Vol. 115: 233–244, 2015
doi: 10.3354/dao02892

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 20

Identification and genetic characterization of *Piscirickettsia salmonis* in native fish from southern Chile

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ABSTRACT: *Piscirickettsia salmonis* is the etiological agent of piscirickettsiosis, a severe disease causing high mortalities in salmonids. This bacterium has been previously identified and isolated in all cultivated salmonids in Chile and worldwide, including *Salmo salar*, *Oncorhynchus kisutch*, and *O. mykiss*, in addition to being found in non-salmonid species such as *Dicentrarchus labrax* and *Atractoscion nobilis*. In this study, the 16S rRNA gene and intergenic spacer ITS-1 of *P. salmonis* were amplified by PCR from DNA samples extracted from the native Chilean fish species *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis*, and *Salilota australis*. Analysis of the 16S rRNA sequences from *O. regia* demonstrated a close phylogenetic relationship with the 16S rRNA gene in the Chilean EM-90 strain. The 16S rRNA sequences from *E. maclovinus*, *S. capensis*, and *S. australis* were related to the Chilean LF-89 sequence and Scottish strains. To confirm these findings, analysis of *P. salmonis* ITS-1 sequences obtained from the 4 sampled native species demonstrated a high degree of identity and a close phylogenetic relationship with Chilean *P. salmonis* sequences, including LF-89 and EM-90. These results suggest a strong relationship between the nucleotide sequences from the 16S rRNA and ITS-1 genes amplified from native fish with those sequences described in the first *P. salmonis* strains to be identified and isolated in Chile.

KEY WORDS: 16S gene · ITS-1 · rDNA sequences · Piscirickettsiosis · Native fish · Salmonids

INTRODUCTION

Piscirickettsia salmonis is the causative agent of piscirickettsiosis. In 1989, this bacterium was first identified in Chile (Bravo & Campos 1989) as the pathogen associated with mortalities in coho salmon *Oncorhynchus kisutch*, and it was subsequently detected in Atlantic salmon *Salmo salar* and rainbow trout *O. mykiss* (Cvitanich et al. 1991, 1995). Fish severely affected by *P. salmonis* present dark coloration, lack of appetite, lethargy, and erratic swimming close to the surface, while less affected fish do not present external abnormalities (Branson & Nieto Diaz-Muñoz 1991, Cvitanich et al. 1991, Brocklebank et al. 1992, Olsen et al. 1997).

The most frequent external lesions include pale gills as a result of severe anemia, abdominal swelling, and petechial and ecchymotic hemorrhages at the base of fins (Rozas & Enríquez 2014). The most common internal signs of infection include splenomegaly, reno-splenomegaly, and hemorrhagic ascites on visceral fat, stomach, swim bladder, and muscle (Schäfer et al. 1990, Cvitanich et al. 1991, Rozas & Enríquez 2014). Moreover, systemic infection also affects other organs such as the liver, ovaries, and brain (Fryer et al. 1990, Wilhelm et al. 2006). Distinguishing mi-

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croscopic lesions can be found on the liver, kidney, spleen, and intestine, in addition to observable pathological changes occurring in the brain, heart, ovaries, and gills (Schäfer et al. 1990, Cvitanich et al. 1991, Palmer et al. 1996, Olsen et al. 1997, Chen et al. 2000a).

P. salmonis is a Gram-negative, non-motile, pleomorphic bacterium which is predominantly coccoid in shape and has an average size between 0.5 and 1.5 µm (Fryer et al. 1992, Rojas et al. 2008). Phylogenetic analysis through 16S rRNA places this bacterium within a new family in the *Proteobacteria*, the gamma subdivision *Gammaproteobacteria* (Fryer et al. 1992, Mauel et al. 1999, Rojas et al. 2008). In relation to this, several research groups have been able to cultivate this bacterium in cell-free, artificial media, leading to the postulation that *P. salmonis* should rather be classified as a facultative intracellular bacterium (Mauel et al. 2008, Mikalsen et al. 2008, Yañez et al. 2012, 2013).

The transmission of this bacterium can occur both horizontally and vertically. In horizontal transmission, infected fish eliminate bacteria through bile, feces, and urine (Almendras et al. 1997, Smith et al. 2004), and these bacteria are capable of penetrating the superficial barriers of the skin, intestine, and gills (Almendras et al. 1997, Smith et al. 1999, 2004). Therefore, this type of transmission is favored, as bacteria have the capacity to survive in seawater for extended periods of time (Lannan & Fryer 1994), with recent studies finding free-living bacteria after up to 40 d in seawater (Olivares & Marshall 2010). Vertical transmission of the bacteria from progenitors to eggs has also been demonstrated *in vitro* and *in vivo* through the detection of bacteria in eggs experimentally infected with the bacteria taken from healthy females. Moreover, in trout infected by intraperitoneal injection of *P. salmonis*, the bacterium was detected in eggs and in coelomic and seminal fluids, and potential mechanisms for transmission have been proposed (Larenas et al. 2003).

While piscirickettsiosis was initially reported in coho salmon (Fryer et al. 1990), evidence of this disease has been found in other, non-salmonid species such as grouper *Epinephelus melanostigma* in Taiwan (Chen et al. 2000b), white sea bass *Atractoscion nobilis* in California, USA (Arkush et al. 2005), and in the muskellunge *Esox masquinongy* (Thomas & Faisal 2009), and in Greece, a confirmed infection of *P. salmonis* was observed in European sea bass *Dicentrarchus labrax* (Athanassopoulou et al. 2004, McCarthy et al. 2005). In all cases, natural infections of *P. salmonis* could be horizontal and without the apparent need for vectors, with natural reservoirs still unknown but possibly including 1 or more fish species or aquatic organisms (Fryer & Hedrick 2003).

Diagnosis of piscirickettsia uses macroscopic examination for clinical symptoms and microscopic examination of tissue smears, preferably of the kidney, liver, and spleen, using a Gram or hematoxylin and eosin stain (Fryer & Lannan 1996). Bacteria can also be cultivated in cell lines (Fryer et al. 1990, Lannan & Fryer 1991) and bacteriological media (Mauel et al. 2008, Mikalsen et al. 2008, Yañez et al. 2012, 2013). Likewise, techniques have also been applied that are based on fluorescent antibodies and enzymatic immunoassays (ELISA) (Lannan & Fryer 1991), PCR analysis, using primers designed to amplify specific sequences of the 16S rRNA gene (Mauel et al. 1996, Karatas et al. 2008), and the intergenic internal transcribed spacer (ITS-1) sequence, located between the 16S and 23S rRNA (Marshall et al. 1998).

Comparative analysis of the gene sequences of the ribosomal operons 16S, 23S, and the intergenic ITS-1 has become routine methodology in studying microbial phylogeny (Woese 1987, Garrity & Holt 2001). In fact, the ribosomal 16S gene can be amplified through PCR, and posterior analysis can play a major role in phylogenetic classification given that this sequence possesses highly conserved and highly variable regions that can be characterized and used for interpreting molecular evolution between bacteria (Clarridge 2004). Along this same line, ITS-1 can contain sequences that code for transfer RNA (tRNA), which is variable according to the species studied and which is especially useful in differentiating subspecies of prokaryotic organisms (Mauel et al. 1999). For example, a comparative analysis of the *P. salmonis* 16S gene isolated from diverse geographical locations such as North America, South America, and Europe demonstrated the formation of a tight monophyletic cluster, with the Chilean EM-90 strain being the most distant, suggesting a unique isolate as compared to the other studied strains (Mauel et al. 1999). Moreover, posterior studies based on the 16S and ITS-1 genes placed Scottish strains in the same group as Norwegian and Canadian strains, while Irish strains formed a new group (Reid et al. 2004). This demonstrates the strength of ITS-1 sequence analysis in finely discriminating between bacterial strains where 16S rRNA analysis has already suggested a close relationship (Ruimy et al. 1994, García-Martínez et al. 1996, Pérez Luz et al. 1998, Austin 2011).

Here we used PCR to amplify DNA fragments coding for the 16S rRNA and ITS-1 genes of *P. salmonis*, as taken from the total DNA of fish native to southern

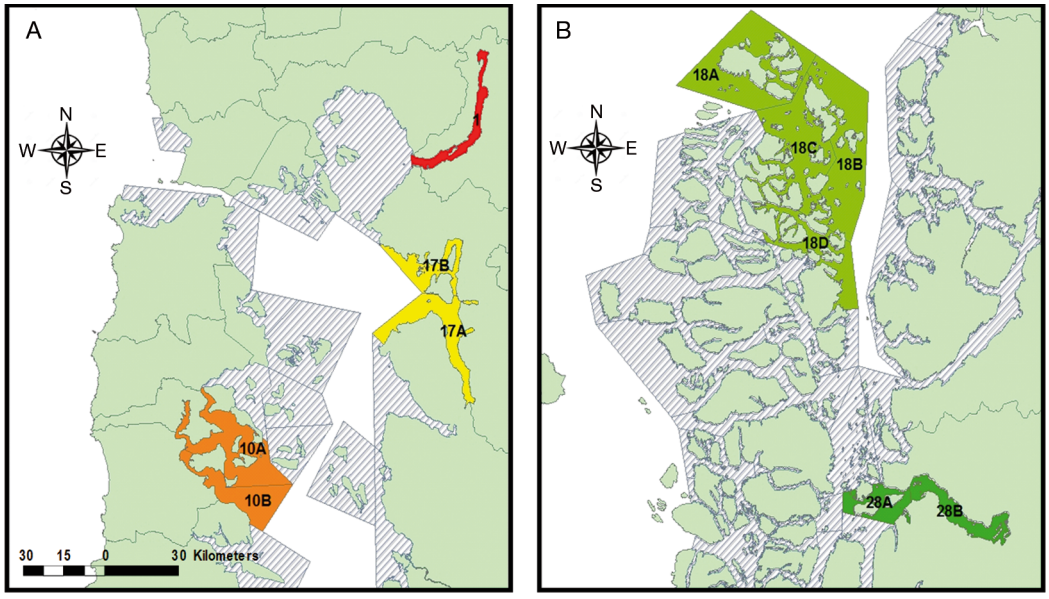

Fig. 1. Native fish sampling sites. From north to south: (A) Región de los Lagos: Reloncaví Estuary (red), Hornopirén (yellow), Castro (orange); (B) Región de Aysén: Melinka (light green) and Aysén (dark green). Numbers correspond to salmonid-culturing concession zones (striped areas: non-sampled zones)

Chile and which tested positive for *P. salmonis*. Phylogenetic comparisons of the amplified fragments were then performed to determine similarity between native fish species.

MATERIALS AND METHODS

Sample collection

The samples used in the present study were obtained through sampling programs organized by the Hydrobiological Health Department of the Institute for Fisheries Development (IFOP) within the framework of the project 'Determining the presence of high-risk diseases in native fish populations' under the SUBPESCA Contract: Integral consultancy for decision making in the fishery and aquaculture industries. Genomic DNA was obtained from a pool of organs (liver, heart, spleen, and kidney) of a single fish and was extracted using the E.Z.N.A® Tissue DNA kit (Omega Bio-tek). The native fish species sampled were the Patagonian blenny *Eleginops maclovinus*, flounder *Paralichthys microps*, chancharro *Helicolenus lengerichi*, pejerrey *Odontesthes regia*, tadpole codling *Salilota australis*, Cape redfish *Sebastes capensis*, Chilean sandperch *Pinguipes chilensis*, Pacific sandperch *Prolatilus jugularis*, Chilean hake *Merluccius gayi gayi*, speckled smooth-hound *Mustelus mento*, Eastern Pacific bonito *Sarda chiliensis*, black cusk eel *Genypterus maculatus*, Chilean jack mackerel *Trachurus symetricus murphyi*, elephant fish *Callorhynchus callorhynchus*, and Southwest Atlantic butterfish *Stromateus brasiliensis*. These fish were captured in sectors close to and far from salmonid cultivation centers in 5 geographic zones corresponding to the Reloncaví Estuary, Hornopirén, Castro, Melinka, and Aysén in 2010 and 2011 (Fig. 1), with a total of 1451 fish sampled.

Amplification of *Piscirickettsia salmonis* 16S gene

P. salmonis detection in native fish samples was performed using a nested PCR assay, following the procedures of Mauel et al. (1996) and using the primers listed in Table 1. In order to amplify a specific fragment of the *P. salmonis* 16S gene, this rRNA gene was first amplified from DNA samples positive for *P. salmonis* using the universal primers for eubacteria, UN and EB (Barry et al. 1990; Table 1, Fig. 2). The PCR product was purified from 1.5% agarose gel using the E.Z.N.A® Gel Extraction kit (Omega Bio-tek). The PCR products of the 16S rRNA gene were utilized as a template for amplification of a smaller section of the 16S rRNA gene, where the primers used were PS2S forward (Mauel et al. 1996) and 1490R reverse (McCarthy et al. 2005). The PCR reac-

Table 1. Sequences and specificity of primers used for 16S rRNA and ITS-1 PCRs and sequencing procedures. F: forward; R: reverse

Primer/location	Sequence (5′–3′)	Amplicon size (bp)	Specificity	Reference
UN (27F)[a]	AGAGTTTGATCCTGGCTCAG	1534	Eubacterial	Barry et al. (1990)
EB (1487R)[a]	ACGGATACCTTGTTACGAGTT			
PS2S (223F)[a]	CTAGGAGATGAGCCCGCGTTG	469	*P. salmonis* 16S	Mauel et al. (1996)
PS2AS (690R)[a]	GCTACACCTGCGAAACCACTT			
PS2S (223F)[a]	CTAGGAGATGAGCCCGCGTTG	1274	*P. salmonis* 16S	Mauel et al. (1996)
1490R (1487R)[b]	CTTCACCCCAGTCATGACCC			McCarthy et al. (2005)
RTS1 (223F)[c]	TGATTTTATTGTTTAGTGAGAATGA	283	*P. salmonis* ITS-1	Marshall et al. (1998)
RTS4 (459R)[c]	ATGCACTTATTCACTTGATCATA			

[a]Numbering indicates the position of *Escherichia coli* 16S rRNA gene
[b]Numbering indicates the position of *Piscirickettsia salmonis* LF-89 16S rRNA gene
[c]Numbering indicates the position of *Piscirickettsia salmonis* LF-89 internal transcribed spacer sequence

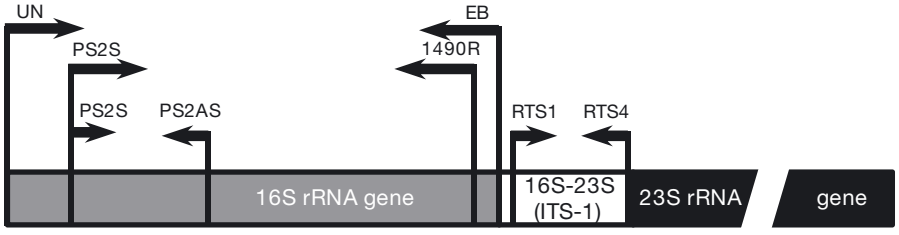

Fig. 2. Region recognized by primers in the ribosomal operon. Arrows indicate the amplification region of primers for the 16S rRNA gene of eubacteria (UN/EB, ca. 1500 bp), for the diagnosis of *Piscirickettsia salmonis* (PS2S/PS2AS, ca. 470 bp), for amplifying the *P. salmonis* 16S rRNA gene (PS2S/1490R, ca. 1270 bp), and for amplifying the *P. salmonis* ITS-1 gene (RTS1/RTS4, ca. 280 bp). In the case of *P. salmonis* primers, positions within the 16S and ITS-1 genes were based on sequences from the LF-89 strain (see Table 2)

tion was carried out using *GoTaq* DNA Polymerase (Promega) in a final volume of 25 µl containing 50 ng of template, 5 µl 5× *GoTaq®* Flexi buffer, 200 mM of $MgCl_2$, 10 mM of dNTPs, and 25 µM of each primer (PS2S and 1490R). The PCR reaction was performed with a MultiGene gradient thermal cycler (Labnet International) using the following settings: 94°C for 5 min; followed by 35 cycles at 94°C for 1 min, 57°C for 1 min, and 1 min at 72°C; with a final extension at 72°C for 10 min.

Amplification of *P. salmonis* intergenic 16S-23S ITS-1

The amplification of *P. salmonis* ITS-1 was performed according to Marshall et al. (1998) with modifications. Briefly, the PCR reaction used *GoTaq* DNA Polymerase (Promega) in a final volume of 25 µl containing 100 ng of genomic DNA from native fish positive for *P. salmonis* as a template, 5 µl of 5× *GoTaq®* Flexi buffer, 200 mM of $MgCl_2$, 10 mM of dNTPs, and 25 µM of each primer (RTS1 and RTS4, Table 1; Marshall et al. 1998). The PCR reaction was performed with a MultiGene gradient thermal cycler (Labnet International), using the following settings: 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 1 min at 72°C; with a final extension at 72°C for 10 min. The PCR products were resolved on 1.5% agarose gel.

DNA sequencing

The PCR products of the *P. salmonis* 16S rRNA and ITS-1 genes were purified from 1.5% agarose gel using the E.Z.N.A® Gel Extraction Kit (Omega Biotek). Each product was cloned in the pGEM-T Easy Vector^{MR} (Promega) according to the manufacturer's instructions. Plasmids with their respective inserts were sequenced using the dideoxy method (Sanger & Coulson 1975) by Macrogen Inc. (South Korea).

Sequences were identified using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov) to compare

against sequences present in the GenBank database. Phylogenetic analysis of the obtained 16S rRNA and ITS-1 sequences was carried out using the MEGA 5.2 software (Tamura et al. 2007), which applied the maximum likelihood method with bootstrap values of 1000, thereby generating a consensus tree for each group of sequences (16S rRNA and ITS-1). For analysis, *P. salmonis* 16S rRNA and ITS-1 sequences obtained from native fish were compared against sequences from Chilean and foreign strains of *P. salmonis* and against 3 unrelated bacterial strains used as an external group (Table 2).

Table 2. Bacterial species and strains used in this study. GenBank accession numbers are listed for all 16S rDNA and internal transcribed spacer (ITS) DNA sequences. (–) no available information

Species/strain	GenBank accession no.	Country	Original isolation source	DNA sequence
Piscirickettsia salmonis LF-89	U36941.1	Chile	*Oncorhynchus kisutch*	16S rDNA
Piscirickettsia salmonis EM-90	U36940.1	Chile	*Salmo salar*	16S rDNA
Piscirickettsia salmonis ATL-4-91	U36915.1	Canada	*Salmo salar*	16S rDNA
Piscirickettsia salmonis NOR-92	U36942.1	Norway	*Salmo salar*	16S rDNA
Piscirickettsia salmonis SLGO-94	U55015.1	Chile	*Oncorhynchus mykiss*	16S rDNA
Piscirickettsia salmonis IRE-91A	AY498633.1	Ireland	*Salmo salar*	16S rDNA
Piscirickettsia salmonis IRE-98A	AY498634.1	Ireland	*Salmo salar*	16S rDNA
Piscirickettsia salmonis IRE-99D	AY498637.1	Ireland	*Salmo salar*	16S rDNA
Piscirickettsia salmonis SCO-02A	AY498635.1	Scotland	*Salmo salar*	16S rDNA
Piscirickettsia salmonis SCO-95A	AY498636.1	Scotland	*Salmo salar*	16S rDNA
Piscirickettsia salmonis CGA/ID655/2012	KC473450.1	Chile	*Oncorhynchus mykiss*	16S rDNA
Piscirickettsia salmonis AL10015	EU289216.1	Chile	–	16S rDNA
Piscirickettsia salmonis SBPLO	AY542956.1	Greece	*Dicentrarchus labrax*	16S rDNA
Piscirickettsia salmonis CGA/8076/2012	KC473425.1	Chile	*Oncorhynchus kisutch*	16S rDNA
Piscirickettsia salmonis P.s1 O. regia	KR136212.1	Chile	*Odontesthes regia*	16S rDNA
Piscirickettsia salmonis P.s3 O. regia	KR136213.1	Chile	*Odontesthes regia*	16S rDNA
Piscirickettsia salmonis P.s6 O. regia	KR136214.1	Chile	*Odontesthes regia*	16S rDNA
Piscirickettsia salmonis P.s8 O. regia	KR136215.1	Chile	*Odontesthes regia*	16S rDNA
Piscirickettsia salmonis P.s45 E. maclovinus	KR136216.1	Chile	*Eleginops maclovinus*	16S rDNA
Piscirickettsia salmonis P.s50 E. maclovinus	KR136217.1	Chile	*Eleginops maclovinus*	16S rDNA
Piscirickettsia salmonis P.s54 S. capensis	KR136218.1	Chile	*Sebastes capensis*	16S rDNA
Piscirickettsia salmonis P.s56 S. australis	KR136219.1	Chile	*Salilota australis*	16S rDNA
Piscirickettsia salmonis LF-89	U36943.2	Chile	*Oncorhynchus kisutch*	ITS-1
Piscirickettsia salmonis EM-90	U36944.2	Chile	*Salmo salar*	ITS-1
Piscirickettsia salmonis ATL-4-91	U36945.2	Canada	*Salmo salar*	ITS-1
Piscirickettsia salmonis NOR-92	U36946.2	Norway	*Salmo salar*	ITS-1
Piscirickettsia salmonis SLGO-94	U62104.2	Chile	*Oncorhynchus mykiss*	ITS-1
Piscirickettsia salmonis IRE-91A	AY498625.1	Ireland	*Salmo salar*	ITS-1
Piscirickettsia salmonis IRE-98A	AY498624.1	Ireland	*Salmo salar*	ITS-1
Piscirickettsia salmonis IRE-99D	AY498631.1	Ireland	*Salmo salar*	ITS-1
Piscirickettsia salmonis SCO-02A	AY498628.1	Scotland	*Salmo salar*	ITS-1
Piscirickettsia salmonis SCO-95A	AY498621.1	Scotland	*Salmo salar*	ITS-1
Piscirickettsia salmonis CGA/ID655/2012	KC473424.1	Chile	*Oncorhynchus mykiss*	ITS-1
Piscirickettsia salmonis AL10015	EU289216.1	Chile	–	ITS-1
Piscirickettsia salmonis SBPLO	AY607585.1	Greece	*Dicentrarchus labrax*	ITS-1
Piscirickettsia salmonis CGA/8076/2012	KC473409.1	Chile	*Oncorhynchus kisutch*	ITS-1
Piscirickettsia salmonis P.s1 O. regia	KR136220.1	Chile	*Odontesthes regia*	ITS-1
Piscirickettsia salmonis P.s3 O. regia	KR136221.1	Chile	*Odontesthes regia*	ITS-1
Piscirickettsia salmonis P.s6 O. regia	KR136222.1	Chile	*Odontesthes regia*	ITS-1
Piscirickettsia salmonis P.s8 O. regia	KR136223.1	Chile	*Odontesthes regia*	ITS-1
Piscirickettsia salmonis P.s45 E. maclovinus	KR136224.1	Chile	*Eleginops maclovinus*	ITS-1
Piscirickettsia salmonis P.s50 E. maclovinus	KR136225.1	Chile	*Eleginops maclovinus*	ITS-1
Piscirickettsia salmonis P.s54 S. capensis	KR136226.1	Chile	*Sebastes capensis*	ITS-1
Piscirickettsia salmonis P.s56 S. australis	KR136227.1	Chile	*Salilota australis*	ITS-1
Vibrio anguillarum M1	JX966409.1	–	–	16S rDNA
Renibacterium salmoninarum ATCC 33209	NR_074198.1	–	–	16S rDNA
Flavobacterium psychrophilum JIP02/86	NR_074630.1	–	–	16S rDNA

RESULTS

Piscirickettsia salmonis 16S RNA and ITS-1 gene amplification in native fish

Table 3 shows the distribution of captured fish according to each geographical zone, with the number of samples per zone being relatively homogeneous. The zones with the greatest number of captured fish were the Reloncaví Estuary and Castro, while those with the lowest number of sampled individuals were Aysén, Melinka, and Hornopirén. In total, 19 fish species were captured, of which the majority (~70%) were *Eleginops maclovinus* and *Odontesthes regia*. Fish were first confirmed positive for *P. salmonis* using a specific PCR reaction described by Mauel et al. (1996). The Reloncaví Estuary registered the highest number of fish positive for *P. salmonis* at 8.7%, followed in descending order by Castro, Hornopirén, Melinka, and Aysén.

Using the universal primers for eubacteria, it was possible to amplify the desired PCR product from samples of total DNA extracted from native fish species positive for *P. salmonis*. This amplicon was 1500 bp in length (data not shown). The PCR product was then used as a template for amplifying a specific region of the *P. salmonis* 16S RNA gene, ca. 1270 bp in length, by using the PS2S and 1490R primers (Table 1, Fig. 2). Furthermore, from samples that generated a product 1270 bp in length, the *P. salmonis* intergenic ITS-1 region was amplified, and, in all cases, yielded a PCR product of approximately 280 bp (data not shown). In some cases, the PCR products of 16S and ITS-1 were acquired through 2 rounds of amplification in order to facilitate cloning and sequencing. Additionally, the PCR products obtained from native fish corresponded in size to products obtained from the DNA of the *P. salmonis* PPT005 strain, which was grown in cell culture (Yañez et al. 2012). Moreover, despite the identification of *P. salmonis* DNA, autopsies of fish from which the samples were taken did not present pathognomonic signs that could be linked to those known for this causative agent in salmonids.

Phylogenetic analysis of *P. salmonis* 16S gene

The PCR products of the 16S gene were obtained by using the PS2S and 1490R primers (Table 1), and sequence analysis of these by the NCBI BLAST program confirmed that products corresponded to *P. salmonis* sequences. Phylogenetic analysis grouped the sequences obtained from native fish in the clade containing *P. salmonis* with 100% bootstrap support. The sequences obtained from *O. regia* samples were grouped in the clade comprising the Chilean EM-90 (U36940.1) strain with a bootstrap support of 92%. Those sequences obtained from *Salilota australis*, *E. maclovinus*, and *S. capensis* samples were grouped in

Table 3. Prevalence (prev.) of *Piscirickettsia salmonis* by species and geographical zone (see Fig. 1). RE: Reloncaví Estuary; Cas.: Castro; Horn.: Hornopirén; Mel.: Melinka

Species	Fish/ species	Positive by zone					Positive by species	
		RE	Cas.	Horn.	Mel.	Aysén	Total	Prev. (%)
Patagonian blenny *Eleginops maclovinus*	763	13	6		2	1	22	2.9
Pejerrey *Odontesthes regia*	275	2	2	7			11	4.0
Chancharro *Helicolenus lengerichi*	91	5					5	5.5
Chilean sandperch *Pinguipes chilensis*	79	2					2	2.5
Cape redfish *Sebastes capensis*	78	3	1		1		5	6.4
Pacific sandperch *Prolatilus jugularis*	39	1					1	2.6
Chilean hake *Merluccius gayi gayi*	35						0	0.0
Tadpole codling *Salilota australis*	19				1		1	5.3
Southwest Atlantic butterfish *Stromateus brasiliensis*	19		1				1	5.3
Speckled smooth-hound *Mustelus mento*	18						0	0.0
Eastern Pacific bonito *Sarda chiliensis*	15						0	0.0
Flounder *Paralichthys microps*	9		1				1	11.1
Black cusk eel *Genypterus maculatus*	5	1					1	20.0
Jack mackerel *Trachurus symetricus murphyi*	5						0	0.0
Elephant fish *Callorhynchus callorhynchus*	1						0	0.0
Total positive by zone		27	11	7	4	1	50	
Prevalence by zone (%)		8.7	3.5	2.4	1.4	0.4		
Total fish by zone		309	313	293	282	254		

the clade containing the Chilean CGA8076 2012 (KC473425.1) strain of *Oncorhynchus kisutch* with a bootstrap support of 59% (Table 2, Fig. 3). It is important to note that this final clade was also grouped with the Scottish strains SCO-02A (AY498635.1) and SCO-95A (AY498636.1).

Phylogenetic analysis of *P. salmonis* ITS-1 gene

Sequencing of 280 bp PCR products, as obtained by amplifying the ITS-1 of native fish total DNA, and analysis through BLAST confirmed that the sequences corresponded to fragments of the 16S-23S internal transcribed spacer of *P. salmonis*. Phylogenetic analysis organized the ITS-1 sequences into clades, where *P. salmonis* ITS-1 sequences obtained from *O. regia* and *E. maclovinus* were grouped in the clade containing the Chilean EM-90 (U36940.1) and al10015 (EU289216.1) strains with a bootstrap support of 99%. On the other hand, *P. salmonis* ITS-1 sequences found in *S. capensis* and *S. australis* were placed in the clade of the Chilean LF-89 (U36941.1) strain with a bootstrap support of 91% (Table 2, Fig. 4).

With this information, a sequence identity matrix was created to estimate the evolutionary divergence

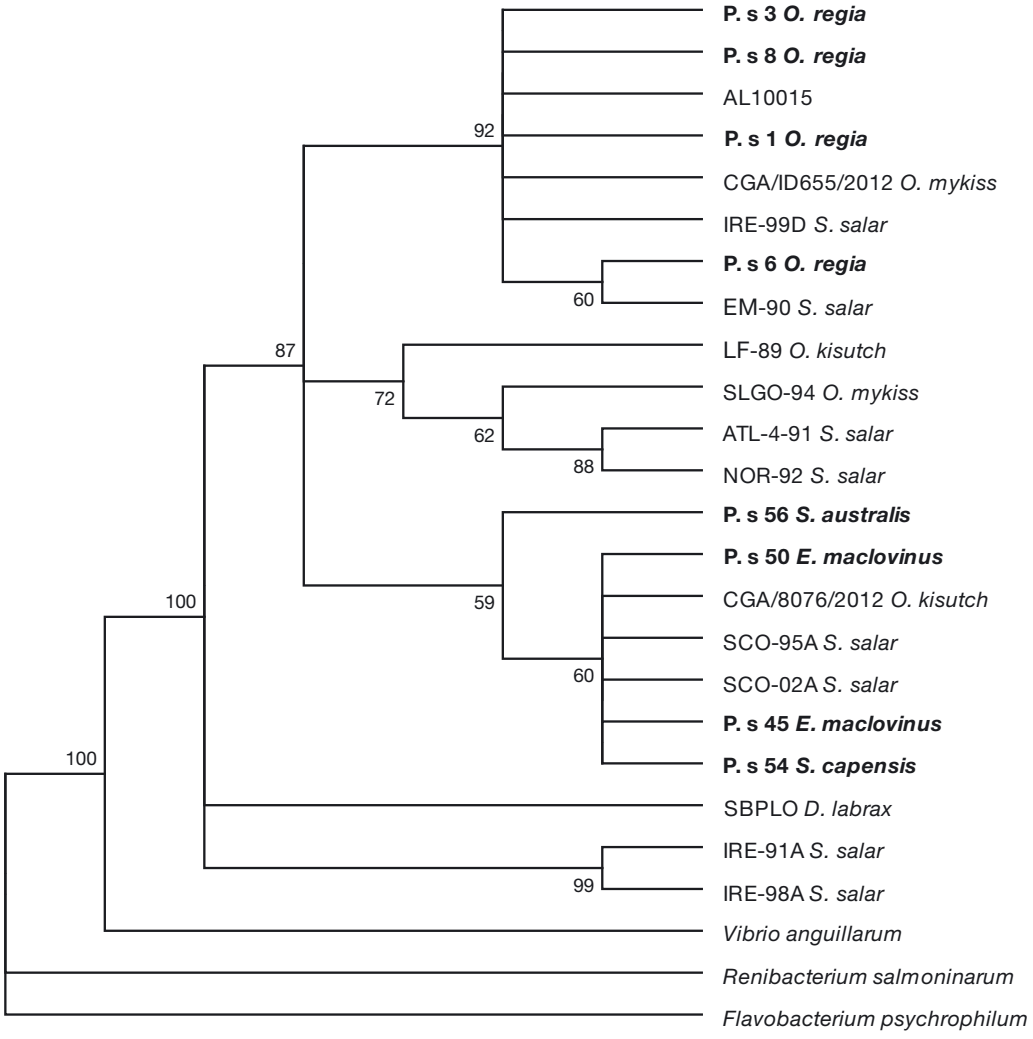

Fig. 3. Phylogenetic relationships of partial 16S rDNA sequences obtained in this study and from *Piscirickettsia salmonis* strains. Inferences were made through the neighbor-joining method, with *Vibrio anguillarum* (GenBank accession no. JX966409.1), *Renibacterium salmoninarum* (GenBank accession no. NR_074198.1), and *Flavobacterium psychrophilum* (GenBank accession no. NR_074630.1) used as outgroups. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 1142 positions between nucleotide positions 233 and 1471 of *P. salmonis* LF-89. See Table 2 for full species names. The number after P.s (*P. salmonis*) identifies the DNA of each sample analyzed

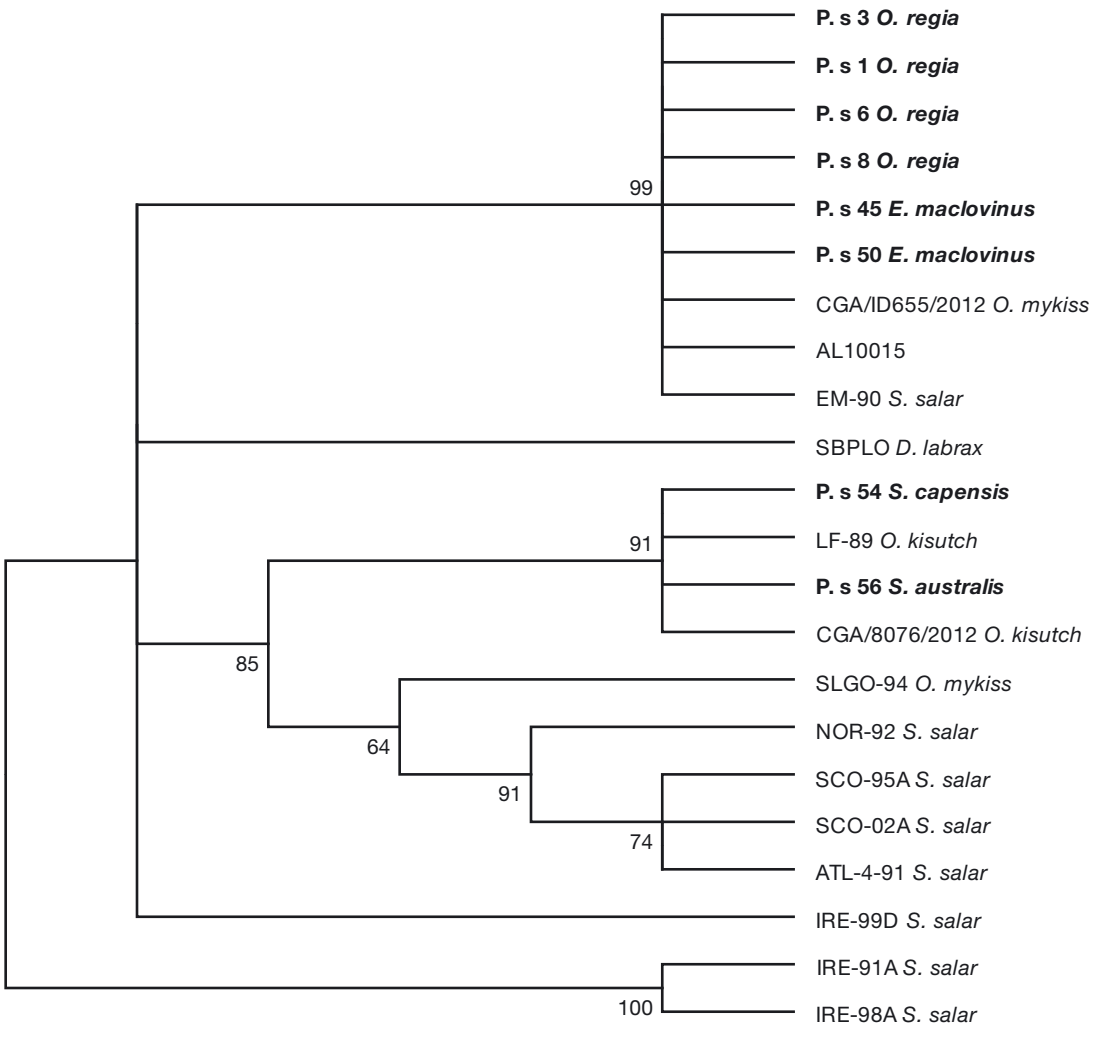

Fig. 4. Phylogenetic relationships of partial internal transcribed spacer sequences obtained in this study and from *Piscirickettsia salmonis* strains. Inferences were made through the neighbor-joining method. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 175 positions between nucleotides 399 and 622 from the 5′ end of the *P. salmonis* LF-89 ITS sequence. See Table 2 for full species names. The number after P.s (*P. salmonis*) identifies the DNA of each sample analyzed

between sequences (Table 4). Analysis involved 22 nucleotide sequences, with 195 positions analyzed in the final data set by using maximum composite likelihood (Tamura et al. 2007) and the MEGA 5 software. All positions containing gaps or missing data were eliminated. Comparison of the ITS-1 sequences demonstrated 100% identity between the sequences analyzed from *E. maclovinus* and *O. regia* to the ITS-1 sequence from the Chilean EM-90 strain. Likewise, the sequences from *S. australis* and *S. capensis* showed 100% and 99.91% identity, respectively, with the ITS-1 sequence of the Chilean LF-89 strain.

DISCUSSION

Studies in a variety of non-salmonid fish species have examined the presence of *Piscirickettsia salmonis* and of *Piscirickettsia*-like organisms (PLOs) using techniques such as bacterial isolation and DNA and antigen identification. In fact, *P. salmonis* has been identified as the causative agent of granulomatous lesions in the liver, spleen, intestine, and gills in farmed white sea bass *Atractoscion nobilis* from southern California (strain WSB-98). It is noteworthy that this strain was first described as a PLO (Chen et al. 2000a), but it was confirmed to be *P. salmonis*

Table 4. Estimates of evolutionary divergence between *Piscirickettsia salmonis* sequences (see Table 2 for sequence details). The number of base substitutions per site between sequences is shown. Analyses were conducted using the maximum composite likelihood model, and analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 195 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. CGA 8076 2012 *O. kisutch*																					
2. P. s 56 *S. australis*	0.000																				
3. P. s 54 *S. capensis*	0.009	0.009																			
4. LF-89 *O. kisutch*	0.000	0.000	0.009																		
5. SCO-02A *S. salar*	0.028	0.028	0.037	0.028																	
6. SCO-095A *S. salar*	0.023	0.023	0.032	0.023	0.004																
7. ATL-4-91 *S. salar*	0.023	0.023	0.032	0.023	0.004	0.000															
8. NOR-92 *S. salar*	0.028	0.028	0.037	0.028	0.009	0.004	0.004														
9. SLGO-94 *O. mykiss*	0.014	0.014	0.023	0.014	0.023	0.018	0.018	0.023													
10. EM-90 *S. salar*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042												
11. AL10015	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000											
12. CGA ID655 2012 *O. mykiss*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000										
13. P. s 50 *E. maclovinus*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000									
14. P. s 45 *E. maclovinus*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000	0.000								
15. P. s 8 *O. regia*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000	0.000	0.000							
16. P. s 6 *O. regia*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000	0.000	0.000	0.000						
17. P. s 3 *O. regia*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
18. P. s 1 *O. regia*	0.037	0.037	0.047	0.037	0.062	0.057	0.057	0.052	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
19. IRE-99D *S. salar*	0.067	0.067	0.077	0.067	0.093	0.088	0.088	0.082	0.083	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067			
20. IRE-91A *S. salar*	0.124	0.124	0.135	0.124	0.146	0.140	0.140	0.133	0.135	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.152		
21. IRE-98A *S. salar*	0.107	0.107	0.118	0.107	0.128	0.122	0.122	0.116	0.118	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.134	0.014	
22. SBPLO *D. labrax*	0.047	0.047	0.057	0.047	0.062	0.057	0.057	0.062	0.047	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.042	0.088	0.122	0.116

through a partial sequence analysis of the 16S rRNA gene (816 bp) and ITS-1 (203 bp) (Arkush et al. 2005). This finding was complemented through experimental infections using this species, which found the bacteria up to 123 d post-infection not only through histology and specific PCR analyses but also through the detection of anti-*P. salmonis* immunoglobulins by ELISA (Arkush et al. 2006). Likewise, this bacterium was also described as a PLO in European sea bass *Dicentrarchus labrax*, but by amplifying and sequencing the 16S rDNA and ITS-1 genes, a close relationship with *P. salmonis* was found. Additionally, this isolate shared antigens with the LF-89 strain, as analyzed through the use of antibodies against *P. salmonis* in an indirect immunofluorescence antibody test and immunohistochemical assay (McCarthy et al. 2005).

Previous studies have established a precedent of finding *P. salmonis* in non-salmonid species. In our study, it is important to mention that it was not possible to isolate the bacterium from the positive species that showed no evident signs of diseases. Likewise, the 2 most-commonly sampled species, comprising >70% of total samples (*O. regia* and *E. maclovinus*; Table 3) corresponded to endemic species that have wide distributions along the southern coastline of Chile (Silva et al. 2001, Licandeo et al. 2006), and these species might be carriers of the bacterium. Building on this, we were able to amplify, clone, and sequence a 1270 bp fragment of the *P. salmonis* 16S rRNA gene from samples of total DNA obtained from *E. maclovinus, O. regia, S. capensis,* and *S. australis*. Combining the specific primers described for the *P. salmonis* 16S gene (Mauel et al. 1996, McCarthy et al. 2005) played an important role in obtaining a specific DNA fragment, which had almost 85% identity to the *P. salmonis* 16S gene sequence. This allowed us to study the phylogenetic relationship of the obtained sequences, taking into consideration that the bacteria could not be isolated from the studied fish. Specifically, the sequences obtained from the 16S gene were aligned with sequences found by other studies to have close relationships (LF-89, ATL-4-91, NOR-92, SGLO-94, and EM-90; Mauel et al.

1999). Interestingly, sequences identified in *O. regia* were those most closely related to the EM-90 strain sequence. The EM-90 and LF-89 strains were first described in Chile in coho salmon and Atlantic salmon, respectively (Fryer et al. 1990, Mauel et al. 1996), suggesting that these isolates have been maintained over time and can also be found in native species.

As previously mentioned, initial studies performed by Mauel et al. (1999) reported the EM-90 strain to be less related to other studied strains, as based on a lesser degree of nucleotide identity in the sequences of ribosomal genes (Rozas & Enríquez 2014). Moreover, restriction sites specific to the EM-90 strain were found, thereby suggesting that restriction fragment length polymorphism could be used to differentiate the EM-90 strain from other strains (Mauel et al. 1999, Rozas & Enríquez 2014). On the other hand, sequences obtained from *E. maclovinus, S. capensis*, and *S. australis* were related to 1 Chilean *P. salmonis* strain and 2 Scottish strains, SCO-95A and SCO-02A, which are also closely related to the Norwegian NOR-92 and Canadian ATL-4-91 strains (Reid et al. 2004).

When analyzing the PCR product of *P. salmonis* ITS-1, a single amplicon of approximately 280 bp was obtained, with no other products observed under the amplification conditions used. Additionally, subsequent studies described 2 ITS-1 regions (A and B) for some isolates of *P. salmonis*, including the EM-90 and LF-89 strains, therefore suggesting that more than 1 ribosomal operon could be present in the genome of this bacterium (Casanova et al. 2001, 2003), where the ITS-1 B sequence was found to be interrupted by 2 tRNA genes ($tRNA^{Ile}$ and $tRNA^{Ala}$). These findings were confirmed by Casanova et al. (2003) using 11 Chilean isolates of *P. salmonis* originating from different species and geographic localities; the results indicated that 2 groups of ITS-1 exist, those with high or low electrophoretic mobility in polyacrylamide gels, including the LF89 and EM-90 strains, respectively (Casanova et al. 2003). In a PLO isolated from European sea bass, 2 regions were found for ITS-1, of which 1 region contained the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, as was found in isolates from salmonid species (Arkush et al. 2005). Finally, the sequence identity matrix in the present study showed that within the sequence size range analyzed, the ITS-1 sequences of the Chilean strains AL10015, IBM001, and EM-90 had 100% identity with sequences from *O. regia* and *E. maclovinus*. This would confirm the close phylogenetic relationship of *P. salmonis* described in native fish and cultured salmonids in Chile.

Taken together, these findings become relevant and suggest a potential mode of infection by *P. salmonis* from native to cultivated fish species and/or vice versa, which could provide an answer to the *P. salmonis* infections found in marine-cultivated salmonids in Chile. It is important to determine whether these native species are passive carriers of *P. salmonis* or are susceptible to infection and multiplication of the bacteria in their organs. Additionally, there is the possibility that ectoparasites present in the marine environment could act as vectors for *P. salmonis* (Fryer et al. 1990). Although a reservoir has not yet been identified, Olivares & Marshall (2010) postulated that marine invertebrates commonly found in association with Chilean salmonid cultivations could play this role. Another important aspect to consider is the capacity of the bacteria to survive in seawater for extended periods of time (Lannan & Fryer 1994), with free-living bacteria having been found after up to 40 d in seawater (Olivares & Marshall 2010). This survival capacity could be related to the development of extracellular aggregates by the bacteria in response to stressful conditions (Marshall et al. 2012). The aggregate compososition is suggested to be exopolysaccharides similar to biofilms; *P. salmonis* was identified embedded in this matrix, suggesting adaptive survival and persistence strategies under marine conditions (Marshall et al. 2012). Finally, our results indicate the presence of *P. salmonis* in native fish species and show that the genomic material has a phylogenetic relationship to DNA sequences of the first *P. salmonis* strains described in Chile and isolated in cultured salmonids.

Acknowledgements. This work was supported by IFOP and Fondap 15110027, Interdisciplinary Center for Aquaculture Research (INCAR), Chile.

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: October 27, 2014; Accepted: June 11, 2015
Proofs received from author(s): August 18, 2015