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Streptococcus phocae, AN EMERGING PATHOGEN FOR SALMONID CULTURE

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Abstract

This work describes the characterization of the causal agent of disease outbreaks that, from 1999, occurred repeatedly during the summer months (temperatures higher than 15ºC) in Atlantic salmon (*Salmo salar*) cage-farmed in Chile affecting both smolts and adult fish cultured in estuary and marine waters, reaching in some occasions a cumulative mortality up to 25% of the affected population. Diseased fish showed exophthalmia with accumulation of purulent and haemorrhagic fluid around eyes, and ventral petechial haemorrhages. At necropsy, haemorrhage in the abdominal fat, pericarditis, and enlarged liver, spleen and kidney are common pathological changes. Gram-stained smears revealed the presence of Gram-positive cocci, □eta-hemolytic, negative for oxidase and catalase tests. Although biochemical characterization of the isolates using the miniaturized system rapid ID 32 Strep suggested their assignation to genus *Gemella*, sequencing and RFLP analysis of the 16S rRNA revealed that bacteria associated with the mortalities belong to *Streptococcus phocae*. Serological studies demonstrated that all the salmon isolates are antigenically homogeneous, which can facilitate the development of preventive measures and, although sharing some antigenical determinants, they belong to a different Lancefield group than the type strain isolated from seals. On the basis of these facts, we conclude that the species *S. phocae* is an emerging pathogen for salmonid culture in Chile, and it should be included as a new member of the warm water streptococcosis.

Key words: Streptococcus phocae, Atlantic salmon, Emerging pathogen

Introduction

Fish stretptococcosis, is currently considered as one of the main limiting factors in the aquaculture industry, due to the significant economic losses (higher than $ 150 million) that these infections cause in different cultured fresh- and seawater fish species worldwide (Austin and Austin, 2007; Ringø and Gatesoupe, 1998; Romalde and Toranzo, 2005). The first description of a streptococcal infection causing rainbow trout (*Oncorhynchus mykiss*) mortalities dates from 1956 (Hoshina *et al.*, 1958). Since then, the disease has increased its host range, including yellowtail (*Seriola quinqueradiata*), salmonids, Japanese eel (*Anguilla japonica*), tilapia (*Oreochromis* spp.), striped bass (*Morone saxatilis*) and turbot (*Scophthalmus maximus*), among others, as well as its geographical distribution (Austin and Austin, 2007; Ringø and Gatesoupe, 1998; Romalde and Toranzo, 2005).

There has been an important controversy about the number and the nature of the bacterial species involved with streptococcosis (Austin and Austin, 2007), probably due to the difficulty of an accurate identification at species level within this bacterial group when using conventional microbiological criteria. With the development of molecular taxonomic techniques, it was possible to more accurately determine the precise taxonomic status of many isolates. Currently, there is general acceptance of the division of streptococcosis into two forms according to the virulence of the agents involved at high or low temperatures (Ghittino, 1999). "Warm water" streptoccosis, causing mortalities at temperatures higher than 15ºC, typically involves species such as *Lactococcus garvieae* (synonym *Enterococcus seriolicida*), *Streptococcus iniae* (synonym *S. shiloi*), *S. agalactieae* (synonym *S. difficilis*) or *S. parauberis*. On the other hand, "cold water" streptococcosis is caused by *Vagococcus salmoninarum* or *L. piscium* and occurs at temperatures below 15ºC.

Streptococcus phocae, a beta-hemolytic streptococcal species, was first isolated from clinical specimens of harbor seal (*Phoca vitulina*) affected by pneumonia (Skaar *et al.*, 2003). Further studies described isolations of this species from other marine mammals including Cape fur seal (*Arctocephalus pusillus pusillus*), ringed seal (*Phoca hispida*) and harbor porpoise (*Phocoena phocoena*) and gray seal (*Halichoerus grypus*) and cetaceans (Henton *et al.*, 1999; Lawson *et al.*, 2004; Raverty and Fiessel, 2001; Raverty *et al.*, 2004; Vossen *et al.*, 2004), associated in general to respiratory infections.

In this paper, the biochemical, serological and genetic characterization of the streptococcal strains isolated during several years from salmon showing septicemic disease is described. In addition, we present data on their implication in the appearance and progression of the disease in Chile from its first observation in 1999, the possible origins, as well as the clinical significance for the Chilean salmon industry.

Materials and Methods

Bacterial strains. A total of 34 strains isolated from internal organs (kidney, spleen or brain) or from ulcers of diseased cage-cultured Atlantic salmon in Chile were studied (Table 1). In addition, the type strain DSMZ 15635 of *Streptococcus phocae*, isolated from seal in Norway, was also analyzed for comparison. The bacteria were routinely grown on Columbia sheep blood agar (CBA)(Oxoid Ltd., Basingstone, England) and on tryptone soy agar supplemented with 1% NaCl (TSA-1) (Difco Laboratoires, Detroit, Mich.) under aerobic conditions at 22-25ºC for 48h. Stock cultures were maintained frozen at -70ºC in criobille tubes (AES Lab., France) or in tryptone soy broth (TSB-1) with 15% glycerol.

Phenotypic tests. All the strains were subjected to taxonomical analysis by standard morphological, physiological, and biochemical plate and tube tests (Facklam, 2002; McFaddin, 1983; Schleifer, 1986). The ability of the strains to grow at 4, 10, 25, 30 and 37ºC was tested in TSA-1 over a period of 10 days. Growth at different salinities was determined in basal medium (neopeptone 4g/L, yeast extract 1g/L, agar 15g/L) supplemented 0, 0.5, 3, 6.5 and 8% NaCl after 1 week incubation.

The commercial Rapid ID 32 Strep and API ZYM miniaturized systems (bioMeriux, Madrid, Spain) were used following the manufacturer's instructions except for the temperature of incubation which was set at 25ºC. Standardized inocula were prepared by suspending cells in tubes containing sterile distilled water and adjusted spectrophotometrically to D.O of 1.0 (A_{580})(Ravelo *et al.*, 2001).

The drug sensitivities of the strains were determined by the disc diffusion method on Muller-Hinton agar (Oxoid) with 1% NaCl. The following chemotherapeutic agents (micrograms per disc) were used: oxolinic acid (10), trimethoprim-sulfametoxazole (23.75/1.25), amoxycillin (25), oxytetracycline (30), erythromycin (15), and florfenicol (30).

Genetic characterization.

Genetic characterization by sequencing of the 16S rRNA gene was also carried out. Isolation of DNA from pure cultures was performed using the "Instagene" matrix (Bio-Rad), following manufacturer's recommendations. 16S rRNA genes were amplified by PCR with universal primers pA and pH (Hutson *et al.*, 1993). Primers (Funke *et al.*, 1995) corresponding to internal conserved regions of this gene were used for the sequencing reactions in an Eppendorf Mastercycler Personal (Eppendorf, Germany), with the CEQ-Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, USA). Sequencing products were analyzed using a CEQ 8000 Sequencer (Beckman Coulter, USA). Comparative sequence analyses were conducted using FASTA3 program. Sequences of the closest relatives were retrieved from Gene bank/EMBL, and the alignments and phylogenetic analysis were performed by the Neighbour-joining method using the ClustalX (Thompson *et al.*, 1997). Bootstraps were calculated with 1000 replications, and *Streptococcus ferus* (AY058218) was used as out-group sequence. Phylogenetic tree was drawn using the software NJPlot (Perrière and Gouy, 1996).

In addition, adscription of the salmon isolates to the species *S. phocae* was also confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene using the restriction enzymes *Ear*I and *Hinc*II (BioLabs, Schalbach, Germany) as described by Vossen *et al.* (2004).

Serological assays

Antiserum against the salmon isolate 2857 was prepared by intravenous injections of rabbits with formalin-killed cells suspended in saline as previously described (Magariños *et al.*, 1992). This protocol was approved by the Ethics Committee for Animal Experiments of the University of Santiago. Whole cell preparations of the isolates were employed in all the serological assays performed. Slide agglutination tests were conducted following the procedures of Toranzo *et al.* (1987) and the dot blot assays were performed as described by Cipriano *et al.* (1985).

The Lancefield streptococcal grouping (Lancefield, 1933) for the fish isolates was confirmed using Diagnostic Reagents (Oxoid Ltd., Basingstone, England) according to the manufacturer's protocol. The strains *Streptococcus agalactiae* S/246 (serogroup B), group G *Streptococcus* S/299 and *Streptococcus mitis* S/290 were included for quality control in every test run and were grown as described above. These strains were obtained from the Instituto de Salud Pública, Chile.

Virulence for fish and mice

In order to determine the virulent capacities of the isolates, infectivity trials were conducted using different fish species and mice. Representative isolates (Au15, 2857, and C4) were intraperitoneally (i.p.) inoculated in Atlantic salmon (20 g) and rainbow trout (15 g). Prior to challenge, fish were randomly sampled to be subjected to microbiological analysis which indicated that they were free of *S. phocae* and other pathogens. Groups of 15 fishes were inoculated (0.1 µl) with different bacterial doses ranging from 10^4 to 10^9 cells/fish. Control fish received 0.1 µl of saline solution (0.85% NaCl). In addition, one group of 15 Atlantic salmon (20 g) was subcutaneously inoculated (0.1 µl/fish) with of two doses (10^5 and 10^6 cells/fish) of the isolate Au15. Fish were maintained in seawater tanks at 19 ± 1ºC with aeration during the course of the experiments (21 days) making a daily 50% water renovation. Fish mortality was daily checked during this period. Dead fish were microbiologically analyzed for re-isolation of the inoculated strain. The degree of virulence was expressed as lethal dose 50% (LD_{50}) calculated as described by Reed and Müench (1938).

To determine the pathogenic potential for homoiotherm animals, a mouse virulence assay was performed including three isolates from Atlantic salmon and the *S. phocae* type strain DSMZ 15635^T. Briefly, groups of 5 BALB/C mice (10-12 weeks old, 21-25 g) were inoculated i.p. with bacterial dose of 10^7 cells (0.1 µl/mouse). Mortalities were recorded daily during a period of 10 days. Strain CDC 2227-81 of *Photobacterium damselae* subsp. *damselae*, previously described as pathogenic for mice (Osorio *et al*., 2000) was included in the assay as positive control.

The Ethics Committee for Animal Experiments of the University of Santiago approved the protocols of this study.

Results

From 1999, disease outbreaks occurred repeatedly during the summer months (temperatures higher than 15ºC) in Atlantic salmon (*Salmo salar*) farmed in Chile affecting both smolts and adult fish cultured in floating cages in estuary and marine waters. The disease presents a rapid time course with appearance of clinical signs in 1 or 2 days, although in acute cases fish can not show any sympthom. Isolates studied in this work were isolated from 28 independent outbreaks, showing cumulative mortalities between 3 and 25% of the affected population. From its first appearance

until 2003, the disease has only been detected in waters of the Reloncaví estuary (X Chilean Region), being further spread to other Chilean Regions, in some cases associated to fish with origin in Reloncaví but sold and transported as smolts.

Diseased fish showed exophthalmia with accumulation of purulent and haemorrhagic fluid around eyes, ventral petechial haemorrhages, skin abscesses and, in some cases, muscle liquefaction with formation of deep ulcerative areas (Fig. 1). At necropsy, haemorrhage in the abdominal fat, pericarditis with accumulation of a white fluid in the pericardic cavity, and enlarged liver (showing a yellowish colour), spleen and kidney are common pathological changes (Fig. 1). Gram-stained smears revealed the presence of Gram-positive cocci in all cases.

The microbiological analysis of internal organs (kidney, spleen and brain) and ulcers revealed the presence on CBA, in pure culture or as major colony type, of pinpoint white colonies (0.5 mm diameter). The isolated bacteria were Gram positive chain-forming cocci (0.6-0.9 μm diameter), beta-haemolytic, negative for the catalase and aesculin hydrolysis tests, growing at 25 and 37ºC and at pH 9.6 but not with 6.5% NaCl or 40% bile salts. These results indicated that the bacteria could be assigned presumptively to the Genus *Streptococcus*.

When the miniaturized system Rapid ID 32 Strep was employed, 30 out of 34 Atlantic salmon isolates rendered the profile 04000000000, showing a positive reaction only for the phosphatase alcaline test. Four strains rendered the same profile than the type strain, 04000001000, being positive also for the utilization of maltose. With both profiles, strains were classified according to the API database as belonging to the Genus *Gemella* (*G. haemolysans* or *G. morbillorum*). On the other hand, all the isolates and the type strain showed the same enzymatic patterns in the API ZYM system, being positive for the production of alkaline and acid phosphatases and leucine arylamidase.

All the isolates showed the same pattern of resistence/susceptibility to chemotherapeutic agents *in vitro*, being susceptible to amoxycillin, oxytetracycline, erythromycin and florfenicol and resistant to flumequine, oxolinic acid and trimethoprim-sulfametoxazole.

Sequencing analysis of the 16S rRNA gene revealed that all the Atlantic salmon isolates studied were identical, and that the sequences obtained showed similarities higher than 98.18% with *S. phocae* (98.18 and 100 % identity with sequences AF235052 and AJ621053, respectively)(Fig. 2). The next relatives were *S. pyogenes*

and *S. agalactiae* with similarities slightly higher than 93%. Moreover, in the RFLP analysis all the isolates rendered the expected fragments employing either *Hinc*II or *Ear*I endonucleases. Restriction with the enzyme *Hinc*II restriction yielded two characteristic fragments with sizes of 180 and 1230 bp) (Fig. 3), whereas *Ear*I rendered three DNA fragments with sizes of 170, 380, and 840 bp (data not shown).

Serological studies using different techniques, such as slide agglutination and dot blot, with specific serum raised against the Atlantic salmon isolate 2857, demonstrated that all the salmon isolates are antigenically homogeneous, sharing antigenic determinants with the type strain isolated from seals (Table 1). However, when the commercial Lancefield streptococcal grouping kit was employed, the isolates from fish were classified as group G, although showing also a weak cross-reactivity with serum from antigenic group A, while the type strain DSMZ 15635^T was assigned to serogroup F.

The three isolates inoculated were pathogenic for Atlantic salmon with LD_{50} values ranging from 1.9 x 10^5 and 5.6 x 10^5 bacteria per fish but not for rainbow trout when i.p. inoculated. Mortalities began two days after inoculation, being observed a dramatical increase by day 6. No mortalities were observed in the control groups. Inoculated bacteria could be recovered from dead fish which showed in all cases the typical necrotic lesions observed in the natural outbreaks. On the other hand, subcutaneously inoculated salmon showed a gross inflammation in the injection area, which progressed to necrosis with caseous material and liquefaction. Mortalities started 4 days after inoculation reaching 80 and 100% for the low and high dose respectively. None of the *S. phocae* isolates, including the type strain, were pathogenic for mice, being observed no mortalities during ten days. Three out of the 5 BALB/C mice inoculated with the strain of *P. damselae* subsp. *damselae* utilized as positive control, died during the same period.

Discussion

Streptococcus phocae, a member of the pyogenic streptococcal group (Köhler, 2007) has been described as an organism associated with disease and mortality of different seal species in Norway, German North and Baltic seas and South Africa (Henton *et al.*, 1999; Skaar *et al.*, 1994; Vossen *et al.*, 2004). More recently Gibello *et al.* (2005), during the development of a PCR protocol for *Streptococcus iniae*, employed some streptococcal strains from the bacterial collection at the FRS marine Laboratory in

Aberdeen, Scotland, originally isolated from Atlantic salmon in Chile which after their genetic characterization were also assigned to the species *S. phocae*. These authors indicated the necessity to determine the real clinical significance of this bacterial species for the Chilean salmon industry. In this work, a collection of 34 strains of *S. phocae* isolated from diseased Atlantic salmon were biochemical, serological and genetically characterized and data on the appearance and progression of the disease in Chile from its first observation in 1999 were presented, in order to get a better understanding of this pathology.

The taxonomical analysis of the Atlantic salmon isolates revealed a great homogeneity, regardless the year of isolation or geographical area of origin, and that they were nearly identical to the type strain of this bacterial species. Such homogeneity has been previously described by other authors for *S. phocae* (Gibello *et al.*, 2005; Henton *et al.*, 1999; Skaar *et al.*, 1994; Vossen *et al.*, 2004). Although the preliminary characterization indicated their assignation to the genus *Streptococcus*, when the miniaturized Rapid ID 32 Strep system was employed, the profiles obtained corresponded to good identification of members of the genus *Gemella*, mainly *G. haemolysans*. However, although it has been reported that differentiation of some streptococcal groups and *Gemella* strains is not easy (Lee *et al.*, 2004), these later organisms are alpha-haemolytic Gram variable cocci forming pairs or tetrads, properties completely different from those of the salmon isolates. Moreover, misidentifications of *S. phocae* strains using this miniaturized system were also reported by Gibello et al. (2005), although these authors observed profiles typical from *Gardnerella vaginalis*. A reason for these misidentifications can be that *S. phocae* is not included in the API Database, which is focused mainly in human pathogens.

The development and application of molecular techniques have greatly facilitated the identification and classification of streptococci (Bentley and Leigh, 1995; Romalde and Toranzo, 2005; Täpp *et al.*, 2003), having the advantages of time-saving and accuracy. The use of molecular methods, such as sequencing or RFLP analysis of the 16S rRNA gene, in the identification of the Atlantic salmon isolates unequivocally demonstrated that all of them belong to the species *S. phocae*.

Serological typing is an effective approach in the epidemiological study of various Gram-positive bacteria. The results from the Lancefield typing, using reagents of the

different antigenic groups, showed that the fish isolates could be assigned to antigenic group G although displaying also a weak reaction with antiserum A. *S. phocae* strains from seals have been described as belonging to Lancefield's groups C and F (Skaar *et al.*, 1994; Vossen *et al.*, 2004). In fact, the reference strain (DSMZ 15635^T) from seal included in the study belonged to the Lancefield's group F. Therefore, the Atlantic salmon isolates represent a distinct serological group within *S. phocae*. On the other hand, all isolates, including the type strain, reacted with the antiserum raised in rabbit against the strain 2857, indicating the existence of some common antigenic components among salmon and seal isolates of *S. phocae*, probably in the cell envelope.

From the data obtained, it seems clear that movement of fish, in particular smolts, between farms located in different geographical areas has played an important role in the spreading of the diseased across Chile. More difficult is to find the origin for the first appearance of the disease in the Reloncaví estuary. We can speculate that a possible origin can be the numerous colonies of marine mammals present in these Chilean regions (Schlatter and Hucke-Gaete, 1999; Sielfeld, 1999). Marine mammals capture cultured salmon through the cage nets, and also cause a great stress with subsequent apetite losses and high susceptibility to diseases (Arnold, 1992). Further studies are needed to confirm this hypothesis.

Although some chemotherapeutants were effective *in vitro*, oral treatments with oxytetracycline (80-120 mg/kg fish for 15-20 days), erythromycin (50-70 mg/kg fish for 7-14 days) or florfenicol (15-20 mg/kg fish for 15 days) reduced mortalities only in part and during the treatment period when applied in the fish farms. The low efficacy of the chemotherapy in controlling this streptococcal disease in Atlantic salmon has led to an effort in developing preventive measures, including the use of auto-vaccines which together with an early detection of clinical signs, maintenance of fish at low densities, cleaning of the cage nets, etc., helped to decrease the impact of *S. phocae* in the cultured fish.

In summary, and on the basis of the facts presented, we conclude that the species *S. phocae* is an emerging pathogen for salmonid culture in Chile, and it should be included as a new member of the warm water streptococcal syndrome.

Nucleotide sequence accession number.

The nucleotide sequence corresponding to the 16S rRNA gene of *S. phocae* 2857 was deposited in the GenBank/EMBL Database as representative of the Atlantic salmon isolates, and it has been assigned the accession number EF599165.

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Figure Legends

Fig. 1.- Clinical signs observed in the diseases Atlantic salmon. a) hemorhages around the eye; b) ventral petechial haemorrhages; c) ventral skin abscesses; d) deep ulceras with muscle liquefation; e) pericarditis with accumulation of a white fluid (arrow) in the pericardic cavity; f) haemorrhage in the abdominal fat and cavity; g) enlarged liver and spleen.

Fig. 2.- Phylogenetic tree of 16S rRNA sequence for the Atlantic salmon isolates and the type strains of the closest streptococci. *Streptococcus ferus* (AY058218) was employed as outgroup. Horizontal branch lengths are proportional to evolutionary divergence. Significant brootstrap values of 1000 replicates appear next tyo the corresponding branch. The bar represents 1% sequence divergence. Sequences from relative species were obtained from GeneBank Database, and their accesion numbers are indicated after the species name.

Fig. 3.- Restriction of the amplified 16S rRNA gene with endocnuclease *Hinc*II. Lanes: M, molecular size marker (50-2000 ladder; Sigma); 1 and 2, amplicon and correspondent restriction from *S. phocae* DSMZ 15635^{T}; 3 to 7, amplicons and correspondent restrictions from Atlantic salmon isolates 2857, C-4 and PF-157, respectively. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified products and restriction fragments in bp.

Table 1.- Origin and serological characterization of the *Streptococcus phocae* isolates used in this study.

Isolate	Origin	Year of Isolation	Reaction with serum against isolate 2857	Lancefield group
Atlantic salmon isolates				
7053-1	Reloncaví Estuary, Chile	1999	+	G
9547-1	Reloncaví Estuary, Chile	1999	+	G
2743-1	Reloncaví Estuary, Chile	2001	+	G
2857	Reloncaví Estuary, Chile	2001	+	G
3385	Reloncaví Estuary, Chile	2001	+	G
3618	Reloncaví Estuary, Chile	2001	+	G
3770	Reloncaví Estuary, Chile	2002	+	G
344	Reloncaví Estuary, Chile	2004	+	G
751	Reloncaví Estuary, Chile	2004	+	G
2880	Reloncaví Estuary, Chile	2004	+	G
5070	Reloncaví Estuary, Chile	2004	+	G
C-4	Reloncaví Estuary, Chile	2004	+	G
Au11	Herradura, Chile	2004	+	G
Au13	Hualaihué, Chile	2004	+	G
Au14	Reloncaví Bay, Chile	2004	+	G
Au15	Curaco de Lin Lin, Chile	2004	+	G
PF-132	Chaitén, Chile	2004	+	G
PF-133	Herradura, Chile	2004	+	G
PF-136	Reloncaví Bay, Chile	2004	+	G
PF-137	Reloncaví Estuary, Chile	2004	+	G
PF-140	Calbuco, Chile	2004	+	G
PF-142	Comau Fiord, Chile	2004	+	G
PF-143	Reloncaví Estuary, Chile	2004	+	G
PF-145	Marimelli, Chile	2004	+	G
PF-146	Marimelli, Chile	2004	+	G
PF-148	Quillaipe, Chile	2004	+	G
PF-153	Quillaipe, Chile	2004	+	G
PF-155	Calbuco, Chile	2004	+	G
PF-030	Punta Venet, Chile	2005	+	G
PF-056	Castro, Chile	2005	+	G
PF-058	Castro, Chile	2005	+	G
PF-059	Castro, Chile	2005	+	G
PF-134	Chaitén Chile	2005	+	G
PF-157	Reloncaví Estuary, Chile	2005	+	G
Reference strain				
DSMZ 15635^T	*Phoca vitulina*, Norway	1994	+	F

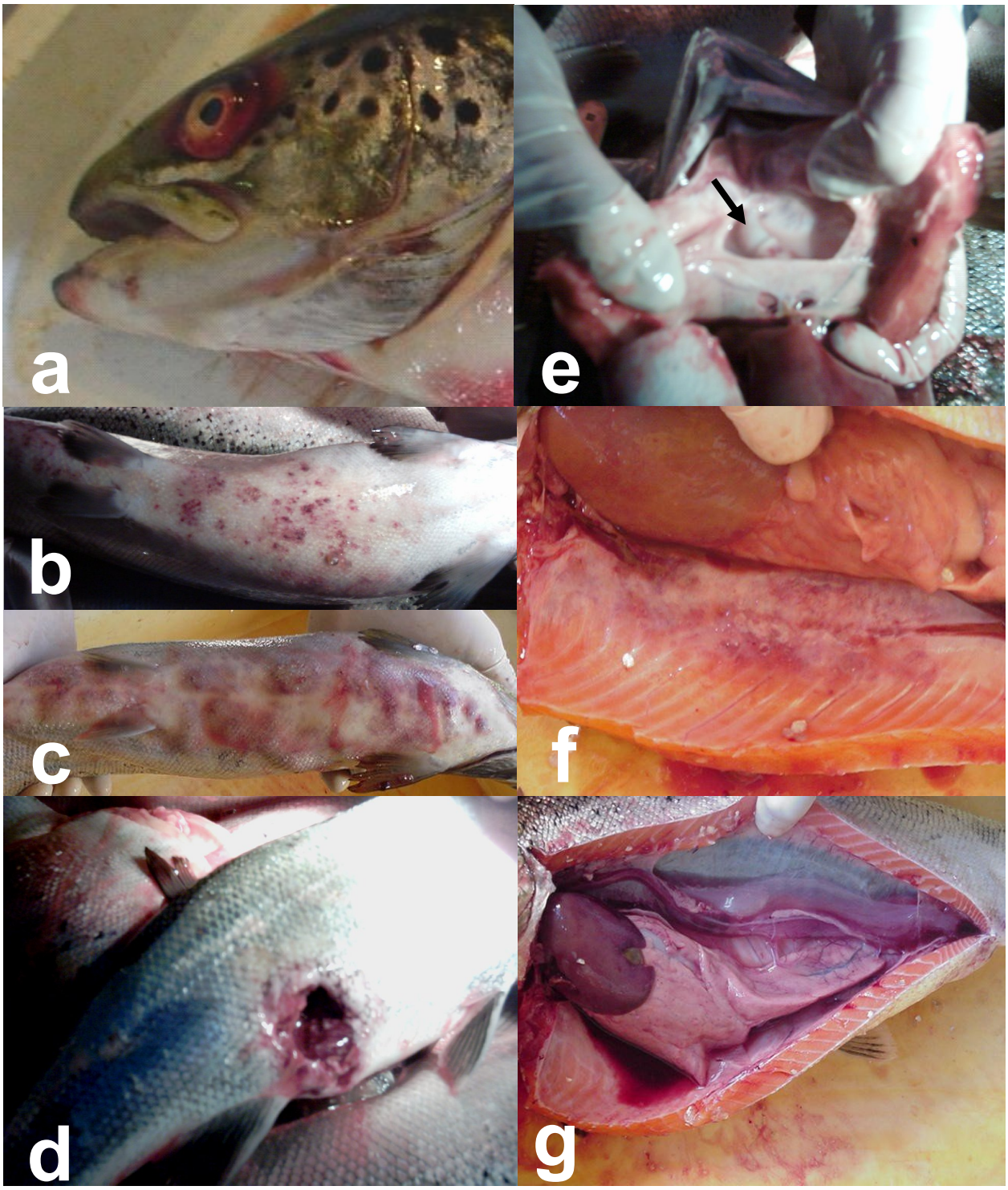

Fig. 1.- Romalde et al.

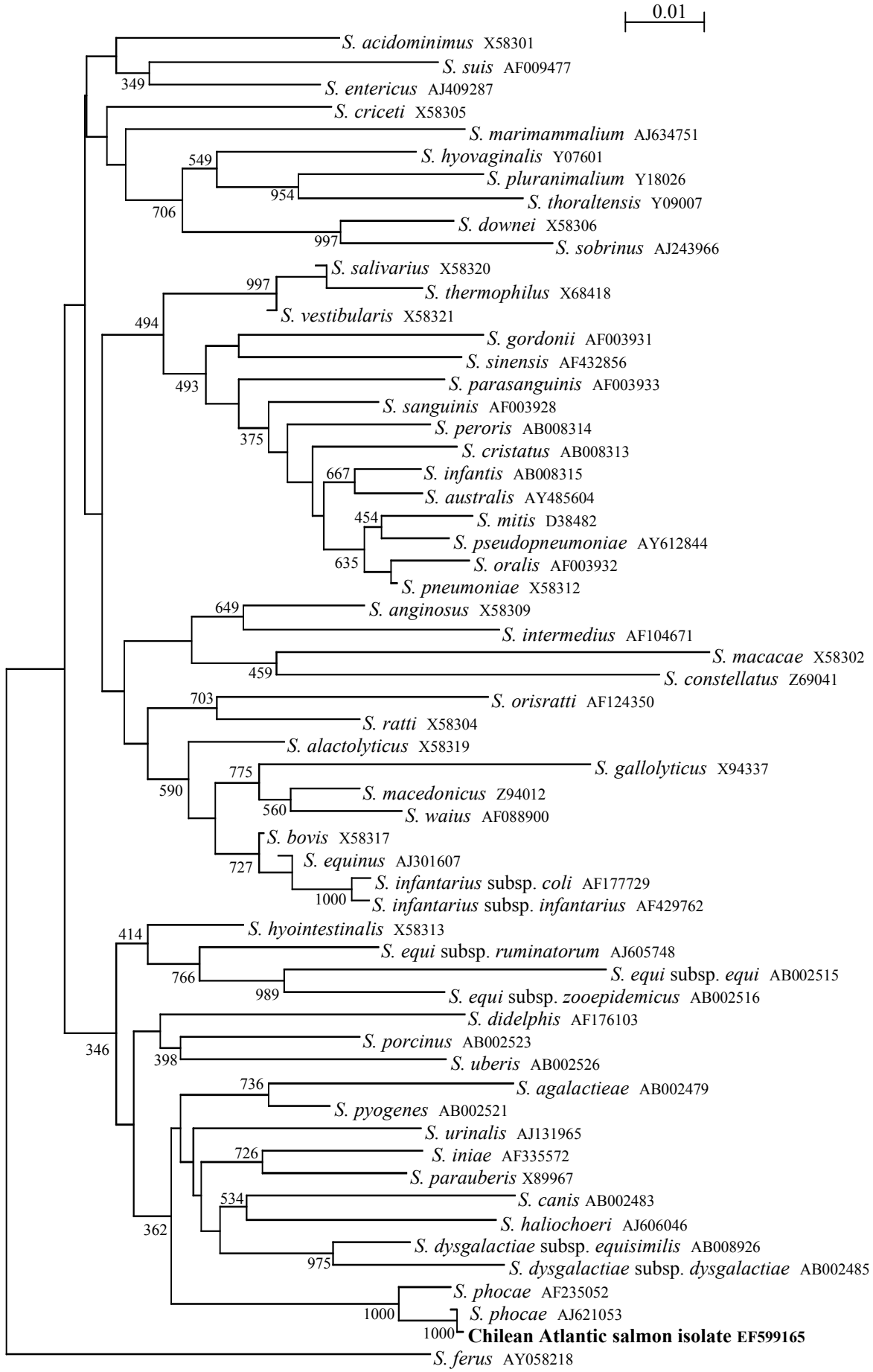

Fig. 2.- Romalde et al.

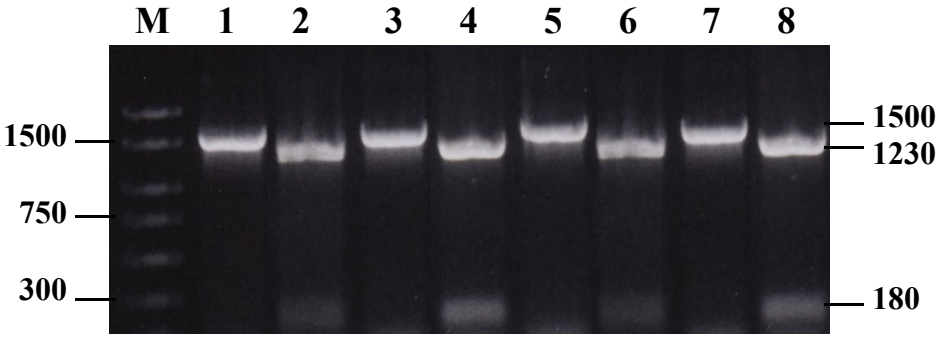

Fig. 3.- Romalde et al.