the titer is reduced by 99% or more. The addition of 10% DMSO to the freezing medium is cyropreservative (Fryer et al 1990).

*Piscirickettsia salmonis* is sensitive to a broad range of antibiotics, but is resistant to penicillin. Penicillin resistance is useful in isolating the bacterium from infected tissue. Extracts from infected fish are frequently contaminated with non-
pathogenic bacteria and penicillin in the medium helps control these contaminates (Fryer personal communication).

Lannan and Fryer (1994) reported that infectivity of partially purified preparations of *P. salmonis* LF-89 dropped to levels below detection (10^2 TCID_{50}/ml) immediately after suspension fresh water. When the preparation of LF-89 was suspended in sea water the bacterium remained viable for at least 14 days, the limit of the experiment.

**Transmission**

No vectors are presently known to be involved in the transmission of *P. salmonis*. There are many types of parasitic crustacea in the marine environment that might serve as rickettsial vectors, the same role performed by arthropods in the terrestrial environment (Weiss and Moulder 1984). A disadvantage to intracellular parasitism is that the organisms are not adapted to survive drying as a result of prolonged exposure to the external environment. This would explain why terrestrial rickettsia need the protection of an arthropod vector to move from host to host. However, the rickettsial agent *Coxiella burnetti* forms a spore-like structure that makes it resistant to drying allowing survival without protection of a vector. It can be transmitted by aerosol dust particles to the respiratory tract of a host.

Two important functions of a vector in the terrestrial environment are to provide protection from desiccation and to facilitate movement from host to host. In the aquatic environment there is little danger of the bacterium drying and water-borne passage may provide a means of transmitting the rickettsia from infected to uninfected hosts. The 14 day survival of *P. salmonis* in sea water (Lannan and Fryer 1994) may be long enough for transmission of the bacterium to occur without the need of a vector.
Cvitanich et al (1991) reported transmission between coho salmon infected artificially with *P. salmonis* and sham-injected coho held in static freshwater and seawater aquaria. In another study transmission did not take place when uninfected coho salmon were suspended in a cage in a tank with flowing freshwater and infected coho (Garces et al. 1991). Results of these experiments suggest that under certain conditions horizontal transmission maybe possible. However the conditions between the experiments differed; flowing freshwater at a mean temperature of 8°C vs static fresh or sea water at 15°C, makes the results difficult to compare.

Vertical transmission has not been demonstrated for *P. salmonis*. If vertical transmission occurs it is believed to be a rare event as demonstrated by the very few reports of fresh water infections (Fryer and Lannan 1996).

**Reports in freshwater**

The first observation of *P. salmonis* in fresh water was reported by Bravo (1994) in rainbow trout. Gaggero et al. (1995) isolated *P. salmonis* from diseased coho, Atlantic salmon and rainbow trout from freshwater fish farms located on Chiloe Island, Chile. The fish displayed the typical signs of piscirickettsiosis and homogenized kidney tissue inoculated onto CHSE-214 cells produced typical *P. salmonis* CPE. The organism present in the cell cultures reacted positively to the *P. salmonis*-specific IFAT confirming identification. The authors speculated that the organism may have been transmitted vertically since the broodstock from which the infected fish were derived were survivors of a salt water epizootic. A fresh water origin of the disease agent was not ruled out however.
Additional *Piscirickettsia salmonis* isolations

A rickettsia-like organism infecting Atlantic, chinook, pink and coho salmon has been reported in British Columbia (Brocklebank et al. 1992). While the mortalities from this disease were much less severe than those associated with the Chilean epizootic, the clinical and internal morphologies were similar to that found in farmed salmonids in Chile. Pleomorphic basophilic intracellular inclusions that stained gram negative were observed. While this disease had not been reported earlier it had been recognized since the early 1970s in pink salmon (*Oncorhynchus gorbuscha*) in experimental tanks and in cultured coho and chinook salmon since the 1980’s.

In Norway (Olsen et al. 1993) and Ireland (Rodger and Drinan 1993) rickettsia-like organisms similar to the Chilean and Canadian organisms were associated with a piscirickettsiosis-like disease. Mortality caused by the disease was low. Using the polyclonal fluorescent antibody test these agents have been identified as *Piscirickettsia salmonis* (Fryer and Lannan 1996). As in Canada piscirickettsiosis was confined to a limited number of fish rearing facilities in Ireland and Norway and did not cause extensive mortalities.

Non-salmonid infecting rickettsia

In the early 1990’s tilapia in Taiwan were affected by a disease causing mass mortality in both fresh and salt water rearing facilities across the island (Chern and Choa 1994, Chen et al 1994). The disease started in a single farm in southern Taiwan and quickly spread to approximately 37 facilities around the island with six species of tilapia affected (Table 2). Infected fish were dark, listless, emaciated and frequently had skin lesions. The diseased fish swam to the surface and collected near the edge of the ponds. The gills were generally pale, the spleen enlarged and occasionally white nodules were
scattered throughout many organs. The pathology exhibited in the diseased tilapia was similar to that caused by *P. salmonis* in salmon.

Attempts to culture the disease agent on nine types of artificial media failed. The tilapia agent was able to replicate and cause CPE (rounding and clustering of infected cells) in EPC and TO2 (Chern and Choa 1994) and CHSE-214 (Chen et al 1994) cell cultures. The cultured bacterium was gram-negative, coccoid to pleomorphic, stained basophilic with hematoxylin and eosin (H&E), was an obligate intracellular parasite and did not react to a monoclonal antibody against the LPS antigen of Chlamydia. It is smaller than *P. salmonis* and measures 0.86 X 0.63 μm. They develop within a cytoplasmic vacuole, have abundant electron-dense ribosome-like particles and occasionally a rippled appearing cell wall. Medium from cell cultures exhibiting CPE was injected into naive fish. The experimental animals contracted the disease and the agent was reisolated from moribund fish thus fulfilling Koch's postulates. Mortalities were higher at 15°C than 30°C in the experimentally infected fish. In cohabitation studies apparent horizontal transmission did occur (Chern and Choa 1994).

Recently a rickettsia-like organism was observed microscopically in moribund specimens of the blue-eyed plecostomus (*Panaque suttoni*) a fresh water tropical fish imported into the United States from Colombia (Khoo et al 1995). In France a rickettsia-like organism was observed in the brain of moribund juvenile sea bass (*Dicentrarchus labrax*) exhibiting abnormal swimming behavior (Comps et al 1996). In Chile an organism similar to *P. salmonis* but smaller (0.2 X 0.8 μm vs 0.5 X 1.5 μm) was isolated in fish cell cultures from Atlantic salmon reared entirely in fresh water (Cvitanich et al. 1995). The organism did not react when tested with the anti-*P. salmonis* IFAT.
Molecular phylogeny

Advances in molecular biology and gene technology have brought the development of new diagnostic methods for the rapid identification and typing of microorganisms, especially pathogens. The detection and identification of bacterial species, subspecies, and biotypes by genetic methods is becoming more important in epidemiology and environmental studies. The basic processes used in molecular phylogenetic analysis between organisms are the cloning and sequencing of genes. The polymerase chain reaction (PCR) has become the standard method used to clone and sequence genes for microbial systematics. PCR has several advantages; it is simple, rapid and able to amplify products from small amounts of DNA or RNA (Mullis and Folloona 1987). The merger of PCR to molecular systematics has made projects involving large numbers of gene sequences possible (Giovannoni 1991). The result of this method is increased quantity and accuracy of the phylogenetic data used for comparisons.

The polymerase chain reaction

There are three steps to the polymerase chain reaction that are sequentially repeated or cycled: 1. melting: the two strands of the DNA molecule are separated at high temperature (94°C); 2. annealing: the primers are joined, or hybridized, to the DNA molecules at locations on opposite strands; 3. extension: the DNA polymerase enzyme attaches to the DNA strands at the primers and copies the gene by nucleotide addition. The cycle of melting-annealing-extension is repeated doubling the number of gene copies with each cycle. In 36 cycles a single copy of a gene will be replicated $10^{11}$ times. Nanogram and microgram quantities of DNA can be synthesized within hours. The copied genes can be directly sequenced, cloned into vectors, cut with restriction
enzymes, or hybridized to nucleic acid probes (Giovannoni 1991). Often only the presence of an amplified product is needed for diagnostic purposes.

Although there are many variables to be optimized when developing a PCR procedure the design of the primers is the most critical (Sharrocks 1994). The sequence of the primers determines the specificity of the PCR by attaching only at sites that are complementary to the primer sequence. This directs where the DNA will be replicated.

Sharrocks proposed a set of general rules for PCR primer design:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. unique oligonucleotide sequence</td>
<td></td>
</tr>
<tr>
<td>2. GC clamp at the 3' end</td>
<td>1-2 G/C nucleotides</td>
</tr>
<tr>
<td>3. No self-complementarity</td>
<td>≤3 contiguous bases</td>
</tr>
<tr>
<td>4. No complementarity to antisense counterpart</td>
<td>≤3 contiguous bases</td>
</tr>
<tr>
<td>5. Random base distribution and composition</td>
<td>45-55% G/C content</td>
</tr>
<tr>
<td>6. Primer length</td>
<td>18-25 bases</td>
</tr>
<tr>
<td>7. Match primer melting temperature</td>
<td></td>
</tr>
</tbody>
</table>

These are general rules and careful adherence to them will increase the probability of success but not guarantee it.

**Genomic classification**

The use of molecular methods for the classification of bacteria needs to be reconciled with the traditional techniques. Wayne et al. (1987) and Murry et al. (1990) agree that all previous classification and nomenclature should reflect genomic relationships as much as possible. It was felt that unless a species that is distinct genomically can also be separated from other species on the basis of phenotypic characters then they should not be designated a separate species. It is now considered essential that any classification, from the species to the Kingdom levels, be based on both phenotypic and genomic characters.
There are several advantages to basing bacterial classification on genomic relatedness:

a) A more unifying concept of a bacterial species is possible.
b) Classifications based on genomic relatedness tend to be stable, that is, they can accommodate new information.
c) Reliable identification schemes can be prepared once organisms have been classified on the bases of genomic relatedness.
d) Information can be obtained that is useful for understanding how various bacterial groups have evolved and how they can be classified according to their ancestral relationships (Johnson 1989).

According to Olsen et al (1986) there is no more fundamental and straightforward method to classify and relate organisms than by nucleic acid sequence comparisons. In order to be useful for phylogenetic comparisons a molecule must meet certain conditions; be universally present, conserved, and have high information content (Giovannoni et al. 1988, Lane 1985, Olsen et al. 1986).

**Ribosomal RNA genes**

The sequences that code for ribosomal RNA (rRNA) are some of the most conserved identified (Woese 1987). Consequently rRNA sequences can be used to determine taxonomic relationships between organisms. In part ribosomal sequences are useful for phylogeny because they evolve slowly so that little or no sequence divergence among strains belonging to one species. This implies that a small difference in gene sequence may indicate that organisms belong to different species (Anderson et al. 1991, Wen 1995). The reliability of the phylogenetic information derived from sequence comparison depends on the number of bases compared, and at least 1000 bases should be analyzed (Murray et al. 1990). The 16S rRNA gene can be easily and specifically
amplified from genomic DNA from small amounts of DNA using primers based on conserved regions identified from the comparison of previously derived sequences.

The 23S rRNA gene can also be sequenced and used for phylogenetic analysis. But for a number of reasons it is not as easily utilized. The molecule is larger in size, there are fewer conserved primer sites and generating full length PCR products of the 23S rRNA gene is often difficult (Lane 1991, Stothard 1994). However, the larger size of the 23S rRNA gene means it contains substantially more phylogenetic information than the 16S rRNA gene and the 23S rRNA gene has diverged 1.9 times faster in the species of Rickettsia studied and 1.67 times faster in the Rhodobacter (Stothard 1994).

The ribosomal 16S and 23S genes code for structural molecules. Subsequently the mutations of these molecules is governed by the need to maintain the structural integrity of the ribosome. Any mutations in the 16S or 23S ribosomal sequences changing the structure of the ribosome would have a high possibility of being lethal and would not be passed on to the next generation. Therefore the 16S and 23S genes may not contain enough phylogenetic information to detect the divergence within a species.

The intragenic spacer (ITS) region that lies between the 16S and 23S rRNA genes does not code for a structural molecule. This region can, however, code for a tRNA molecule, but not all ITS regions contain a tRNA sequence and if present that sequence is flanked by non-structure coding regions. The ITS region mutation rate is not governed in the same manner as the 16S and 23S genes and so evolutionary divergence between sequences of closely related organisms will take place at a faster rate than with the 16S and 23S rRNA genes. This allows the ITS region to be utilized in differentiating organisms that are members of the same species (East and Collins 1993, Frothingham and Wilson 1993, Gurtler 1993). PCR can also be used to amplify non-coding regions of the DNA such as the intragenic spacer region between the 16S rRNA gene and the 23S rRNA gene by using conserved primer sites within the flanking 16S and 23S genes.
Analysis of sequences

All 16S rRNAs have the same core structure (Gutell 1985). This structure contains areas that are hyper variable, semi-conserved, and conserved in 90% of the bacterial sequences known. The conserved regions provide the means to align newly produced sequences with the known sequences of bacteria previously assembled. To properly determine the relationships between sequences you must align homologous positions not just homologous sequences. Erroneous phylogeny and similarity analysis will result if there is an improper alignment. Sequences can be aligned in a linear manner and then transferred, either manually or with the program gRNAID (Giovannoni personal communication), onto a previously known secondary structure. The alignment of the sequence onto the secondary structure makes it easier to predict which bases are positionally homologous. The secondary structure alignment also permits consideration of base-pairing across the helices which should be preserved by compensatory base changes on the opposite position.

If the alignment of a region is ambiguous the region should be left out of phylogenetic analysis. However regions that are ambiguous between unrelated organisms can still be useful for the design of species/group specific primers/probes and for the phylogenetic comparisons of closely related organisms.

Because the length of 16S rRNA sequences can vary between organisms there will necessarily be gaps inserted into some sequences in order to achieve homology between the positions. These gaps are usually omitted from comparisons because the phylogenetic meaning of the gaps are not obvious. If, for instance, one sequence is missing a region of 10 bases found in another sequence was the gap cause by one event or ten separate events? If there is no means of determining what the past event(s) were the region is usually removed from the analysis.
Methods to infer phylogenetic relationships

Several methods for inferring phylogenetic relations from sequences are distance matrix, parsimony, and maximum likelihood. In a distance matrix analysis the phylogenetic tree is constructed from a matrix of pairwise comparisons generated for each pair of sequences in the group. The lengths of the lines connecting the sequences are adjusted to fit the original similarity computations most closely optimizing the tree. The sum of the adjusted lines connecting any two organisms is referred to as the evolutionary distance and is based on the number of differences between the sequences.

Finding the minimum number of mutations required to produce a phylogenetic tree is the goal of parsimony analysis. The most parsimonious tree is the one that requires the fewest number of mutations to produce. Maximum likelihood evaluates the net likelihood that a given tree will yield the sequences. This method considers the probability of each nucleotide occurring at each node in the tree as a function of both branch lengths and branching order.

Determining a tree that exactly fits the data is usually impossible, therefore a best fit tree is determined. Often the best fit tree is only slightly better than the other trees considered leading to a need for a means to evaluate the significance of the best fit tree. One method to evaluate the significance of a tree is bootstrap analysis.

In a bootstrap analysis positions are sampled at random, recorded and then replaced. Positions may be sampled repeatedly or not at all. Repeated sampling and analysis of the data results in variation among the estimates indicating the size of error involved in making the tree. A particular relationship in a tree is considered significant if it is present in 95% of the trees. Combining several methods of inferring trees in the analysis increases the confidence in a particular tree.
Problems associated with phylogenetic analysis

Any analysis is only as good as the original data. Taq polymerase has an error rate of 0.1-0.3%, which can lead to 2-3 nucleotide errors in a 16S rDNA sequence after 35 cycles of PCR (Innis et al. 1988, Dunning et al. 1988). Since the similarities observed between microbial species is much larger than the highest rates of error reported it is unlikely that Taq polymerase errors would result in mistakes in phylogenetic inference (Giovannoni 1991). Eckert and Kunkel (1991) recommended several parameters to increase Taq polymerase fidelity:

a. keep the number of cycles to the minimum required,
b. reaction conditions - keep the four deoxynucleotide triphosphate precursors at equal concentrations and at the lowest concentration necessary to support the desired amount of DNA synthesis, keep reaction times as short as possible, keep the MgCl₂ concentration as low as possible to support the amount of desired synthesis and the MgCl₂ concentration should not be in large excess over the dNTP concentration, minimize reaction time at high temperatures,
c. verify the DNA sequence to ensure that the original sequence was maintained.

Contamination is another source of error. The polymerase chain reaction is sensitive enough to detect a single copy of a gene. Any contamination of reagents, plasticware or pipetting devices could easily result in the sequencing of non target bacteria genes. According to Yap et al. (1994) there are several sources and types of contamination (given in decreasing order of importance):

- carry-over products from previous PCR, by far the most significant, due to the amount of amplification (10⁶ to 10¹²-fold)
- vectors containing cloned DNA, especially where used as probes for hybridization experiments
- positive controls (cloned DNA, highly infected tissue)
• cross-contamination between samples
• contamination from other sources during collection of samples
• contamination of Taq polymerase with bacterial DNA, an important consideration when amplifying conserved ribosomal RNA sequences.

Negative controls, replicate samples and sequence analysis may be necessary to verify the PCR results, especially for diagnostic purposes.

Because of the sensitivity of PCR the preparation of the reaction and reagents must be carried out with extreme cleanliness to prevent contamination. The physical separation of pre- and post-PCR reaction, including reagents, equipment (pipettes, gloves, microcentrifuges, pens, iceboxes, etc.) can reduce the risk or amount of contamination. Also the addition of negative no target DNA controls with every set of PCR reactions will reveal and monitor possible contamination (Kawasaki, E.S. 1990, M. H. Mahbubani and A. K. Bei 1994).

In summary

It is now clear that rickettsia-like bacteria are an important group of fish pathogens. These agents affect a number of fish species (Table 2) in both freshwater and marine environments and are capable of causing severe epizootics. Further research will likely increase the number of disease agents and hosts species known. These experiments will undoubtedly use molecular phylogeny to clarify the relationships between the rickettsial species now known and those that may be discovered in the future.

The objectives of this study were:

1. to develop a highly specific, sensitive assay which can differentiate *P. salmonis* from other rickettsia-like bacteria,
2. to confirm the placement of *P. salmonis* within the gamma proteobacteria,

3. to develop and understanding of the phylogenetic relationship between

   the available Piscirickettsia isolates by sequencing and analysis the

   16S, ITS and 23S ribosomal genes/regions.

The following chapters describes the efforts to achieve these objectives.
References


CHAPTER 2

Development of Polymerase Chain Reaction assays for Detection, Identification, and Differentiation of *Piscirickettsia salmonis*

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Abstract

A nested polymerase chain reaction (PCR) was developed to detect genomic DNA of *Piscirickettsia salmonis*, the causative agent of an epizootic disease in salmonids. The nested PCR assay, which used general bacterial 16S rDNA primers in the first amplification reaction, and *P. salmonis* -specific primers in a second reaction, allowed detection of less than one *P. salmonis* tissue culture infectious dose 50 (TCID$_{50}$). Using the *P. salmonis* -specific primers in a single PCR reaction allowed the detection of 60 TCID$_{50}$. The specificity of the PCR was assessed with a panel of four salmonid and 15 bacterial genomic DNA preparations. Amplification products were produced only with *P. salmonis* DNA. Restriction fragment length polymorphism (RFLP) analysis of the complete 16S gene PCR products demonstrated that one isolate, EM-90, was unique. Two additional primers were developed and used in PCR assays that differentiated EM-90 from the four other *P. salmonis* isolates tested.
Introduction

Salmonid mariculture in Chile has undergone rapid growth and development during the last two decades. In the late 1980's a number of mariculture facilities began experiencing extensive losses to an infectious disease of unknown etiology. Signs of the disease became evident 6-8 weeks after fish were moved to salt water netpens. In 1989 mortality up to 90% was reported in some coho salmon (Oncorhynchus kisutch) facilities in the Puerto Montt area of southern Chile (Bravo & Campos 1989). The etiologic bacterium of this disease was isolated (Fryer et al. 1990) and characterized (Fryer et al. 1992). The causative agent was designated Piscirickettsia salmonis (gen. nova, sp. nova), an intracellular parasite and the first such rickettsial-like agent isolated from fish. The disease was designated piscirickettsiosis. Since P. salmonis first appearance in Chile, Piscirickettsia or Piscirickettsia-like organisms have been reported from salmonids in Canada (Evelyn 1992), Norway (Olsen et al. 1993) and Ireland (Rodger & Drinan 1993).

Presumptive diagnoses of piscirickettsiosis are made by observing gross signs of disease, histological staining of smears or tissue sections, electron microscopy, isolation in fish cell cultures and consideration of the previous disease history in the rearing facility. Currently, confirmation of P. salmonis is accomplished by indirect fluorescent antibody test (IFAT; Lannan et al 1991). While the IFAT is invaluable, it uses polyclonal antibodies that do not differentiate between isolates of P. salmonis. Electron microscopy is time consuming, expensive and organisms of similar morphology are difficult to differentiate. Piscirickettsia salmonis is sensitive to many of the antibiotics commonly used in cell culture media making isolation the agent from tissues of infected fish difficult and time consuming due to contamination. The problems associated with
the above diagnostic methods demonstrate the need for a highly specific, sensitive and rapid assay for diagnosis of piscirickettsiosis.

In addition to the isolates from salmonids rickettsia-like organisms have recently been isolated or reported from several species of tilapia (*Oreochromis* and *Tilapia* sp.) in Taiwan (Chern & Chao 1994), and Japan (Wada et al. 1995). Other rickettsia-like organisms have been reported in the blue-eyed plecostomus (*Panaque suttoni*) from Columbia, SA (Khoo, Dennis & Lewbart 1995) and juvenile sea-bass (*Dicentrarchus labrax*) in France (Comps, 1996). The discovery of these agents demonstrates the need for a highly specific, sensitive assay which can differentiate *P. salmonis* from other rickettsia-like agents.

This report describes the development of a nested PCR for detecting *P. salmonis* genomic DNA from cultured fish cells and the tissues of infected fish. This assay proved to be both specific and sensitive. Two additional PCR primers were developed that differentiated between *P. salmonis* isolate EM-90 and the other four isolates of *P. salmonis* examined in this study.

**Material and Methods**

**Bacterial strains and culture.** The species examined, their strain designations and isolation sources are noted (Table 2.1). The bacterium *Neorickettsia helminthoeca* was obtained along with the metacercariae of its host *Nanophyetus salmincola* from kidney tissue of adult steelhead trout (*Oncorhynchus mykiss*) (Gebhard et al. 1966). No attempt was made to isolate the bacterium from the metacercariae.

*Piscirickettsia* isolates were grown at 15°C in chinook salmon embryo cell line (CHSE 214, Lannan et al. 1984) in antibiotic-free Eagle’s minimum essential medium (MEM) with Earle’s salts (Sigma Chemical Co., St. Louis, Missouri) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) until lysis of the cell
Table 2.1 Bacteria examined in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain designation</th>
<th>Original isolation source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Piscirickettsia salmonis</em></td>
<td>LF-89^T ATCC</td>
<td>coho salmon (<em>Oncorhynchus kisutch</em>)</td>
</tr>
<tr>
<td></td>
<td>VR 1361</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EM-90</td>
<td>Atlantic salmon (<em>Salmo salar</em>)</td>
</tr>
<tr>
<td></td>
<td>SLGO-94</td>
<td>rainbow trout (<em>Oncorhynchus mykiss</em>)</td>
</tr>
<tr>
<td></td>
<td>ATL-4-91</td>
<td>Atlantic salmon (<em>S. salar</em>)</td>
</tr>
<tr>
<td></td>
<td>NOR-92</td>
<td>Atlantic salmon (<em>S. salar</em>)</td>
</tr>
<tr>
<td></td>
<td>LS174</td>
<td>chinook salmon (<em>Oncorhynchus tshawytscha</em>)</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-13-78</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>ATCC 15948</td>
<td>human</td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td>ATCC 33202</td>
<td>channel catfish (<em>Ictalurus punctatus</em>)</td>
</tr>
<tr>
<td><em>Edwardsiella ictaluri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella piscicida</em></td>
<td>7-1-77</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Carnobacterium piscicola</em></td>
<td>LBK1-70</td>
<td>rainbow trout (<em>O. mykiss</em>)</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>ATCC 19435</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>ATCC 14715</td>
<td>coho salmon (<em>O. kisutch</em>)</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em></td>
<td>ATCC 33685</td>
<td>Atlantic salmon (<em>S. salar</em>)</td>
</tr>
<tr>
<td><em>Yersinia ruckerii</em></td>
<td>HI-70</td>
<td>rainbow trout (<em>O. mykiss</em>)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 35548</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Renibacterium salmoninarum</em></td>
<td>D6</td>
<td>coho salmon (<em>O. kisutch</em>)</td>
</tr>
<tr>
<td><em>Flexibacter psychrophilus</em></td>
<td>SR1-77</td>
<td>coho salmon (<em>O. kisutch</em>)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 33430</td>
<td>human</td>
</tr>
<tr>
<td><em>Lactococcus piscicum</em></td>
<td>7-77</td>
<td>coho salmon (<em>O. kisutch</em>)</td>
</tr>
</tbody>
</table>

^a All non *P. salmonis* species were provided by the Oregon State University Department of Microbiology Fish Pathogen Culture Collection

sheet was near completion (approximately 14 d).

**DNA isolation.** *Piscirickettsia salmonis* DNA was isolated using DNA-STAT 60 (Tel-Tex, Inc., Friendswood, TX) following the manufacturer's protocol. Briefly, 7 ml of DNA-STAT 60 was added to 25 cm² cultures near complete lysis and the cells and supernatant passed through a pipette five times to disrupt the remaining intact CHSE 214 cells and lyse the bacterial cells. Next, 1.5 ml of chloroform was added, the mixture was shaken for 30 sec and then centrifuged at 16000 g for 15 min at 4°C in a microcentrifuge. The upper layer was transferred to a second tube and DNA precipitated with 500 µl of 100% isopropanol. After 10 min at room temperature the DNA was pelleted by centrifugation at 16000 g at 4°C for 10 min in a microcentrifuge. The supernatant was
then removed, the pellet vacuum dried for 10 min and resuspended in 200 μl TE (100 mM Tris pH 8.0, 10 mM EDTA).

The Norwegian isolate, NOR-92, was contaminated with a mycoplasma. To overcome this, amplification was performed on a plasmid (pCRII®, Invitrogen, San Diego, CA. USA) that contained the NOR-92 16S gene as an insert. Due to limited DNA availability isolates LF-89 and EM-90 were also amplified from plasmid inserts.

Chromosomal DNA from each of the non- \textit{P. salmonis} bacterial strains was prepared as above using DNA-STAT 60 with the following modifications: five ml Lennix L broth cultures were incubated, with shaking, at either 16 or 37°C; the bacterial cells were pelleted, resuspended in 1.5 ml DNA-STAT 60; 300 μl of chloroform was added. DNA from rainbow trout (\textit{Oncorhynchus mykiss}), chinook (\textit{Oncorhynchus tshawytscha}), coho, and Atlantic salmon (\textit{Salmo salar}) were provided by J. Leong (Dept. of Microbiology, Oregon State University, Corvallis, OR.). As a confirmation of the quality of the bacterial DNA, 16S genes were amplified prior to performing the nested PCR (data not shown).

Infected coho salmon kidney and spleen tissues fixed in ethanol were provided by P. Smith (Veterinary Sciences, University of Chile, Santiago, Chile) from culture facility in Chile that was experiencing an epizootic of piscirickettsiosis. \textit{Piscirickettsia salmonis} infection was confirmed by observation and histological staining for the presence of rickettsia-like organisms in host cells. Healthy coho salmon tissues were obtained from the Oregon Department of Fish and Wildlife, Fall Creek Hatchery served as negative controls, since there is no history of \textit{P. salmonis} in Oregon.

Fish infected with \textit{Nanophyetus salmincola}, the host of \textit{Neorickettsia helminthoea}, were obtained from a location where the salmon poisoning syndrome is endemic (Gebhard et al. 1966). The genomic DNA of \textit{N. helminthoea}, its host \textit{N. salmincola} and the \textit{N. salmincola} fish host, steelhead trout, were isolated together from
kidney tissue using DNA-STAT 60 as previously described. DNA was also isolated from infected tissue by concentration of the *N. salmincola* metacercariae as follows: one gram of infected steelhead kidney tissue was homogenized in 50 ml PBS, the metacercariae were allowed to settle for 4 hr (Gebhard et al. 1966), the supernatant removed and the DNA extracted with DNA-STAT 60. The number of metacercariae per gram of host tissue was determined by counting in a haemocytometer. Staining with Giemsa was used to establish the presence of rickettsia-like bodies in the metacercaria.

Salmonid kidney or spleen tissues (100 mg), negative control or *P. salmonis* infected, were digested for 3 hr at 65°C in 250 μl of lysis buffer (50 mM KCl, 10 mM Tris pH 7.8, 2.5 mM MgCl₂, 0.1% gelatin, 0.45% NP40, 0.45% Tween 20 with 1 mg/ml proteinase K added just before use), followed by boiling for 10 min, quenching on ice and centrifugation to remove cell debris. Five microliters of supernatant was used for PCR.

**Oligonucleotide design and synthesis.** The *Piscirickettsia* specific primers (Table 2.2) were designed by comparing the published sequence (Fryer et al. 1992, EMBL accession no. X60783) and 20 other published bacterial sequences using the Genetic Data Environment (GDE version 2.2) software package. The proposed primers were further tested for specificity using a commercially available software package (Amplify; University of Wisconsin, Madison, WI) and the Ribosomal Data Base project program Check_Probe (Maidak et al. 1994). From 16S rDNA sequence data of five *P. salmonis* isolates (GenBank accession numbers: LF-89⁹ U36941, EM-90 U36940, ATL-4-91 U36915, NOR-92 U36942, SLGO U55015) two additional primers, PS3AS and EM90AS were developed (Table 2.2). The design of the universal primers EubA and EubB has been described (Giovannoni, 1991). The oligonucleotides were synthesized at the Oregon State University Center for Gene Research and Biotechnology Central Services facility.
Table 2.2 Sequences and specificity of primers utilized for the identification or differentiation of *Piscirickettsia salmonis* strains

<table>
<thead>
<tr>
<th>Primer/Location&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5'-3')</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EubB (27F)</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>eubacterial</td>
</tr>
<tr>
<td>PS2S (223F)</td>
<td>CTAGGAGATGAGCCCGCGTTG</td>
<td><em>P. salmonis</em> (all strains)</td>
</tr>
<tr>
<td>PS2AS (690R)</td>
<td>GCTACACCTGCAGAAACCCTT</td>
<td><em>P. salmonis</em> (all strains)</td>
</tr>
<tr>
<td>PS3AS (1032R)</td>
<td>TCCCGAAGGCACTTCCCGCATCTC</td>
<td><em>P. salmonis</em> (LF-89 type strain)</td>
</tr>
<tr>
<td>EM90AS (1032R)</td>
<td>TCCCGAAGGCACTCATAATATCTCTATC</td>
<td><em>P. salmonis</em> (EM-90)</td>
</tr>
<tr>
<td>EubA (1518R)</td>
<td>AAGGAGGTGATCCANCCRC</td>
<td>eubacterial</td>
</tr>
</tbody>
</table>

<sup>a</sup> F, forward; R, reverse; numbering corresponds to *Escherichia coli* 16S gene.
45 µl of reaction mixture consisting of 1X PCR buffer (Promega, Madison, WI; 10 mM Tris HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X 100), 200 µM each of dATP, dCTP, dTTP, dGTP, 1 µM EubA primer, 1 µM EubB primer, 2.5 U Taq DNA polymerase (Promega) and covered with 50 µl of mineral oil. The mixture was denatured at 94°C for 2 min and amplification was performed with 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The tubes were then held at 4°C.

The second amplification was performed by adding 3 µl of the first PCR products to the reaction mixture containing: 2 µM each of PS2S, PS2AS primers instead of the EubA and EubB primers. Reaction conditions were: 35 cycles of 94°C for 1 min, 61°C for 2 min, and 72°C for 3 min. Amplifications with the primer sets PS2S-PS3AS or PS2S-EM90AS were performed by adding 2 µM of each primer to the above reaction mixture. The reaction conditions were modified as follows: 35 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min. Amplifications from plasmid DNA were achieved with the same reaction conditions as the genomic DNA. Aliquots (10 µl) of the PCR reaction mixture were electrophoresed in 2% agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50 ml ethidium bromide and photographed under UV transillumination. Aliquots (10 µl) of the PCR reaction products were digested with EcoRI or PstI (Promega) following the manufacturers instructions and visualized as described.

**PCR sensitivity.** To define the sensitivity of the PCR assay *P. salmonis* DNA was serially diluted and 6 x 10⁷ tissue culture infectious dose 50 (TCID₅₀) to 0.6 TCID₅₀ of DNA was added to the reaction mixture. The *P. salmonis* cells were stained with Giemsa and counted with a haemocytometer for comparison with the TCID₅₀ counts.
Results

Development of the PCR

Amplification of *P. salmonis* genomic DNA with primers PS2S and PS2AS resulted in a product with the predicted 476-bp length (Fig. 2.1A). Verification that the product was from the 16S gene of *P. salmonis* was determined by sequencing the LF-89\(^T\) PCR product and aligning it to the *P. salmonis* LF-89\(^T\) sequence (Fryer et al 1992, EMBL accession no. X60783). Amplification using PS2S and either PS3AS or EM90AS resulted in the appearance of the predicted 816-bp PCR products (Fig. 2.2).

Detection Threshold of the PCR

Amplification of 10-fold serial dilution’s of *P. salmonis* DNA indicated that less than one TCID\(_{50}\) could be detected in a 2% agarose gel with the nested PCR (Fig. 2.3A). However, for reasons that remain unclear, amplification of the 467-bp product from 6 \(\times 10^4\) TCID\(_{50}\) was consistently less than that obtained from 6 \(\times 10^3\) TCID\(_{50}\) or less (Fig. 2.3A). When using DNA isolated from infected fish cell cultures or purified *P. salmonis* (Lannan et al 1991) a single amplification step with the PS2S and PS2AS primers was sufficient to detect the *P. salmonis* 16S gene at 60 TCID\(_{50}\) (Fig. 2.3B).

---

Fig. 2.2 Differentiation of *Piscirickettsia salmonis* isolates by PCR. Both assays produce a 816-bp product. Isolates: Lf - LF-89\(^T\), Em - EM-90, At - ATL-4-91, No - NOR-92, SI - SLGO-94. PS3AS indicates amplification using the primer pair PS2S-PS3AS. EM90AS indicates amplification using the primer pair PS2S-EM90AS. STD - pGEM DNA markers. Neg - no DNA negative control.
Performing two sequential amplifications using the species-specific primers PS2S and PS2AS produced variable yield and was not consistently observed in agarose gels (data not shown).

**Restriction Fragment Length Polymorphism (RFLP)**

All five isolates produced a product of approximately 1540 bp when amplified with the universal primers EubA and EubB. When these products were cut with *EcoRI* LF-89, ATL-4-91, NOR-92 and SLGO-94 all produced two bands (994 bp and 546 bp). EM-90 was not cut by the restriction endonuclease *EcoRI*. The restriction endonuclease *PstI* produced three bands (541 bp, 519 bp, and 480 bp) from the 16S PCR products of isolates LF-89, ATL-4-91, NOR-92 and SLGO-94 and two bands (1058 bp and 482 bp).

---

**Fig. 2.3** Detection thresholds of the nested (A)(EubA-EubB primer pair first round, PS2S-PS2AS primer pair second round) and single round (B)(PS2S-PS2AS primer pair) PCRs. Each lane represents the equivalent DNA, in each 50 µl PCR, from the following number of tissue culture infectious dose 50 (TCID_{50}): (1) 6 x 10^7 TCID_{50}, (2) 6 x 10^6 TCID_{50}, (3) 6 x 10^5 TCID_{50}, (4) 6 x 10^4 TCID_{50}, (5) 6 x 10^3 TCID_{50}, (6) 600 TCID_{50}, (7) 60 TCID_{50}, (8) 6 TCID_{50}, (9) 0.6 TCID_{50}. STD - pGEM DNA markers (Promega). Neg - no DNA negative control.
from EM-90 (Fig. 2.4). Using RFLP demonstrated that EM-90 was distinguishable from the other 4 isolates of *P. salmonis*.

![PCR-RFLP patterns of 16S rDNA from Piscirickettsia salmonis isolates digested with EcoRI or PSTI. Lf - LF-89T, Em - EM-90, At - ATL-4-91, No - NOR-92, Sl - SLGO-94. The 16S rDNA genes for isolates LF-89, EM-90 and NOR-92 were amplified from inserts in the pCRII plasmid. STD - pGEM DNA markers. Neg - no DNA negative control.](image)

**Specificity of the PCR**

When using the primer combination of PS2S and PS2AS with the 18 bacterial and four salmon DNAs tested, the PCR produced a product only with the 5 isolates of *P. salmonis* (Fig. 2.1A,B,C). PCR performed with the *P. salmonis*-specific primers did not produce products when *Neorickettsia/Nanophyetus* DNA from infected kidney tissues or concentrated metacercaria were used as templates (Fig. 2.5). The nested assay distinguished between *P. salmonis* when compared with other bacteria including 12 species of fish pathogens, and no products were produced from the DNA from the 4 species of salmon examined (Fig. 2.1A,B,C). When using PS2S and PS3AS as primers a product was produced with all isolates of *P. salmonis* examined except EM-90. Amplification using PS2S and EM90AS produced a product only with isolate EM-90 (Fig. 2.2).
Fig. 2.5 Specificity of *Piscirickettsia salmonis* PCR using DNA from adult steelhead trout (*Oncorhynchus mykiss*) kidney tissue infected with *Nanophyetus salmonicola* metacercariae the host for *Neorickettsia helmintheoca*. Lanes marked K use infected kidney tissue. Lanes marked M use DNA from concentrated metacercaria. The metacercaria averaged $6.8 \times 10^5$ per gram of kidney tissue. STD - pGEM DNA markers (Promega). Lf - LF-89T positive control. Neg - no DNA negative control.

Fig. 2.6 Agarous gel electrophoresis of *Piscirickettsia salmonis* specific nested PCR using infected fish tissue. Lns 1-3, and 6-10 were *P. salmonis* positive fish tissue samples. Lns 4, 5, 11, 12 were *P. salmonis* negative fish tissue samples. 467-bp indicates the expected PCR product. STD - pGEM DNA markers. Neg - no DNA negative control.

**Amplification from infected tissue**

With a single amplification of the infected fish tissue, using the *P. salmonis*-specific primers, 50% of the reactions produced the expected product (gel not shown). All tissue infected with *P. salmonis* revealed the expected 467-bp product when the nested PCR amplification was performed (Fig. 2.6). Close examination of the gel was required to observe the bands in lanes 4 and 5 and they could easily have been
improperly identified as negative. The 467-bp band was not observed in any of the non-infected tissue or the preparations containing no DNA.

Discussion

The polymerase chain reaction has shown potential for improving the diagnosis of infectious diseases caused by fastidious or slowly growing microorganisms (Eisenstein 1990). PCR assays have been used for the detection of a number of other rickettsiae; *Coxiella burnetii* (Mallavia et al. 1990), *Rickettsia rickettsii* (Tzianabos et al. 1989), and *Rickettsia typhi* (Carl et al. 1990).

Here we have described the development of PCR assays for the detection of *P. salmonis* DNA in fish cell cultures and tissues of infected fish. Amplification using the primer pair PS2S-PS2AS can distinguish *P. salmonis* from its fish host and other bacteria. The PCR assays using PS2S and either PS3AS and EM9OAS distinguished between EM-90 and the other isolates of *P. salmonis*. The nested PCR was more sensitive and reliable than two sequential amplification using the *P. salmonis*-specific primers.

The nested PCR approach increased the sensitivity of the assay from 60 TCID\(_{50}\) to less than one TCID\(_{50}\) which should allow detection early in the infection when the quantity of bacteria in the tissue is low. The variation in the amount of product produced with the nested PCR is due to the variation in the quantity of *P. salmonis* in the infected tissue. Therefore, replicate amplifications are recommended to confirm negative results. One TCID\(_{50}\) may represent infection with more than one bacterial cell leading to an underestimate of the number of *P. salmonis* cells present. Therefore, the detection of less than one TCID\(_{50}\) does not mean that less than one *P. salmonis* bacterium can be detected; instead it probably reflects the affinity of *P. salmonis* to host cell membranes, which leads to clumping. DNA from non-viable *P. salmonis* bacterial cells will be
present in the preparation further complicating the quantification of the number of infectious bacterial cells represented by the DNA preparation. We attempted to overcome this by measuring the TCID$_{50}$, staining the cells with Giemsa and counting by haemocytometer. The numbers agreed within 15% between the two methods.

Currently, preliminary diagnosis of *P. salmonis* relies on the presence of gross signs of disease and Giemsa staining of infected fish tissue, with confirmation by IFAT (Lannan et al 1991). However, *P. salmonis* infections which are in the early stages when few bacterial cells are present or from fish which are asymptomatic carriers could go undetected by these test. The sensitivity of the nested PCR assay will allow identification of the infectious agent below the detection limits of microscopic examination. Using the nested PCR will also allow the tissue distribution of the bacterium to be studied early in the infection prior to the onset of disease signs, thus resulting in a better understanding of the transmission and pathogenesis of *P. salmonis*.

The entire process of tissue preparation, DNA isolation, amplification and visualization of the PCR products can be accomplished in 1-2 days. The PCR/RFLP procedures take considerable less time than culturing *Piscirickettsial* agents and can differentiate between strains which cannot be distinguished by polyclonal IFAT.

A number of rickettsia-like bacteria have been recently observed in a variety fish hosts (Chern & Chao 1994, Wada et al. 1995, Khoo, Dennis & Lewbart 1995, Comps, 1996). The discovery of these agents makes it likely that *P. salmonis* is only the first aquatic rickettsia-like bacterium to be characterized. Using the nested PCR assay described here will allow *P. salmonis* to be rapidly distinguished from the other agents and help in understanding the classification of this emerging group of microorganisms.

The PCR methods presented here are rapid, sensitive and specific tests for *P. salmonis* and will be useful in defining the biology, taxonomy and ecological characteristics of this agent. The sensitivity and specificity of these PCR procedures will
be useful for defining the mode(s) of transmission, the natural host(s), the reservoir(s),
and the geographical distribution of *P. salmonis* and other related rickettsia-like
organisms.

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Traxler (Pacific Biological Station, Nanaimo, B.C.) and H. P. Melby (National
Veterinary Institute, Oslo, Norway) for providing *P. salmonis* isolates EM-90, SLGO-
94, ATL-4-92 and NOR-91 respectively. We thank Dr. Tony Amandi and Craig Banner
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CHAPTER 3

Phylogenetic analysis of *Piscirickettsia salmonis* isolates
by 16S ribosomal DNA sequencing

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Abstract: Piscirickettsia salmonis is the etiologic agent of piscirickettsiosis, a systemic disease of salmonids. Variation in salmonid mortality between isolates from different geographic locations have been observed. To assess the genetic variability in this species or species complex, the 16S ribosomal DNA of five isolates from three different host and three geographic origins were amplified using the polymerase chain reaction. The PCR products were sequenced and compared with other previously published bacterial small subunit rRNA sequences. The results of this analysis confirm that P. salmonis is a member of the gamma subgroup of the proteobacteria and show that the five isolates form a tight monophyletic cluster. Within P. salmonis three groups of sequences were identified. Two of the isolates, LF-89 and SLGO-94 are highly related (>99.7% similarity) and have similar geographic but different host species origins. Another group, composed of isolates ATL-4-91 and NOR-92, were both isolated from Atlantic salmon but from different geographic areas in the northern hemisphere and show a sequence similarity value of 99.7%. The sequence of isolate EM-90 was unique with similarities ranging from 98.5-98.9% when compared to the other four isolates.
Introduction

*Piscirickettsia salmonis* is an obligate intracellular bacterium and the first rickettsia-like organism isolated and characterized from fish. The bacterium is the causative agent of piscirickettsiosis, a systemic disease of salmonids that has been reported in Chile (Fryer and Lannan 1994, Fryer et al. 1990, 1992), Norway (Olsen et al. 1993), Ireland (Rodger et al. 1993) and the Pacific coast of Canada (Evelyn 1992).

The mortality associated with piscirickettsiosis has varied with geographic area and host species. Mortalities between 30-90% in netpen reared salmon have been recorded in Chile (Bravo and Compos 1989), while those from the Pacific coast of Canada, Norway and Ireland vary between 0.6-15% (Evelyn 1992, Olsen et al. 1993, Rodger et al. 1993). Differences in mortality may be due to variation in virulence factors intrinsic to the bacterium, host species, environmental factors or the mariculture practices that differ between the areas. To date no controlled studies have been reported comparing the characteristics of these isolates.

Isolation of *P. salmonis* has proven difficult, no doubt in part due to the need to use antibiotic free media with the tissue culture. This has helped to limit the number of isolates available for examination and comparison. At present the molecular phylogenetic placement of *P. salmonis* has been based on the 16S rRNA gene sequence of a single isolate, LF-89 the type strain (Fryer et al. 1992).

The nucleotide sequence that codes for the small ribosomal subunit (16S rRNA) is universally present in organisms, easy to clone and sequence, and one of the most conserved known (Woese 1987). These characteristics of the 16S rRNA gene make it a useful tool in determining the evolutionary relationships between organisms. Comparisons of 16S rDNA sequences have become routine methodology for study of microbial phylogeny and have been used previously to classify bacteria in the *Rickettsiaceae* (Anderson et al. 1991, Weisburg et al. 1989, Wen et al. 1995) and other
microorganisms difficult to culture by conventional means (Berchtold et al. 1994, Giovannoni 1991).

To investigate the hypothesis that genetic differences might be found among isolates from different geographical locations, the sequences of approximately 95% of the 16S rDNA genes from five isolates of *P. salmonis* derived from various host and geographic origins were determined. Three groups of isolates could be identified by nucleotide sequence at the 16S rDNA gene locus.

**Material and Methods**

*Piscirickettsia isolates.* *Piscirickettsia salmonis* type strain LF-89 (ATCC VR 1361) was isolated from coho salmon (*Oncorhynchus kisutch*) in Chile (Fryer et al 1990, Fryer and Lannan 1994). Chilean isolate EM-90 was cultured from Atlantic salmon (*Salmo salar*) and provided by E. Madrid (Marine Harvest, Puerto Montt, Chile). ATL-4-91 was isolated from Atlantic salmon collected off the east coast of Vancouver Island, British Columbia, Canada (Brocklebank et al. 1992). This bacterium was provided by G. Traxler (Pacific Biological Station, Nanaimo, Canada). Norwegian isolate NOR-92 was obtained from Atlantic salmon and provided by H. P. Melby (National Veterinary Institute, Oslo, Norway). Chilean isolate SLGO-94 was cultured from rainbow trout (*Oncorhynchus mykiss*) and supplied by P. Smith (University of Chile, Santiago, Chile).

**Bacterial culture conditions and DNA isolation.** *Piscirickettsia salmonis* was grown at 15°C in chinook salmon embryo cells (CHSE-214) in antibiotic-free Eagle’s minimum essential medium (MEM) with Earle’s salts (Sigma Chemical Co., St. Louis, Missouri) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) until 90% of the cell sheet was lysed (approximately 14 d) (Lannan et al. 1984). Genomic DNA was isolated using DNA-STAT 60 (Tel-Tex, Inc.,
Friendswood, TX.) following the manufacturer’s protocol. Briefly 7 ml of DNA-STAT 60 was added to 25 cm² cultures near complete lysis and the cells and supernatant passed through a pipette five times to disrupt the remaining intact CHSE 214 cells and to lyse the bacterium. Chloroform (1.5 ml) was added, the mixture shaken for 30 sec and then centrifuged at 12000 x g for 15 min at 4°C in a microcentrifuge. DNA from the upper layer was transferred to a second tube and precipitated with 500 μl of 100% isopropanol. After 10 min at room temperature the DNA was pelleted by centrifugation at 12000 x g at 4°C for 10 min in a microcentrifuge. The supernatant was removed and the pellet vacuum dried for 10 min and resuspended in 200 μl TE (100 mM Tris pH 8.0, 10 mM EDTA). A volume of 5μl of the DNA preparation was used in each 50μl PCR. Precise quantification of the DNA was not attempted as amplification is possible over a wide range of template DNA concentrations.

**Oligonucleotide design and synthesis.** The eubacterial 16S primers, EubA and EubB, the 16S sequencing primers, 519F, 519R, 1100F, 1100R, 1406F, 1406R, and the *Piscirickettsia* specific primers, PS2S and PS2AS, have been described (Giovannoni 1991, Lane 1991, Mauel et al. 1996) (Table 3.1). The oligonucleotides were synthesized at the Oregon State University Center for Gene Research and Biotechnology, Central Services Laboratory.

**PCR amplification of the 16S rDNA gene.** To amplify the 16S gene, 5 μl DNA lysate was added to 45 μl of reaction mixture consisting of 1X PCR buffer (Promega, Madison, WI - 10 mM Tris HCl (pH 8.4), 1.5 mM MgCl₂, 50 mM KCl, 100 mg/ml gelatin, 0.05% NP-40), 200 μM each of dATP, dCTP, dTTP, dGTP, 1 μM EubA primer, 1 μM EubB primer, 2.5 U Taq DNA polymerase (Promega) and covered with 50 μl mineral oil. The mixture was denatured at 94°C for 2 min and amplification was achieved with 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. Amplification was followed by an extension period of 72°C for 7 min and the tubes were
then held at 4°C. Negative controls having no DNA template were included whenever samples were amplified. Aliquots (10 µl) of the PCR reaction mixture were fractionated in a 2% agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50 ml ethidium bromide and photographed under UV transillumination to confirm amplification of the correct size PCR products.

Table 3.1 Sequences and specificity of primers utilized for 16S rDNA PCR and sequencing

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<th>Sequence (5'-3')</th>
<th>Specificity</th>
<th>Reference</th>
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<td>519F</td>
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<td>EubA (1518R)</td>
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<td>11</td>
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</table>

a F, forward; R, reverse, numbering corresponds to Escherichia coli 16S gene

Cloning of the 16S rDNA gene. The 16S PCR products were purified using the PCRquick Kit (Quigen). The purified PCR products were cloned into the TA cloning vector pCRJ® using the manufacturers protocol (Invitrogen Corporation, San Diego, Ca.). Vector plasmids were digested with EcoRI (Promega) to confirm insertion of DNA fragments of the expected size. Digested and undigested plasmids were fractionated in a 2% (w/v) agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50 ml ethidium bromide.
Sequence determination. Sequencing of the 16S rDNA inserts was carried out on an ABI 373 automated sequencer (Perke and Elmer, Applied Biosystems, Inc. Foster City, Ca.) using the dye-primer (T7 and SP6 primer sites on the pCRII plasmid) and dye-terminator (Table 3.1 for primers and locations) kits supplied by Applied Biosystems, Inc.

The sequences determined in this investigation have been deposited in GenBank nucleotide sequence data base and given the following accession numbers: LF-89 U36941, EM-90 U36940, ATL-4-91 U36915, NOR-92 U36942, SLGO-94 U55015.

Additional taxa. The 16S gene sequences of Proteobacteria representing diversity within the phylum were compared to the Piscirickettsia isolates. These organisms and the GenBank accession for the nucleotide sequences used in this study are: Rickettsia rickettsii M21293, Ehrlichia risticii M21290, Cowdria ruminantium X61659, Ehrlichia canis M73221, Anaplasma marginale M60313, Ehrlichia equi M73223, Afipia clevelandensis M69186, Bartonella quintana M11927, Brucella abortus X13695, Wolbachia persica M21292, Francisella tularensis Z21932, Coxiella burnetii M21291, Legionella pneumophila M36025, Pseudomonas aeruginosa X06684, Vibrio anguillarum X16895, and Escherichia coli rRNA operon J01695. Since Chlamydial infections have been reported in fish (Fryer and Lannan 1994), Chlamydia trachomatis M59178 was included in the analysis. The Bacillus subtilis rRNA operon M10606 was used as an outgroup.

Phylogenetic analysis. Each Piscirickettsia sequence was confirmed from the products of three separate PCR reactions that were sequenced in both forward and reverse directions. Sequences were aligned manually and phylogenetic trees were constructed from the sequence data using programs in PHYLIP, phylogenetic inference package version 3.5 (Felsenstein 1989). A matrix of evolutionary distances was constructed from the aligned data by the method of Jukes and Cantor (1969), which
assumes that independent changes occur at all sites with equal probability. In addition, a cladistic reconstruction of the phylogenetic relationships of the taxa was performed using the parsimony program PAUP version 3.0r (Swofford 1993). Phylogenetic trees were also produced using a least squares fitting algorithm from the De Soete Tree Fit program (De Soete 1983). Only data for sequence positions that were known for all the isolates used in the analysis were compared. This resulted in the use of 1527 bases for comparisons among the *P. salmonis* isolates and 1313 bases for comparison between *P. salmonis* and the selected non-*P. salmonis* sequences.
Table 3.2. Levels of similarity between 16S rDNA gene sequences

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<th>P. salmonis</th>
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<th>R. rickettsii</th>
<th>E. canis</th>
<th>C. ruminantium</th>
<th>E. risticii</th>
<th>A. marginale</th>
<th>Ehrlichia equi</th>
<th>A. clevelandensis</th>
<th>B. quintana</th>
<th>B. abortus</th>
<th>W. persica</th>
<th>F. tularensis</th>
<th>C. burnetii</th>
<th>L. pneumophila</th>
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\(^a\) Regions of uncertain homology and ambiguity were eliminated leaving 1313 positions for comparison.
Results

Using the flanking primers (EubA and EubB), we amplified only one band (~1500 bases) from each of the five isolates studied. The sequence of Piscirickettsia salmonis LF-89T was aligned with the 16S rDNA sequences of Clamydia trachomitis, representative members from the alpha and gamma proteobacteria, and Bacillus subtilis. The similarity values derived for the various taxa used in the phylogenetic
| Figure 3.2(a). 16S rDNA nucleotide sequences of *Piscirickettsia salmonis* strains; LF (LF-89), EM (EM-90), ATL (ATL-4-91), NOR (NOR-92), SLGO (SLGO-94). Points indicate nucleotide identity with LF-89. Lowercase letters indicate uncertainty in the sequence determination. Restriction enzyme sites used in this study are indicated by a bar above or below the sequences. |
Figure 3.2(b). Secondary structure model of the 16S rRNA gene of *Piscirickettsia salmonis* LF-89 (Gutell et al. 1985). Sequence variation between LF-89 and the other strains are indicated by the following; EM-90 (○), ATL-4-91 (+), NOR-92 (○), SLGO-94 (>), two or more isolates (•). The circled area indicates the stem loop of greatest diversity. Lowercase letters indicate uncertainty in the sequence determination. Numbering corresponds to that of the *Escherichia coli* 16S rRNA.
Comparison indicate that *P. salmonis* is a member of the gamma subgroup of the proteobacteria distantly related to the genera *Coxiella* and *Legionella* (Table 3.2). Analysis of the 16S rDNA gene data by either the Jukes-Cantor or the parsimony methods produced the same tree (Fig. 3.1). Bootstrap replicates (number = 100) in the Phylip package produced confidence estimates of 100% for all nodes.

Each of the five *Piscirickettsia salmonis* 16S rDNA sequences comprise a continuous length of 1540 nucleotides or approximately 99.4% of the gene sequence (Fig 3.2). The nucleotide similarity values used in the phylogenetic comparison of the *P. salmonis* isolates represented 1527 nucleotide positions and were unambiguously determined for each of the five isolates (Table 3.3).

<table>
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<tr>
<th>Isolate</th>
<th>% Similarity to:</th>
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<td>SLGO-94</td>
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</table>

1527 nucleotides were used for the comparison after elimination of ambiguous nucleotides.

Comparison of the 16S sequence alignments of the five *P. salmonis* isolates reveals very high levels of similarity (≥98.5%) showing that they are a monophyletic group. Isolates LF-89 and SLGO-94 differ by only two nucleotides and are 99.7% similar. LF-89 and SLGO-94 differed from the sequences of ATL-4-91 and NOR-92 by 6 and 7 nucleotides respectively (level of similarity 99.4%). ATL-4-91 and NOR-92 differ by 5 nucleotides (99.7% similarity). EM-90 differed from LF-89 by 22 nucleotides (98.5%
similarity) and from ATL-4-91 by 18 nucleotides, NOR-92 by 19 nucleotides, and SLGO-94 by 20 nucleotides (98.9, 98.7 and 98.5% similarity respectively). Nine of the base differences between EM-90 and the other isolates are found between bases 1003-

Figure 3.3. Phylogenetic relationships among *Piscirickettsia salmonis* isolates. Evolutionary distances were calculated by the method of Jukes and Cantor (1969). After elimination of ambiguous nucleotides, 1527 were used for comparison.
1020 and correspond to matching positions on a stemloop (Figure 3.2a, b). Analysis of the 16S rDNA gene data by either the Jukes-Cantor or the parsimony methods produced the same tree (Fig. 3.3). The bootstrap values for all nodes were 100%.

Discussion

The isolation of *Piscirickettsia salmonis* from different geographical regions prompted the examination of the 16S rRNA gene sequences from multiple isolates. It seemed plausible that piscirickettsiosis might be a disease with a long evolutionary history and that phylogenetic diversity might exist between isolates of varied geographic origins.

Small subunit rDNA gene sequences have been recognized as one of the most useful types of data available to determine the phylogenetic relationships between bacteria (Berchtold et al. 1994, Weisburg et al. 1989, Wen et al. 1995b, Woese 1987). The use of the polymerase chain reaction (PCR) to amplify the 16S rDNA gene for analysis has greatly facilitated the identification of new isolates and or species of obligate intracellular organisms, where growing and purifying enough material for other genetic and biochemical studies is often difficult (Anderson et al. 1991, Chen et al. 1994, Wen et al. 1995a, Wen et al 1995b).

The amount of nucleotide sequence variation which distinguished between the 16S rDNA for LF-89' from EM-90 was small. The evolutionary significance of this variation of *P. salmonis* is uncertain. Previous research with obligate intracellular parasites indicates differences in 16S rDNA similarity levels ranging from 99.9 to 84% have been used as criteria for assigning strains of novel species (Anderson et al. 1991). Wen (1995a) suggested that two strains of *Ehrlichia risticii* may represent new species on the basis of distinct antigenic profiles and similarity values of 99.2-99.3% (Wen 1995a).
However, Fox (1992) states that while 16S rDNA data is a powerful tool for determining the species a strain belongs, it may not by itself be enough to define a species.

Although the amount of variation between LF-89\textsuperscript{T} and EM-90 is relatively small, the evidence clearly demonstrates it is the result of evolutionary divergence. The conclusion that this variation was due to differences between strains and not artifacts associated with the methods used was supported by the results. Each isolate was amplified and cloned from three separate PCR amplifications. In addition the restriction enzymes \emph{EcoRI} and \emph{PstI} where consistently able to distinguish isolate EM-90 from the other four \emph{P. salmonis} isolates studied.

The 16S rDNA data demonstrates that the five isolates of \emph{P. salmonis} form a monophyletic group exhibiting high (>98.5\%) nucleotide similarities (Table 3.3). In agreement with the results of the previous study (Fryer et al. 1992), the 16S rDNA sequence data indicate that \emph{P. salmonis} is a member of the gamma subgroup of the proteobacteria and is distantly related to the genera \emph{Coxiella} and \emph{Legionella} with similarities of 89.2 and 89.0\% respectively.

Three groups can be identified within the genus \emph{Piscirickettsia} on the basis of these data (Figure 3.3). One group consists of LF-89\textsuperscript{T} and SLGO-94. Isolate SLGO-94 was obtained from an area in close proximity to the location where LF-89\textsuperscript{T} the first isolate, was cultured. However, SLGO-94 was isolated from rainbow trout five years after LF-89\textsuperscript{T} was obtained from coho salmon. The 16S sequence data indicates that SLGO-94 differs from strain LF-89\textsuperscript{T} by only 2 nucleotides (similarity 99.8\%). The second group consists of ATL-4-91 and NOR-92, both isolated in the northern hemisphere from the same host species, but from different waters. Despite being from bodies of water distant from each other ATL-4-91 and NOR-92 are 99.7\% similar. The third group is represented by a single member, EM-90, which was cultured from Atlantic salmon in southern Chile. However, even though EM-90 was isolated from the same
geographic area it only exhibits a 98.5% similarity to LF-89 and SLGO-94. EM-90 differs from ATL-4-91 and NOR-92 by 19 and 18 nucleotides giving similarities of 98.9 and 98.7% respectively.

The differences in sequence between EM-90 and the other four *P. salmonis* sequences has allowed the development of isolate-specific PCR assays which can be used to differentiate EM-90 from LF-89, ATL-4-91, NOR-92 and SLGO-94 (Mauel et al. 1996). It was also found that *EcoRI* and *PstI* restriction sites located in the variable stem-loop region permitted the use of restriction fragment length polymorphism (RFLP) to differentiate EM-90 (Mauel et al. 1996).

It is not clear if the sequence diversity described here is sufficient to warrant designating these isolates new species of the genus *Piscirickettsia*. Other studies have linked virulence, phenotypic, biochemical and antigenic characteristics with the 16S rDNA data as criteria in establishing a novel species.

Information on the genus *Piscirickettsia* is still emerging. The diversity within the 16S rDNA gene suggest that there maybe more than one species, but this requires confirmation by additional research on the biology and biochemistry of the bacterium. Research is required to determine if the apparent variation in virulence between the isolates is caused by factors intrinsic to the bacterium or by environmental conditions.

In conclusion, comparative 16S rDNA sequence analysis of *Piscirickettsia salmonis* indicates the genus is a monophyletic group and a member of the gamma proteobacteria. Within the species three groups can be identified with one isolate, EM-90, showing genetic divergence from the other *P. salmonis* isolates.

**Acknowledgments**

The authors thank P. Caswell-Reno and M. Whipple (Oregon State University, Hatfield Marine Science Center) for maintaining the *P. salmonis* cultures. We also thank
D. Mourich, Dr. J. Leong, and D. Gordon (Department of Microbiology, Oregon State University) for their comments and advice. This work was supported by Sea Grant No. NA36RG0451.

References


CHAPTER 4

Phylogenetic analysis of *Piscirickettsia salmonis* isolates
by Internal Transcribed Spacer (ITS) and 23S ribosomal DNA sequencing

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Abstract:

*Piscirickettsia salmonis* is the etiologic agent of piscirickettsiosis a systemic disease of salmonids. To assess the genetic variability in this genus the internal transcribed spacer (ITS) and 23S ribosomal DNA of six isolates from three different host and three geographic origins were amplified using the polymerase chain reaction. The PCR products were sequenced and indicate that the six isolates form a tight monophyletic cluster. One spacer sequence was identified per strain and the ITS region did not contain tRNA genes. The ITS sequences were 311-bp and varied between isolates (95.2 to 99.7% similarity). Along with the ITS region about two thirds of the 23S rDNA gene was sequenced from five of the isolates with similarities ranging from 97.9 to 99.8%. Phylogenetic trees were constructed based on the sequence data and compared these trees with each other and a tree based on 16S rDNA sequences previously reported by this laboratory. The trees were similar topographically, suggesting that the three types of molecules provided similar phylogenetic information. Within *P. salmonis* three groups of sequences were identified. Three of the isolates, LF-89, C1-95, and SLGO-94 are closely related (99.1-99.7% ITS and 99.3-99.8% 23S rDNA similarities) and have similar geographic but different host species origins. Another group, composed of isolates ATL-4-91 and NOR-92, were both isolated from Atlantic salmon but from different geographic areas in the northern hemisphere and show a sequence similarity value of 99.4% ITS and 98.7% 23S. The sequence of isolate EM-90 was unique with similarities ranging from 95.2 - 96.9% ITS and 97.6 - 98.5% 23S rDNA when compared to the other five isolates. The ITS region has diverged on average 3.15 times faster than the 16S rDNA gene, while the 23S rDNA gene has diverged on average 1.6 times faster than the 16S rDNA gene in *P. salmonis*. 
Introduction

The bacterium *Piscirickettsia salmonis* is an obligate intracellular parasite and the causative agent of piscirickettsiosis, a systemic disease of salmonids that has been reported in Chile (Bravo and Compos 1989, Fryer et al. 1990), Norway (Olsen et al. 1993), Ireland (Rodger and Drinan 1993) and the Pacific coast of Canada (Evelyn 1992). *Piscirickettsia salmonis* is the first rickettsia isolated and characterized from fish and is a member of the multiphylogenetic family, Rickettsiaceae, a group which includes other intracellular bacteria such as *Rickettsia, Ehrlichia, Cowdria, Anaplasma*, and *Wolbachia*. The obligate intracellular and fastidious nature of the Rickettsiae has made them difficult to study.

The nucleotide sequences that code for the ribosomal subunits (16S and 23S rRNA) are universally present in organisms, easy to clone and sequence, and two of the most conserved (Woese 1987). The characteristics of the 16S and 23S rDNA genes make them useful in determining the evolutionary relationships between organisms and their use as indicators of evolutionary divergence is now widespread. While 16S rDNA sequence analysis is a powerful and accurate means for determining inter- and intragenetic relationships as the evolutionary distances are reduced, a point is reached where there is insufficient nucleotide differences to differentiate between isolates/strains within a species. The 23S rDNA gene has an advantage as an evolutionary chronometer, over the 16S rDNA gene, because it is larger in size and having a greater number of variable regions offering more data points for analysis. The larger 23S gene contains about twice the number of nucleotide bases as the smaller 16S gene and may provide information on phylogenetic relationships between closely related organisms not available in the 16S rDNA gene (Ludwig and Schleifer 1994).

Many bacterial 16S and 23S rDNA genes are separated by internally transcribed spacer regions. These spacer regions are known to show a large number of sequence and
length variations that are useful for differentiating within species of prokaryotic organisms (Barry et al. 1991, Navarro et al. 1992, Trevisanato et al. 1996, Leblond-Bourget et al. 1996). In addition, the ITS region is easier to sequence because of its smaller size than are the 16S or 23S genes.

Problems associated with the isolation of *P. salmonis* has limited the number of isolates available for examination. Analysis within the genus based on a comparison of the 16S rDNA sequences of 5 isolates from varied host and geographic origins has been previously accomplished (Mauel et al. in press). Here the sequence and phylogenetic analysis of the complete internal transcribed spacer (ITS) for six isolates of *P. salmonis* and for approximately two thirds of the 23S rDNA gene for 5 *P. salmonis* isolates is described.

**Material and Methods**

*Piscirickettsia isolates.* *Piscirickettsia salmonis* type species LF-89 ATCC VR 1361 was isolated from coho salmon (*Oncorhynchus kisutch*) in Chile (Fryer et al. 1990, 1992). Chilean isolate EM-90 was cultured from Atlantic salmon (*Salmo salar*) and provided by E. Madrid (Marine Harvest, Puerto Montt, Chile). ATL-4-91 was isolated from Atlantic salmon collected off the east coast of Vancouver Island, British Columbia, Canada (Brocklebank et al. 1992) and was provided by G. Traxler (Pacific Biological Station, Nanaimo, B.C.). Norwegian isolate NOR-92 was obtained from Atlantic salmon and provided by H. P. Melby (National Veterinary Institute, Oslo, Norway). Chilean isolates SLGO-94 and C1-95 were supplied by P. Smith (University of Chile, Santiago, Chile). SLGO-94 was obtained from rainbow trout (*Oncorhynchus mykiss*) and C1-95 was isolated from coho salmon.

**Culture of Rickettsia and isolation of DNA.** *Piscirickettsia salmonis* isolates were grown at 15°C in chinook salmon embryo cells, CHSE-214 (Lannan et al.
1984) in antibiotic-free Eagle’s minimum essential medium (MEM) with Earle’s salts (Sigma Chemical Co., St. Louis, Missouri) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) until approximately 90% of the cell sheet was lysed (about 14 d). Genomic DNA was isolated using DNA-STAT 60 (Tel-Tex, Inc., Friendswood, TX) following the manufacturer’s protocol. Briefly 7 ml of DNA-STAT 60 was added to 25 cm² cultures near complete lysis and the cells and supernatant passed through a pipette five times to disrupt the remaining intact CHSE 214 cells and to lyse the bacterium. Chloroform (1.5 ml) was added, the mixture shaken for 30 sec and then centrifuged at 12000 x g for 15 min at 4°C in a microcentrifuge. DNA from the upper layer was transferred to a second tube and precipitated with 500 µl of 100% isopropanol. After 10 min at room temperature the DNA was pelleted by centrifugation at 12000 x g for 10 min in a microcentrifuge at 4°C. The supernatant was removed and the pellet vacuum dried for 10 min and resuspended in 200 µl TE (100 mM Tris pH 8.0, 10 mM EDTA). A volume of 5 µl of the DNA preparation was used in each 50 µl PCR. Precise quantification of the DNA was not attempted as amplification is possible over a wide range of template DNA concentrations.

Oligonucleotide design and synthesis. The ITS PCR primer pair (PS16SA - PS23SB, Table 4.1) are modifications of previously published primers (East and Collins 1993, Frothingham and Wilson 1993, Gurtler 1993). The ITS sequencing primers (PS16SH and PS23SB, Table 4.1) were designed using 16S and 23S sequence data derived in the course of this study. The 23S PCR and sequencing primers are modifications of primers previously described by Stothard et al. (1994) (Table 4.1). The oligonucleotides were synthesized at the Oregon State University Center for Gene Research and Biotechnology, Central Services Laboratory.

PCR amplification. To amplify the ITS gene, 5 µl DNA lysate was added to 45 µl of reaction mixture consisting of 1X PCR buffer (10 mM Tris HCl (pH 8.4), 1.5
mM MgCl₂, 50 mM KCl, 100 mg/ml gelatin, 0.05% NP-40, Promega, Madison, WI), 200 μM each of dATP, dCTP, dTTP, dGTP, 2 μM PS16SA primer, 2 μM PS23SC primer, 2.5 U Taq DNA polymerase (Promega, Madison, WI) and covered with 50 μl mineral oil. The mixture was denatured at 94°C for 2 min and amplification was achieved with 35 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 3 min. Amplification was followed by an extension period of 72°C for 7 min and the tubes were then held at 4°C.

Table 4.1 Sequences of primers utilized for Internal Transcribed Spacer (ITS) and 23S rDNA gene PCR and sequencing

<table>
<thead>
<tr>
<th>Primer/Location&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS16SA (1387F)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GCCTTGTACACAACCGCCC</td>
</tr>
<tr>
<td>PS16SH (1516F)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CCTGCGGCTGGATTACCT</td>
</tr>
<tr>
<td>PS23SB (507R)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTTTCCCCACCGGTCTAC</td>
</tr>
<tr>
<td>PS23SC (203R)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TAGATGTTTCAGTTCCCC</td>
</tr>
<tr>
<td>PS441F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GTGAACCTAGTACCGGTAGG</td>
</tr>
<tr>
<td>PS761F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GATGACTTTGTGGTGITGGG</td>
</tr>
<tr>
<td>PS1104F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>AGCGTAATAGCTCAC</td>
</tr>
<tr>
<td>PS1623F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>AAACCGACACAGGTAG</td>
</tr>
<tr>
<td>PS1948F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GTAGCGAAATTCTCTTGC</td>
</tr>
<tr>
<td>PS2253F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GGTACAAAGTGACCAC</td>
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<tr>
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<td>CCTCGATGTCGGCTC</td>
</tr>
<tr>
<td>PS2758F&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTGAAAGCATCTAA</td>
</tr>
<tr>
<td>PS716R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CACCAIAACCACACTCATCC</td>
</tr>
<tr>
<td>PS1091R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>AGTGAGCTATTACACG</td>
</tr>
<tr>
<td>PS1608R&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>PS1930R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CGACAAGGAATTTCGCTAC</td>
</tr>
<tr>
<td>PS2241R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ACCGCCCGACGTGAAACT</td>
</tr>
<tr>
<td>PS2498R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GAGGCACATCGAGG</td>
</tr>
<tr>
<td>PS2747R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GCTGATGCGTTTC</td>
</tr>
</tbody>
</table>

<sup>a</sup>F, forward, R, reverse
<sup>b</sup>numbering corresponds to *Escherichia coli* 16S rDNA gene
<sup>c</sup>Number of bases from the 5' end of *P. salmonis* 23S rDNA gene
No DNA negative controls were included whenever samples were amplified. Aliquots (10 μl) of the PCR reaction mixture were fractionated in a 2% agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50ml ethidium bromide and photographed under UV transillumination to confirm amplification of the correct size PCR products.

**Amplification of 23S rDNA genes.** To amplify the 23S gene, 5 μl DNA lysate was added to 45 μl of reaction mixture consisting of 1X PCR buffer (10 mM Tris HCl (pH 8.4), 3.5 mM MgCl₂, 50 mM KCl, 100 mg/ml gelatin, 0.05% NP-40, Promega, Madison, WI), 200 μM each of dATP, dCTP, dTTP, dGTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI) and covered with 50 μl mineral oil. The first 457 nucleotides of the 5' end of the 23S gene was amplified and sequenced with the ITS region. The 23S gene was amplified in two sections by using the primer pairs PS441F - PS1930R and PS1623F -PS2498R which are modification of primers previously described by Stothard et al. (1994)(Table 4.1). The mixture was denatured at 94°C for 2 min and amplification was achieved with 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. Amplification was followed by an extension period of 72°C for 7 min and the tubes then held at 4°C. No DNA negative controls were included whenever samples were amplified. Aliquots (10 μl) of the PCR reaction mixture were fractionated in a 2% agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50ml ethidium bromide and photographed under UV transillumination to confirm amplification of the correct size PCR products.

**Cloning the ITS region.** The ITS PCR products were purified using the PCRquick Kit (Quigen). The purified PCR products were cloned into the TA cloning vector pCRII® using the manufacturers protocol (Invitrogen Corporation, San Diego, CA). Vector plasmids were digested with EcoRI (Promega, Madison, WI) to confirm insertion of DNA fragments of the expected size. Digested and undigested plasmids
were fractionated in a 2% (w/v) agarose 1X TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg/50 ml ethidium bromide.

**Sequence determination.** ITS sequences were determined by sequencing the PCR product inserted into the pCRII® vector. The 23S rDNA gene sequences were determined by direct sequencing of the PCR products. Sequencing was carried out on an ABI 373 automated sequencer (Perke and Elmer, Applied Biosystems, Inc. Foster City, CA) using the dye-primer (T7 and SP6 primer sites on the pCRII plasmid) and dye-terminator (Table 4.1 for primers and locations) kits supplied by Applied Biosystems, Inc.

The sequences determined in this investigation have been deposited in GenBank nucleotide sequence data base and given the following accession numbers: LF-89 U36943, EM-90 U36944, ATL-4-91 U36945, NOR-92 U36946, SLGO-94 U62104, C1-95 U62103.

**Phylogenetic analysis.** Each *Piscirickettsia* ITS and 23S sequence was confirmed from the products of three separate PCR reactions that were sequenced in both forward and reverse directions. Sequences were aligned manually and phylogenetic trees constructed from the sequence data using programs in PHYLIP, phylogenetic inference package version 3.5 (Felsenstein 1989). A matrix of evolutionary distances was constructed from the aligned data by the method of Jukes and Cantor (1969), which assumes that independent changes occur at all sites with equal probability. In addition, a cladistic reconstruction of the phylogenetic relationships of the taxa was performed using the parsimony program PAUP version 3.0r (Swofford 1993). Phylogenetic trees were also produced using a least squares fitting algorithm from the De Soete Tree Fit program (De Soete 1983). Only data for sequence positions that were known for all the isolates used in the analysis were compared. This resulted in the use of 309 bases for the
comparisons of the ITS region between the *P. salmonis* isolates and 1902 bases for comparison of the 23S rDNA gene between the isolates.

**Results**

The complete ITS rDNA sequences of six isolates of *Piscirickettsia salmonis* were determined by sequencing plasmid inserts derived with PCR using primers to conserved areas in the 16S and 23S rDNA genes. Only one ITS sequence was obtained from each isolate (Figure 4.1) with two lengths of ITS spacers being observed. ATL-4-91 and NOR-92 have ITS regions of 309 base pairs (bp). LF-89, EM-90, SLGO-94 and C1-96 had an ITS of 308-bp in length. The similarity values used in the phylogenetic comparison of the *P. salmonis* isolates represented 311 nucleotide positions that were unambiguously determined for each of the six isolates and included insertions and deletions (Table 4.2).

Comparison of the ITS sequence alignments of the six *P. salmonis* isolates reveals high levels of similarity (ranging from 95.2 - 99.4%). A tree was constructed from the data

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Similarity to:</th>
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<tbody>
<tr>
<td></td>
<td>LF-89</td>
</tr>
<tr>
<td>LF-89</td>
<td>96.5</td>
</tr>
<tr>
<td>EM-90</td>
<td>97.8</td>
</tr>
<tr>
<td>ATL-4-91</td>
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<td>C1-95</td>
<td>99.1</td>
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<tr>
<td>SLGO-94</td>
<td>97.8</td>
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309 nucleotide positions were used for the comparison after elimination of ambiguous nucleotides.
<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>EM</th>
<th>ATL</th>
<th>C1</th>
<th>SL</th>
<th>NOR</th>
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<td>100</td>
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</table>

Figure 4.1. Comparison of the nucleotide sequences of the 16S-23S internal transcribed spacer (ITS) for six isolates of Piscirickettsia salmonis. Regions indicated by boxes show homology with sequences in *E. coli* which are thought to be involved in processing of rRNA molecules (Young 1979). Points indicate nucleotide identity with LF-89. LF = LF-89, EM = EM-90, AT = ATL-4-91, C1 = C1-95, SL = SLGO-94, NOR = NOR-92.
Figure 4.2. Phylogenetic relationships among *Piscirickettsia salmonis* isolates using the internal transcribed spacer (ITS) sequence. Evolutionary distances were calculated by the method of Jukes and Cantor (1969). After elimination of ambiguous nucleotides, 309 were used for comparison.

revealing the presence of three groups within *P. salmonis* with LF-89, SLGO-94 and C1-95 forming a group, NOR-92 and ATL-4-91 a second group and EM-90 forming its own group (Figure 4.2).

Approximately 2500-bp of the 23S rDNA gene was amplified and directly sequenced for all of the isolates except NOR-92. The NOR-92 culture was contaminated with a mycoplasma and was not usable to clone the 23S gene. However, 460-bp of the 5' end of the 23S gene was cloned and sequenced along with the ITS region.

The 23S rDNA gene was analyzed in two ways. The first 460-bp down stream from the 5' end was analyzed for all six isolates. In addition, five of the isolates were amplified and sequenced to position 2495 downstream from the 5' end, with NOR-92 omitted for reasons noted. A similarity matrix was constructed from each analysis (Tables 4.3, 4.4) and phylogenetic trees produced (Figures 4.3, 4.4). Overall, the
Table 4.3. Sequence similarities of the first 450 nucleotides of the 5' end of the 23S rDNA gene between isolates of *Piscirickettsia salmonis*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Similarity to:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LF-89 EM-90 ATL-4-91 C1-95 SLGO-94</td>
</tr>
<tr>
<td>LF-89</td>
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</tr>
<tr>
<td>EM-90</td>
<td>97.6</td>
</tr>
<tr>
<td>ATL-4-91</td>
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</tr>
<tr>
<td>C1-95</td>
<td>99.3 97.8 98.9</td>
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</tr>
<tr>
<td>NOR-92</td>
<td>99.1 98.5 98.7 99.3 99.1</td>
</tr>
</tbody>
</table>

457 nucleotide positions were used for the comparison after elimination of ambiguous nucleotides.

Phylogenetic relationships observed in the two 23S and the ITS trees were similar. The 23S tree constructed from analysis of the first 460-bp contained the same groups as the other trees but differed in the branching pattern within the group containing isolates LF-89, SLGO-94 and C1-95 (Figure 4.3). Analysis of the ITS and 23S rDNA gene data by either the Jukes-Cantor or the parsimony methods produced the same trees. The bootstrap values for all nodes were 100%. The ITS region has diverged on average 3.15 times faster between isolates than the divergence reported previously for the *P. salmonis* 16S rDNA gene (Mauel et al. 1996), while the 23S rDNA gene has diverged on average 1.6 times faster between isolates than the 16S rDNA gene in *P. salmonis*. 
Table 4.4. 23S rDNA gene sequence similarities between 5 isolates of *Piscirickettsia salmonis*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>LF-89</th>
<th>EM-90</th>
<th>ATL-4-91</th>
<th>C1-95</th>
</tr>
</thead>
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<tr>
<td>LF-89</td>
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<td>ATL-4-91</td>
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<tr>
<td>C1-95</td>
<td>99.5</td>
<td>97.9</td>
<td>98.5</td>
<td></td>
</tr>
<tr>
<td>SLGO-94</td>
<td>99.6</td>
<td>98.1</td>
<td>98.7</td>
<td>99.8</td>
</tr>
</tbody>
</table>

1902 nucleotide positions were used for the comparison after elimination of ambiguous nucleotides.

Figure 4.3. Phylogenetic relationships among *Piscirickettsia salmonis* isolates using the first 450 nucleotides of the 5' end of the 23S rDNA gene sequence. Evolutionary distances were calculated by the method of Jukes and Cantor (1969). After elimination of ambiguous nucleotides 457 positions were used for comparison.
Figure 4.4. Phylogenetic relationships among *Piscirickettsia salmonis* isolates using the 23S rDNA sequence. Evolutionary distances were calculated by the method of Jukes and Cantor (1969). After elimination of ambiguous nucleotide positions 1902 positions were used for comparison.

**Discussion**

The use of ITS sequence divergence between isolates or strains belonging to the same species can help to clarify relationships within a species. According to Leblond-Bourget (1996) as an approximation most species groups exhibit 16S-23S sequence divergence of <13%. The maximum divergence in this study was 4.8% (Table 4.1). That only one ITS sequence was obtained for each of the *P. salmonis* isolates suggests the presence of one rRNA operon which would agree with reports from other slow growing organisms (Frothingham and Wilson 1993). There are limitations to the applications of ITS sequence-based strain differentiation. It is well suited to slow-growing organisms that often have one copy of the rRNA operon. The use of ITS sequencing would be more complex for species that have more than one rRNA operon because of sequence variation between the operons (Frothingham and Wilson 1993).
Although the amount of variation between LF-89 and EM-90 is relatively small, the evidence clearly demonstrates it is the result of evolutionary divergence. The conclusion that this variation was due to differences between strains and not artifacts associated with the methods used was supported by the results. The ITS region from each isolate was amplified and cloned from three separate PCR amplifications. To avoid incorrect sequence data, which may result from cloning artifacts, and to minimize errors introduced by the DNA polymerase, direct sequencing of the amplified DNA was performed. In addition, the three trees generated in this study demonstrated similar topography which was in agreement with the 16S tree produced in a previous study (Mauel et al. 1996).

Three groups (LF-89T, C1-95, SLGO-94; ATL-4-91, NOR-92; and EM-90) can be identified within the genus Piscirickettsia on the basis of this data. One group consists of LF-89T, C1-95 and SLGO-94. Isolates C1-95 and SLGO-94 were obtained from an area in close proximity to the location where LF-89T, the first isolate, was cultured. However, SLGO-94 was isolated from rainbow trout five years after LF-89T was obtained from coho salmon and C1-95 was also isolated from coho but 6 years later. The second group consists of ATL-4-91 and NOR-92, both isolated in the northern hemisphere from the same host species, but from different waters. Despite being from bodies of water distant from each other, ATL-4-91 and NOR-92 differ from each other by one position within the ITS region. The third group is represented by a single member, EM-90, which was cultured from Atlantic salmon in southern Chile. However, even though EM-90 was isolated from the same geographic area it only exhibits a evolutionary divergence when compared to the other Chilean isolates LF-89T, C1-94 and SLGO-94 and the northern hemisphere ATL-4-91 and NOR-92.

The processing of rRNA into the ribosomal subunits involves cleavage with RNase III (Young et al. 1979). In Escherichia coli, one position at which this cleavage
occurs is after the sequence GCUCACACA, 33 nucleotides downstream from the 3' end of the 16S rRNA gene (Young et al. 1979). In *P. salmonis*, this sequence is present with only one base change (GUUCACACA) and is located one position farther from the 16S rRNA gene than in *E. coli*. This sequence is also found in *Aeromonis hydrophila* (East and Collins 1993) and *Plesiomonas shigelloides* (East et al. 1992) and they are identical to the sequence found in *P. salmonis*. Another region of sequence homology is present in *E. coli*, *A. hydrophila* and *P. shigelloides* upstream from the 23S rDNA gene. This region is 12 nucleotides in length and is also present in *P. salmonis* (Figure 4.1). The presence of these regions in *E. coli*, *P. salmonis*, *A. hydrophila*, and *P. shigelloides* implies that a similar mechanism for the processing and maturation of rRNA exists in all four organisms.

It is not clear if the sequence diversity described here is sufficient to warrant designating these isolates new species of the genus *Piscirickettsia*. Information on the genus *Piscirickettsia* is still emerging. The diversity within the 16S rDNA gene suggest that there maybe more than one species, but this requires confirmation by additional research on the biology and biochemistry of the bacterium. Research is required to determine if the apparent variation in virulence between the isolates is caused by factors intrinsic to the bacterium or by environmental conditions.

In conclusion, comparative ITS, 23S and 16S rDNA sequence analysis of *Piscirickettsia salmonis* indicates the genus is a monophyletic group and a member of the gamma proteobacteria. Within the genus three groups can be identified: a Chilean group consisting of LF-89, C1-96 and SLGO-94; a northern hemisphere group composed of ATL-4-91 and NOR-92; and a third group composed of one Chilean isolate, EM-90, that exhibits considerable genetic divergence from the other *P. salmonis* isolates.
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SUMMARY

A nested polymerase chain reaction (PCR) was developed to detect genomic DNA of *P. salmonis*. The specificity of the PCR was assessed with a panel of four salmonid and 15 bacterial genomic DNA preparations and amplification products were observed only from *P. salmonis* DNA. A single amplification using *P. salmonis*-specific primers allowed detection of 60 tissue culture infectious dose 50 (TCID$_{50}$) while a nested PCR assay allowed detection of less than one *P. salmonis* TCID$_{50}$.

One isolate, EM-90, was found to be unique by restriction fragment length polymorphism (RFLP) analysis of the 16S products from 6 isolates of *P. salmonis*. The genetic variability of this species or species-complex was assessed by amplifying, sequencing and analyzing the 16S ribosomal DNA of five isolates from three different geographical locations. The results of this analysis confirm that *P. salmonis* is a member of the gamma subgroup of the proteobacteria and that the six isolates form a tight monophyletic cluster. From this data two additional primers were developed for use in a PCR assay and were used to differentiate EM-90 from the five other *P. salmonis* isolates tested.

Three groups of sequences were identified from the 16S rDNA sequences. Two of the isolates, LF-89 and SLGO-94, were closely related (>99.7% similarity) and were isolated from different hosts but the same geographic location. Another group was composed of ATL-4-91 and NOR-92 both isolated from Atlantic salmon taken from different geographic areas (99.7% similarity). EM-90 was found to form its own group with similarity values ranging from 98.5 - 98.9% when compared to the other four isolates. To further access the genetic variability of the *P. salmonis* isolates, the internal transcribed spacer (ITS) and 23S rDNA were amplified, sequenced and analyzed. The analysis again indicated that the six isolates form a tight monophyletic cluster. One
spacer sequence was identified per isolate and the ITS region did not contain a tRNA gene. The ITS sequences were 311-bp in length and varied between the isolates (95.2-99.7% similarity). Sequences homologous to areas in *E. coli* thought to be involved in the maturation and processing of ribosomal RNA were identified in the ITS region. The presence of these regions in *E. coli*, *P. salmonis*, *A. hydrophila*, and *P. shigelloides* implies that a similar mechanism for the processing and maturation of rRNA exists in all four organisms.

Approximately 1900-bp of the 23S rDNA gene was amplified from the six isolates and similarities ranged from 97.9 to 99.8% between the isolates. Phylogenetic trees were constructed with the 16S, ITS and 23S rDNA data and were of similar topography.

Three groups of ITS and 23S rDNA sequences were identified. The isolates LF-89, C1-95, and SLGO-94 are closely related (99.1% - 99.7% ITS and 99.3 - 99.8% 23S rDNA similarities) and have similar geographic but different host species origins. Isolated from Atlantic salmon but from different geographic areas in the northern hemisphere, ATL-4-91 and NOR-92 show a sequence similarity values of 99.4% ITS and 98.7% 23S rDNA. The sequence of isolate EM-90 was again unique with similarities ranging from 95.2 - 96.9% ITS and 97.6 - 98.5% 23S rDNA when compared to the other five isolates. The *P. salmonis* ITS region has diverged on average 3.15 times faster than the 16S rDNA gene, while the 23S rDNA gene has diverged 1.6 times faster than the 16S rDNA gene.

The isolates of *Piscirickettsia salmonis* examined in this study have been shown to form a tight monophyletic group within the gamma proteobacteria. The ribosomal operon has been found to be of the 16S-ITS-23S-ITS-5S type and no tRNA sequences were observed in the 16S-23S ITS region. While EM-90 is divergent when compared to
the other isolates examined, further studies are needed to determine the exact relationships between EM-90 and the other *Piscirickettsia salmonis* isolates.
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