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Streptococcus phocae, AN EMERGING PATHOGEN FOR SALMONID

3

CULTURE

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29 **Abstract**

30

31 This work describes the characterization of the causal agent of disease outbreaks that,
32 from 1999, occurred repeatedly during the summer months (temperatures higher than
33 15°C) in Atlantic salmon (*Salmo salar*) cage-farmed in Chile affecting both smolts
34 and adult fish cultured in estuary and marine waters, reaching in some occasions a
35 cumulative mortality up to 25% of the affected population. Diseased fish showed
36 exophthalmia with accumulation of purulent and haemorrhagic fluid around eyes, and
37 ventral petechial haemorrhages. At necropsy, haemorrhage in the abdominal fat,
38 pericarditis, and enlarged liver, spleen and kidney are common pathological changes.
39 Gram-stained smears revealed the presence of Gram-positive cocci, α -hemolytic,
40 negative for oxidase and catalase tests. Although biochemical characterization of the
41 isolates using the miniaturized system rapid ID 32 Strep suggested their assignation to
42 genus *Gemella*, sequencing and RFLP analysis of the 16S rRNA revealed that
43 bacteria associated with the mortalities belong to *Streptococcus phocae*. Serological
44 studies demonstrated that all the salmon isolates are antigenically homogeneous,
45 which can facilitate the development of preventive measures and, although sharing
46 some antigenical determinants, they belong to a different Lancefield group than the
47 type strain isolated from seals. On the basis of these facts, we conclude that the
48 species *S. phocae* is an emerging pathogen for salmonid culture in Chile, and it should
49 be included as a new member of the warm water streptococcosis.

50

51 **Key words:** *Streptococcus phocae*, Atlantic salmon, Emerging pathogen

52 Introduction

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

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

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

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

91

92 **Materials and Methods**

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

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

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

113 The drug sensitivities of the strains were determined by the disc diffusion method on
114 Muller-Hinton agar (Oxoid) with 1% NaCl. The following chemotherapeutic agents
115 (micrograms per disc) were used: oxolinic acid (10), trimethoprim-sulfamethoxazole
116 (23.75/1.25), amoxicillin (25), oxytetracycline (30), erythromycin (15), and
117 florfenicol (30).

118 **Genetic characterization.**

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

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

138 **Serological assays**

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

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

152 **Virulence for fish and mice**

153 In order to determine the virulent capacities of the isolates, infectivity trials were
154 conducted using different fish species and mice. Representative isolates (Au15, 2857,
155 and C4) were intraperitoneally (i.p.) inoculated in Atlantic salmon (20 g) and
156 rainbow trout (15 g). Prior to challenge, fish were randomly sampled to be subjected
157 to microbiological analysis which indicated that they were free of *S. phocae* and other
158 pathogens. Groups of 15 fishes were inoculated (0.1 µl) with different bacterial doses
159 ranging from 10^4 to 10^9 cells/fish. Control fish received 0.1 µl of saline solution
160 (0.85% NaCl). In addition, one group of 15 Atlantic salmon (20 g) was
161 subcutaneously inoculated (0.1 µl/fish) with of two doses (10^5 and 10^6 cells/fish) of
162 the isolate Au15. Fish were maintained in seawater tanks at $19 \pm 1^\circ\text{C}$ with aeration
163 during the course of the experiments (21 days) making a daily 50% water renovation.
164 Fish mortality was daily checked during this period. Dead fish were microbiologically
165 analyzed for re-isolation of the inoculated strain. The degree of virulence was
166 expressed as lethal dose 50% (LD₅₀) calculated as described by Reed and Muench
167 (1938).

168 To determine the pathogenic potential for homoiotherm animals, a mouse virulence
169 assay was performed including three isolates from Atlantic salmon and the *S. phocae*
170 type strain DSMZ 15635^T. Briefly, groups of 5 BALB/C mice (10-12 weeks old, 21-
171 25 g) were inoculated i.p. with bacterial dose of 10^7 cells (0.1 µl/mouse). Mortalities
172 were recorded daily during a period of 10 days. Strain CDC 2227-81 of
173 *Photobacterium damsela* subsp. *damsela*, previously described as pathogenic for
174 mice (Osorio *et al.*, 2000) was included in the assay as positive control.

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

177

178 **Results**

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

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

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

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

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

212 All the isolates showed the same pattern of resistance/susceptibility to
213 chemotherapeutic agents *in vitro*, being susceptible to amoxicillin, oxytetracycline,
214 erythromycin and florfenicol and resistant to flumequine, oxolinic acid and
215 trimethoprim-sulfamethoxazole.

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

220 and *S. agalactiae* with similarities slightly higher than 93%. Moreover, in the RFLP
221 analysis all the isolates rendered the expected fragments employing either *HincII* or
222 *EarI* endonucleases. Restriction with the enzyme *HincII* restriction yielded two
223 characteristic fragments with sizes of 180 and 1230 bp) (Fig. 3), whereas *EarI*
224 rendered three DNA fragments with sizes of 170, 380, and 840 bp (data not shown).
225 Serological studies using different techniques, such as slide agglutination and dot blot,
226 with specific serum raised against the Atlantic salmon isolate 2857, demonstrated that
227 all the salmon isolates are antigenically homogeneous, sharing antigenic determinants
228 with the type strain isolated from seals (Table 1). However, when the commercial
229 Lancefield streptococcal grouping kit was employed, the isolates from fish were
230 classified as group G, although showing also a weak cross-reactivity with serum from
231 antigenic group A, while the type strain DSMZ 15635^T was assigned to serogroup F.
232 The three isolates inoculated were pathogenic for Atlantic salmon with LD₅₀ values
233 ranging from 1.9×10^5 and 5.6×10^5 bacteria per fish but not for rainbow trout when
234 i.p. inoculated. Mortalities began two days after inoculation, being observed a
235 dramatical increase by day 6. No mortalities were observed in the control groups.
236 Inoculated bacteria could be recovered from dead fish which showed in all cases the
237 typical necrotic lesions observed in the natural outbreaks. On the other hand,
238 subcutaneously inoculated salmon showed a gross inflammation in the injection area,
239 which progressed to necrosis with caseous material and liquefaction. Mortalities
240 started 4 days after inoculation reaching 80 and 100% for the low and high dose
241 respectively. None of the *S. phocae* isolates, including the type strain, were
242 pathogenic for mice, being observed no mortalities during ten days. Three out of the 5
243 BALB/C mice inoculated with the strain of *P. damselae* subsp. *damselae* utilized as
244 positive control, died during the same period.

245

246 **Discussion**

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

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

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

278 The development and application of molecular techniques have greatly facilitated the
279 identification and classification of streptococci (Bentley and Leigh, 1995; Romalde
280 and Toranzo, 2005; Täpp *et al.*, 2003), having the advantages of time-saving and
281 accuracy. The use of molecular methods, such as sequencing or RFLP analysis of the
282 16S rRNA gene, in the identification of the Atlantic salmon isolates unequivocally
283 demonstrated that all of them belong to the species *S. phocae*.

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

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

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

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

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

317
318

319

320 **Nucleotide sequence accession number.**

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

324

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423 **Figure Legends**

424

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

430

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

438

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

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

448	Isolate	Origin	Year of	Reaction with serum against	
449			Isolation	isolate 2857	Lancefield group
450	<hr/>				
451	Atlantic salmon isolates				
452	7053-1	Reloncaví Estuary, Chile	1999	+	G
453	9547-1	Reloncaví Estuary, Chile	1999	+	G
454	2743-1	Reloncaví Estuary, Chile	2001	+	G
455	2857	Reloncaví Estuary, Chile	2001	+	G
456	3385	Reloncaví Estuary, Chile	2001	+	G
457	3618	Reloncaví Estuary, Chile	2001	+	G
458	3770	Reloncaví Estuary, Chile	2002	+	G
459	344	Reloncaví Estuary, Chile	2004	+	G
460	751	Reloncaví Estuary, Chile	2004	+	G
461	2880	Reloncaví Estuary, Chile	2004	+	G
462	5070	Reloncaví Estuary, Chile	2004	+	G
463	C-4	Reloncaví Estuary, Chile	2004	+	G
464	Au11	Herradura, Chile	2004	+	G
465	Au13	Hualaihué, Chile	2004	+	G
466	Au14	Reloncaví Bay, Chile	2004	+	G
467	Au15	Curaco de Lin Lin, Chile	2004	+	G
468	PF-132	Chaitén, Chile	2004	+	G
469	PF-133	Herradura, Chile	2004	+	G
470	PF-136	Reloncaví Bay, Chile	2004	+	G
471	PF-137	Reloncaví Estuary, Chile	2004	+	G
472	PF-140	Calbuco, Chile	2004	+	G
473	PF-142	Comau Fiord, Chile	2004	+	G
474	PF-143	Reloncaví Estuary, Chile	2004	+	G
475	PF-145	Marimelli, Chile	2004	+	G
476	PF-146	Marimelli, Chile	2004	+	G
477	PF-148	Quillaipe, Chile	2004	+	G
478	PF-153	Quillaipe, Chile	2004	+	G
479	PF-155	Calbuco, Chile	2004	+	G
480	PF-030	Punta Venet, Chile	2005	+	G
481	PF-056	Castro, Chile	2005	+	G
482	PF-058	Castro, Chile	2005	+	G
483	PF-059	Castro, Chile	2005	+	G
484	PF-134	Chaitén Chile	2005	+	G
485	PF-157	Reloncaví Estuary, Chile	2005	+	G
486	Reference strain				
487	DSMZ 15635 ^T	<i>Phoca vitulina</i> , Norway	1994	+	F
488	<hr/>				

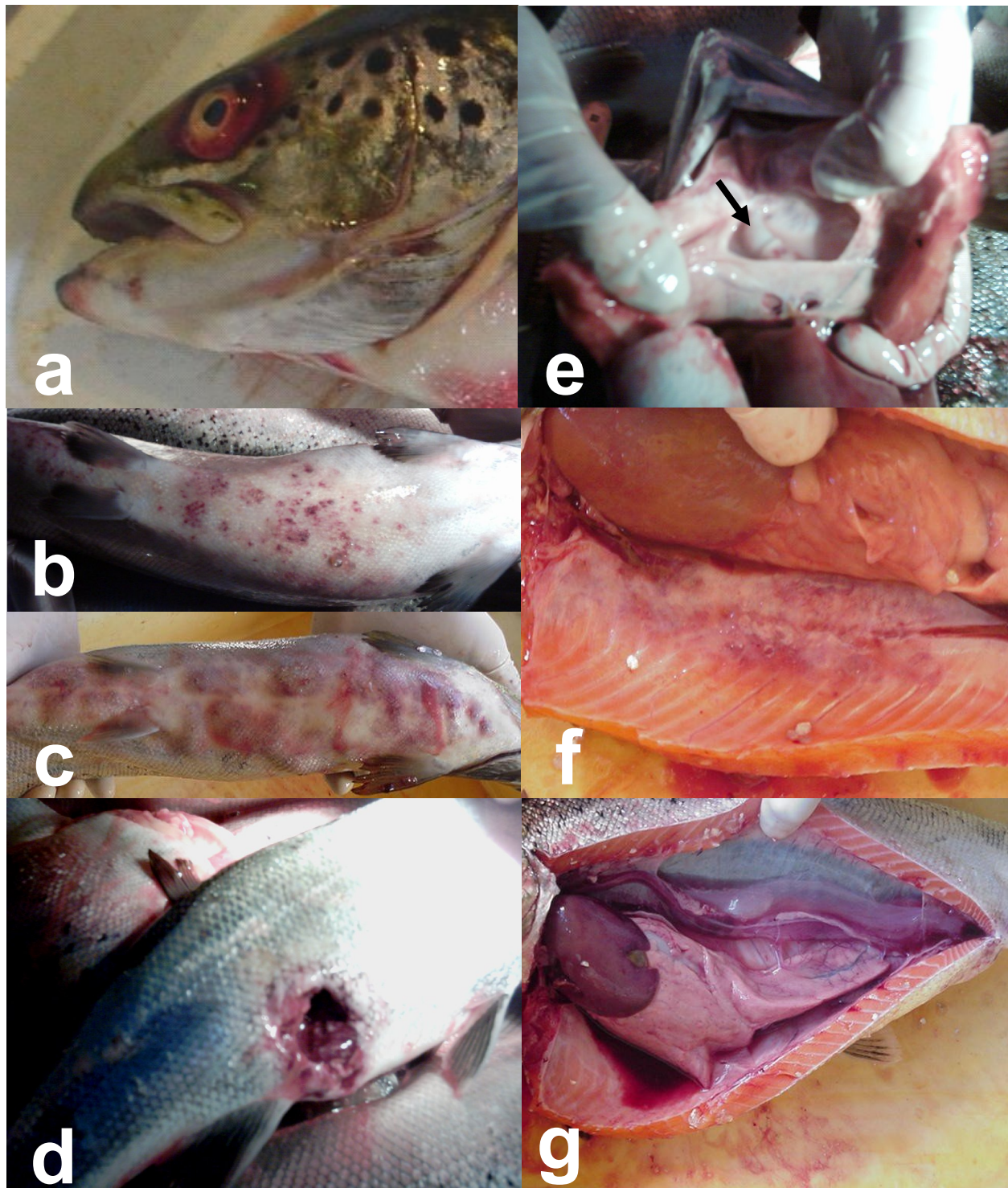


Fig. 1.- Romalde et al.

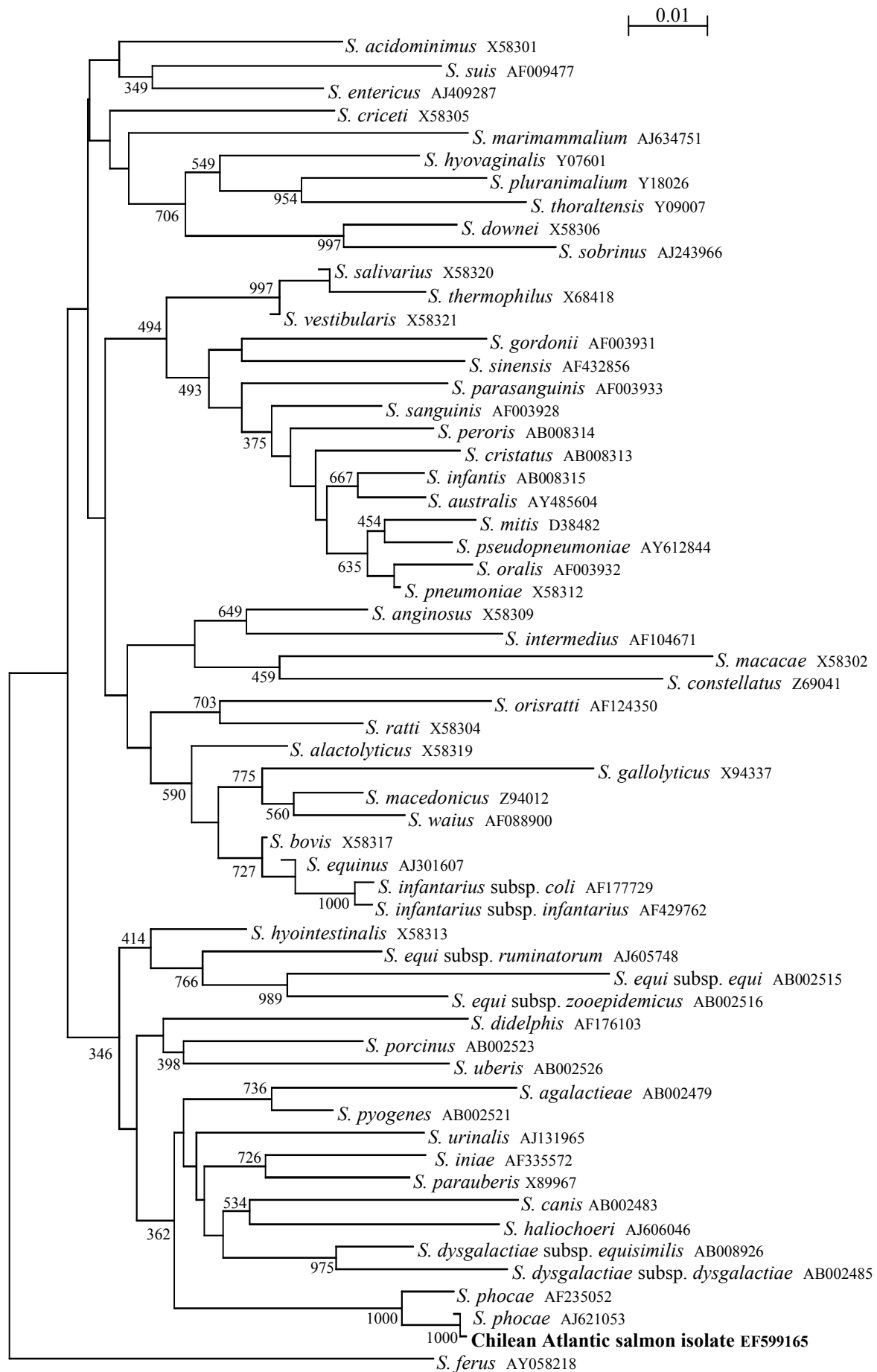


Fig. 2.- Romalde et al.

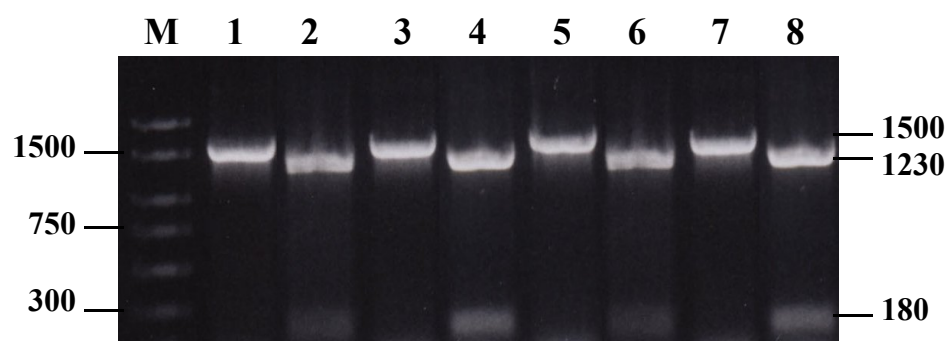


Fig. 3.- Romalde et al.