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Confirmation of *Piscirickettsia salmonis* as a pathogen in European sea bass *Dicentrarchus labrax* and phylogenetic comparison with salmonid strains

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ABSTRACT: European sea bass *Dicentrarchus labrax* from the Mediterranean were diagnosed with a severe encephalitis. Rickettsia-like organisms (RLOs) were associated with brain lesions in routine paraffin sections. These were found to share common antigens with the *Piscirickettsia salmonis* type-strain, LF-89, by indirect fluorescent antibody test (IFAT) and by immunohistochemistry (IHC). In addition, we compared the DNA sequences of the 16S rDNA and 16S-23S internal transcribed spacer region (ITS) with those published for *P. salmonis* strains and found that the sea bass piscirickettsia-like organism (SBPLO) was another strain of *P. salmonis*, closely related to the salmonid pathogens. Furthermore, we showed that the SBPLO possessed at least 2 ITS regions, 1 of which contained tRNA genes.

KEY WORDS: *Piscirickettsia salmonis* · European sea bass *Dicentrarchus labrax* · IFAT · Immunohistochemistry · rDNA · Phylogeny

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INTRODUCTION

Piscirickettsia salmonis, the causative agent of Piscirickettsiosis or Salmonid Rickettsial Syndrome, was first identified as a pathogenic agent in disease outbreaks amongst farmed coho salmon *Oncorhynchus kisutch* in Chile during 1989 (Fryer et al. 1990, Branson & Diaz-Munoz 1991, Cvitanich et al. 1991). Subsequently, the organism was confirmed as the agent responsible for clinical and chronic disease amongst farmed salmonids from both the Pacific and Atlantic coasts of Canada, Ireland, Norway and Scotland (Brocklebank et al. 1992, Palmer et al. 1996, Olsen et al. 1997, Birrell et al. 2003). The disease has appeared primarily to affect salmonids; chinook salmon *O. tshawytscha*, sakura salmon *O. masou*, rainbow trout *O. mykiss*, pink salmon *O. gorbuscha* and Atlantic salmon *Salmo salar* are all susceptible, albeit to differing degrees (House et al. 1999). While rickettsia-like organisms (RLOs) have been observed in non-salmonid hosts such as the blue-eyed plecostomus *Panaque suttoni* (Khoo et al. 1995), 5 species of cultured tilapia in Taiwan (Chern & Chao 1994, Chen et al. 1994) and in Hawaiian tilapia (Mauel 2003), to date the only non-salmonids in which a piscirickettsia-like organism (PLO) has been confirmed using serological methods are the white seabass *Atractoscion nobilis* in California, USA (M. F. Chen et al. 2000) and the grouper *Epinephelus melanostigma* in Taiwan (S. C. Chen et al. 2000). RLOs have also been identified in European sea bass (Comps et al. 1996) but, while antigenic similarities have been confirmed (Steiropoulos et al. 2002), the relatedness of these organisms to *P. salmonis* has not yet been determined.

Based on the sequence of its 16S rRNA gene, the *Piscirickettsia salmonis* type-strain, LF-89 (ATCC VR 1361),

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was placed among the Gammaproteobacteria (Fryer et al. 1992). Subsequently, Mauel et al. (1999) used comparisons of 16S rRNA gene sequences and internal transcribed spaces (ITS) sequences to determine the relatedness of other *P. salmonis* isolates from Chile, Canada and Norway. They established that these strains formed a monophyletic group within the Gammaproteobacteria, although 1 Chilean isolate, EM-90, had diverged sufficiently to allow differentiation from the other isolates based on restriction fragment length polymorphism (RFLP) (Mauel et al. 1996). This group found only 1 ITS sequence in the isolates examined but, following polyacrylamide gel analysis of amplified ITS regions, Casanova et al. (2001) have suggested that *P. salmonis* may contain at least 2 rRNA (*rrn*) operons, as is commonly the case for other Gram-negative bacteria (Gürtler & Stanisich 1996, Crosby & Criddle 2003). More recently, Reid & Birkbeck (2003) have extended the information on regional variation of *P. salmonis* isolates through their comparison of 16S rDNA and ITS sequences from Scottish and Irish isolates.

In this study, routine diagnostic histopathological examination was conducted on European sea bass presenting with clinical signs of nervous disease. In paraffin sections an RLO was seen to be associated with the encephalitic lesions. Serological analyses, indirect fluorescent (IFAT) and immunohistochemistry (IHC) were then used to confirm the tentative diagnosis and to confirm whether or not the organism was antigenically related to the *Piscirickettsia salmonis* type-strain, LF-89. The DNA sequences of the 16S rDNA and ITS region were then compared with those of published *P. salmonis* strains to establish whether or not the sea bass piscirickettsia-like organism (SBPLO) might be another strain of *P. salmonis* and how closely genetically related it was to the salmonid pathogens. Furthermore, it was sought to establish if the sea bass isolate possessed at least 2 ITS regions, 1 of which contained tRNA genes in the 16S-23S spacer region.

MATERIALS AND METHODS

Fish. Juvenile sea bass *Dicentrarchus labrax* from a farm in Greece which were exhibiting abnormal swimming and whirling behaviour, and experiencing low levels of mortality, were euthanized by terminal anaesthesia in 2-phenoxyethanol and sampled for histological analysis and screening by polymerase chain reaction (PCR) for suspected *Piscirickettsia salmonis* infection. Samples of whole fish were fixed in 10% neutral buffered formalin for histology, and samples of brain and mid-gut were preserved in 100 and 70% ethanol for subsequent extraction of genomic DNA for DNA analysis.

Examination of tissue sections. *Histology:* Formalin fixed tissues for histological examination were embedded in paraffin, sectioned at 5.0 µm and stained with haematoxylin and eosin.

Serological analyses: Slides were prepared from 5.0 µm paraffin-wax embedded tissue sections taken from the brain and midgut of infected sea bass. Sections prepared from the liver and kidney of rainbow trout *Oncorhynchus mykiss* experimentally infected with *Piscirickettsia salmonis* LF-89 (type-strain ATCC VR 1361) were used as a positive control, while sections from non-infected sea bass were used as negative controls. Sections were dewaxed in 2 successive xylene baths (5 min each) and rehydrated in a 100% ethanol bath for 5 min followed by a 70% ethanol bath for 3 min. After rinsing with distilled water, tissue sections were encircled with wax using a PAP pen (VWR International).

Indirect fluorescent antibody test (IFAT): Sections were subjected to additional fixing in 95 % methanol for 5 min, washed with 0.01 M phosphate buffered saline (PBS), pH 7.4 and non-specific binding sites were blocked by incubation in goat serum (Sigma-Aldrich) (diluted 1/10 in PBS) for 10 min at room temperature (RT; approx. 21°C). After washing in PBS, tissue sections were incubated with rabbit anti-*Piscirickettsia salmonis* serum (kindly provided by Professor J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, OR, USA), diluted 1/100 in PBS. Tissue sections incubated with PBS only were also used as additional negative controls. Slides were washed again in PBS, then incubated with fluorescein isothiocyanate (FITC)-labelled, goat, anti-rabbit serum (Diagnostics Scotland) diluted 1/100 in PBS, for 30 min at RT. After a final washing step, slides were mounted in 50% (v/v) glycerol and examined under oil immersion with an Olympus BX50 fluorescent microscope.

Immunohistochemistry (IHC): The IHC procedure was carried out essentially as described by Alday-Sanz et al. (1994). The sections were incubated in 10% (v/v) hydrogen peroxide in methanol for 10 min at RT to block endogenous peroxidase activity. After washing with Tris-buffered saline (0.2 M TBS, pH 7.2), non-specific binding sites were blocked by incubation in goat serum, diluted 1/10 in TBS, for 10 min at RT. Slides were washed in TBS, tapped dry and incubated with anti-*Piscirickettsia salmonis* polyclonal antibody (PAb), diluted 1/100 in TBS, for 60 min in a humid chamber. Excess primary antibody was removed by washing in TBS and slides were incubated in goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, diluted 1/50 in TBS, for 30 min in a humid chamber. Sections were then incubated with 1% (v/v) hydrogen peroxide in 1.5 mg 3,3-diaminobenzidine tetrahydrochloride (DAB) and 10 ml TBS for 10 min. The reaction was

stopped by immersing the slides in tap water and slides were then counterstained with haematoxylin for 3 min. Excess stain was removed by incubating the slides in tap water for 10 min, followed by dehydration in 70% ethanol (3 min), 100% ethanol (5 min) and 2 successive xylene baths (2 × 5 min). Slides were mounted in Pertex and examined under a light microscope.

Concurrently, infected sea bass tissues were screened with an anti-*Photobacterium damselae* subsp. *piscicida* monoclonal antibody (mAb) (Aquatic Diagnostics) in IFAT and IHC. Tissues from sea bass, positive and negative for *Ph. damselae* subsp. *piscicida*, were used as controls. The monoclonal antibody was used according to the manufacturer's instructions, and either goat anti-mouse IgG-HRP (Diagnostics Scotland) or goat anti-mouse IgG-FITC (Diagnostics Scotland) diluted 1/100 in PBS was used as secondary antibody. The incubations with these antibodies were as described above.

DNA analysis. ***Isolation of genomic DNA:*** Brain tissue (0.5 g), preserved in 70 or 100% ethanol, was excised using sterile scalpels and genomic DNA was extracted using a Nucleon ST Kit (Tepnel Life Sciences PLC). Brain tissue from 2 fish was combined for each sample and 2 samples were extracted on 2 different days. As a control for possible contamination during the extraction process, concurrent extraction procedures were carried out in the absence of any tissues. Genomic DNA, extracted from cell monolayers infected with *Piscirickettsia salmonis* LF-89 or with *P. salmonis* strain SLGO-95 (kindly provided by Dr. P. A. Smith, Faculty of Veterinary Sciences, University of Chile, Santiago) was used as a positive control. Extracted DNA was stored at –20°C until required for PCR.

PCR amplification of 16S rDNA: Initially, a nested PCR amplification was carried out using the bacterial 16S primers, EubA and EubB, in the first-round amplification and *Piscirickettsia*-specific primers, PS2S and PS2AS, in the second-round amplification (Table 1) (Mauel et al. 1996). PCR amplifications were performed using 0.2 ml Ready-to-Go PCR beads (Amersham Biosciences UK), following the manufacturer's instructions, with a final concentration of 1 µM of the appropriate primer pair and 1 µl template DNA. For the second-round amplification, 1 µl of the first-round reaction was used as template. Cycling conditions were modified from Corbeil et al. (2003). Briefly, for primary PCR the mixture was denatured at 95°C for 5 min and amplified with 35 cycles of 94°C for 30 s, 50°C for 40 s, 72°C for 40 s with a final extension step of 72°C for 5 min. For nested PCR, the mixture was denatured at 95°C for 5 min followed by amplification with 35 cycles of 94°C for 30 s, 61°C for 40 s, 72°C for 40 s and a final extension step of 72°C for 5 min. Subsequently, the nested PCR was carried out using primer pairs (Table 1) designed to amplify the region between nucleotides 225 and 1475, namely PS2S and 860R (nucleotides 226–860), PS2S and 1283R (226–1283) and 860F and 1470R (860–1476). The numbering corresponds to the published sequence for the *P. salmonis* type-strain LF-89 (GenBank accession number U36941) (Mauel et al. 1999). For these amplifications, cycling parameters were as described above, except that each primer was used at 1 µM final concentration, and an annealing temperature of 56°C was used in the PCR cycle.

All amplifications were performed using a Biometra T Gradient thermocycler (Anachem) and 5 µl of each amplification reaction was examined for specificity by electrophoresis on a 1% agarose gel containing 0.5 mg ml^{-1} ethidium bromide. Molecular weight markers (DNA Molecular Weight Marker VI or DNA Molecular Weight Marker XIV, Roche Diagnostics) were added to the gel as a reference.

PCR amplification of the ITS region: A nested PCR amplification was carried out using conditions and cycling parameters as described above for the 16S rDNA PCR. However, primers PS16SA and PS23SB were used in the first round reaction, and primers PS16SH and PS23SC were used in the second round as

Table 1. Sequences and specificity of primers used for 16S rRNA gene and internal transcribed spacer (ITS) region PCR and sequencing. F: forward; R: reverse

Primer/location	Sequence (5′-3′)	Specificity
Eub B (27F)[a]	AGAGTTTGATCMTGGCTCAG	Eubacterial
Eub A (1518R)[a]	AAGGAGGTGATCCANCCRCA	Eubacterial
PS2S (223F)[a]	CTAGGAGATGAGCCCGCGTTG	*P. salmonis* 16S
PS2AS (690R)[a]	GCTACACCTGCCAAACCACTT	"
850F (851F)[b]	GGATTCCCTTGAGGAGTTTAGTGG	"
850R (828R)[b]	CCACTAAACTCCTCAAGGGACTCC	"
1280R (1283R)[b]	CTTTCTCAGGTTCGCTCCAC	"
1490R (1487R)[b]	CTTCACCCCAGTCATGACCC	"
PS16SA (1387F)[a]	GCCTTGTACACAACCGCCC	*P. salmonis* ITS
PS23SB (507R)[c]	CCTTTCCCTCACGGTCAT	"
PS16SH (1519F)[b]	CCTGCGGCTGGATTACCT	"
PS23SC (203R)[c]	TAGATGTTTCAGTTCCCC	"
ITSUF (1430F)[b]	AGTGAATTGCACCAGAAGGG	"
ITSUR (303R)[c]	ATCACCCTCTATCGCCACAC	"

[a]Numbering corresponds to *Escherichia coli* 16S rRNA gene
[b]Numbering corresponds to *Piscirickettsia salmonis* LF-89 16S rRNA gene
[c]Number of bases from the 5′ end of the *Piscirickettsia salmonis* 23S rRNA gene

previously described by Mauel et al. (1999) (Table 1). Subsequently, using primers ITSUF and ITSUR, complementary to *Piscirickettsia salmonis* LF-89 16S and 23S rDNA sequences (Table 1), direct PCR amplification was carried out on DNA isolated from infected sea bass. These primers were also used for nested PCR amplification of first round products obtained with primers PS16SA and PS23SB. Amplification conditions were as described above for nested 16S rDNA PCR except that each primer was used at 0.5 µM with an annealing temperature of 58°C. A faint secondary product produced by this amplification was excised from the gel and re-amplified using 30 cycles and either primer pair PS16SH/PS23SC or primer pair ITSUF/ITSUR.

DNA sequencing: In order to obtain template DNA for sequencing, the GFX™ PCR DNA and Gel Band Purification Kit was used to clean amplification reactions or to clean fragments excised from gels. Sequencing reactions were carried out using a DYEnamic™ ET terminator kit Cycle Sequencing Kit (Amersham Biosciences) and following the manufacturer's instructions. Primers used for the nested PCR reactions were diluted to 5 pmol and used as sequencing primers. Each segment of DNA was sequenced in the forward and reverse direction, following at least 3 separate PCR amplifications on different days, with the exception of the secondary ITS fragment which was amplified in a single run using 2 different primer pairs, as described above. Sequencing reactions were run on the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems), were viewed using BioEdit software (Hall 1999) and aligned using Clustal X (Thompson et al. 1994) with manual editing. Sequences obtained for the SBPLO 16S rDNA were compared with published *Piscirickettsia salmonis* sequences, sequences from fish pathogens or from other members of the Gammaproteobacteria (Table 2), while sequences obtained for ITS DNA were compared amongst *P. salmonis* strains only. Phylogenetic trees were constructed using PHYLIP version 3.6 (Felsenstein 1989). Distance matrices generated by DNADIST were determined using the assumptions of Kimura (1980). These matrices were used to generate dendrograms using the neighbour-joining method (Saitou & Nei 1987). Dendrograms were also constructed using the parsimony program DNAPARS and the maximum-likelihood program DNAML, in the PHYLIP software package. The bootstrap values were obtained from 1000 trees generated with SEQBOOT and CONSENSE within PHYLIP.

Table 2. Bacterial species and strains used in this study and the GenBank accession numbers for their 16S rDNA and ITS DNA sequences

Species/strain	GenBank accession number	DNA sequence
Piscirickettsia salmonis SBPLO	AY542956	16S rDNA
Piscirickettsia salmonis LF-89	U36941	"
Piscirickettsia salmonis SLGO-94	U55015	"
Piscirickettsia salmonis NOR-92	U36942	"
Piscirickettsia salmonis EM-90	U36940	"
Piscirickettsia salmonis ATL-4-91	U36915	"
Piscirickettsia salmonis IRE-91A	AY498633	"
Piscirickettsia salmonis IRE-98A	AY498634	"
Piscirickettsia salmonis IRE-99D	AY498637	"
Piscirickettsia salmonis SCO-95A	AY498636	"
Piscirickettsia salmonis SCO-02A	AY498635	"
Piscirickettsia salmonis SBPLO (ITS_0)	AY607584	ITS and 23S rDNA
Piscirickettsia salmonis SBPLO (ITStRNA)	AY607585	"
Piscirickettsia salmonis LF-89	U36943	"
Piscirickettsia salmonis EM-90	U36944	"
Piscirickettsia salmonis ATL-4-91	U36945	"
Piscirickettsia salmonis NOR-92	U36946	"
Piscirickettsia salmonis SLGO-94	U62104	"
Piscirickettsia salmonis C1-95	U62103	"
Piscirickettsia salmonis IRE-91A	AY498625	"
Piscirickettsia salmonis IRE-98A	AY498624	"
Piscirickettsia salmonis IRE-99C	AY498632	"
Piscirickettsia salmonis IRE-99D	AY498631	"
Piscirickettsia salmonis SCO-95A	AY498621	"
Piscirickettsia salmonis SCO-98B	AY498630	"
Piscirickettsia salmonis SCO-98C	AY498629	"
Piscirickettsia salmonis SCO-02A	AY498628	"
Piscirickettsia salmonis SCO-02D	AY498622	"
Piscirickettsia salmonis SCO-02E	AY498623	"
Piscirickettsia salmonis SCO-02F	AY498626	"
Piscirickettsia salmonis SCO-02G	AY498627	"
Vibrio anguillarum	X16895	16S rDNA
Photobacterium damselae	AB026844	"
Aeromonas salmonicida	X60405	"
Methylophaga marina	X95459	"
Beggiatoa sp.	AF035956	"
Piscirickettsia grp. Clone LA7-B48N	AF513949	"
Tilapia parasite	AF206675	"

RESULTS

Histology

In general, lesions found in infected tissue sections were necrotizing and granulomatous. Liver, kidney, gastro-intestinal tract, pancreas, muscle and subder-

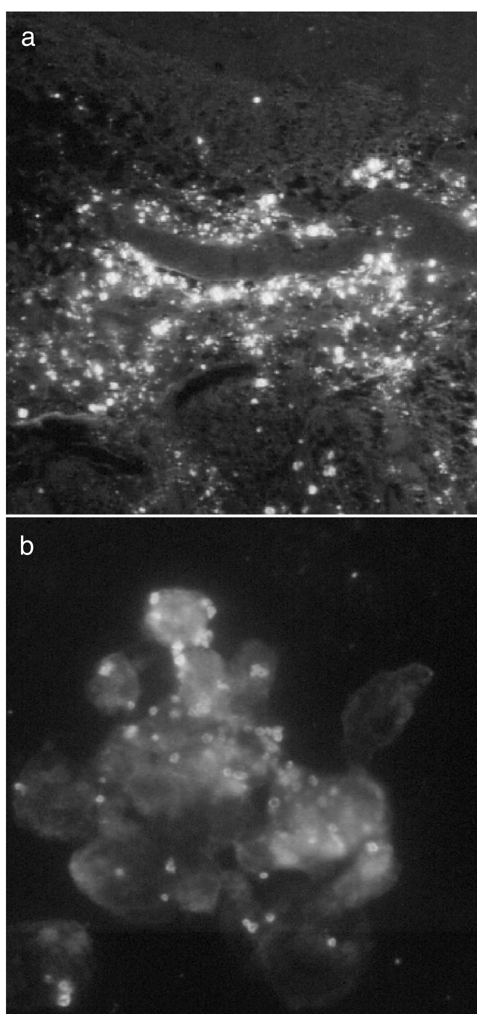

Fig. 1. *Piscirickettsia salmonis*. Indirect fluorescent antibody test, using anti-*P. salmonis* polyclonal serum to detect (a) sea bass *Piscirickettsia*-like organisms (SBPLO) in the brain of European sea bass; magnification = ×100. (b) *P. salmonis* strain SLGO-95 in CHSE-214 cell culture smear. Magnification = ×1000

mis were involved to variable degrees from one fish to another, but the organ most consistently affected was the brain. Here, lesions were also necrotizing and granulomatous, involving mostly brain stem, but in some fish they extended also to involve the tegmentum and olfactory lobes and tracts. In most fish, lesions were present in the third ventricle, involving the ciliated ependymal cells lining this, or those cells immediately beneath. The ventricular lumen sometimes was filled with debris and large numbers of eosinophilic foamy macrophages, some of which contained dense, basophilic, spherical, 1 µm-sized RLOs. These large foamy gemistocyte-like cells dominated the inflammatory response in the brain.

Serological analyses

In sections of sea bass brain tissue, ring-shaped organisms were detected by IFAT in the tegmentum using anti-*Piscirickettsia salmonis* PAb as primary antibody (Fig. 1a). The appearance of these organisms was similar to the *P. salmonis* type-strain LF-89 seen in IFAT of cell culture smears (Fig. 1b). No organisms were detected in the non-infected tissue sections or in sections incubated with anti-*Photobacterium damselae* subsp. *piscicida* MAb (not shown). In IHC, DAB deposition corresponded with areas of necrosis in the medulla oblongata (Fig. 2b) and the sensory epithelium of the olfactory organ (Fig. 2c,d). No organisms were detected in the non-infected tissue or in sections incubated with anti-*Ph. damselae* subsp. *piscicida* MAb (Fig. 2a).

Nested PCR and sequence analysis

16S rDNA

Nested PCR of DNA extracted from sea bass samples, using *Piscirickettsia salmonis*-specific primers PS2S and PS2AS, amplified a product of approximately 470 bp. A fragment of similar size was amplified from DNA extracted from cell-cultures of LF-89 and SLGO-95. Sequence analysis confirmed the sea bass product to be 16S rDNA, corresponding to nucleotides 226–673 of the published LF-89 sequence (GenBank accession number U36941) and differing from this sequence at positions 448, 450–454, 464–468, 470 and 641. Subsequent nested PCR and sequencing of the region between nucleotides 226 and 1475 confirmed the initial findings and revealed that the sea bass RLO 16S rDNA also differed from LF-89 rDNA at a further 10 positions (859, 876, 918, 928, 960, 1005, 1289, 1402, 1084 and 1103). A sequence identity matrix, constructed from the 16S rDNA sequence of the SBPLO, and *P. salmonis* strains LF-89, SLGO-95, NOR-92, ATL-4-91, EM-90, SCO-95A, SCO-02A, IRE-91A, IRE-98A and IRE-99D is shown (Table 3). This matrix was calculated from 1297 positions (including gaps) of the *P. salmonis* strains and the SBPLO. Sequence similarity between the SBPLO 16S rDNA gene and the type-strain LF-89 is 98.2%, which compares well with the sequence similarities of the Canadian strain, ATL-4 91 (98.7%) and the Norwegian strain, NOR-92 (98.9%). The similarity of the divergent Chilean strain, EM-90, is 97.1%. Both Scottish isolates (SCO-95A and SCO-02A) show a 16S rDNA sequence similarity of 99.3% with the type-strain, while the Irish strains (IRE-99D, IRE-98A and IRE-91A) reveal similarities of 98.0 to 97.1%. Dendrogram analysis of the SBPLO, *P. salmonis*

Fig. 2. *Dicentrarchus labrax*. Immunohistochemical staining of sea bass brain sections using (a) anti-*Photobacterium damselae* subsp. *piscicida* monoclonal antibody (MAb); and (b,c) rabbit anti-*Piscirickettsia salmonis* serum. Scale bar = 500 µm. Arrows indicate areas of diaminobenzidine tetrahydrochloride (DAB) deposition. Box in (c) indicates area enlarged in (d). Scale bar = 100 µm

Table 3. *Piscirickettsia salmonis*. Sequence similarities of partial ITS DNA sequences between 18 isolates of *P. salmonis* and the sea bass piscirickettsia organism (SBPLO) isolate. The comparison was made using 269 nucleotide positions, including gaps

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. LF-89	1.000	0.962	0.965	0.958	0.958	0.958	0.877	0.950	0.950	0.950	0.946	0.950	0.943	0.954	0.954	0.768	0.774	0.942	0.931
2. SLGO-94	–	1.000	0.988	0.973	0.973	0.939	0.867	0.966	0.965	0.965	0.962	0.965	0.958	0.969	0.969	0.755	0.761	0.920	0.909
3. C1-95	–	–	1.000	0.969	0.969	0.935	0.856	0.962	0.962	0.962	0.958	0.962	0.954	0.965	0.965	0.751	0.757	0.916	0.905
4. ATL-4-91	–	–	–	1.000	0.992	0.931	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.755	0.761	0.924	0.912
5. NOR-92	–	–	–	–	1.000	0.939	0.867	0.984	0.984	0.984	0.981	0.984	0.977	0.988	0.988	0.763	0.768	0.931	0.920
6. EM-90	–	–	–	–	–	1.000	0.877	0.924	0.924	0.924	0.920	0.924	0.916	0.928	0.928	0.761	0.759	0.938	0.927
7. SBPLO	–	–	–	–	–	–	1.000	0.867	0.867	0.867	0.863	0.871	0.859	0.871	0.871	0.833	0.830	0.858	0.847
8. SCO-95A	–	–	–	–	–	–	–	1.000	0.992	0.992	0.988	0.992	0.984	0.996	0.996	0.760	0.765	0.931	0.920
9. SCO-98B	–	–	–	–	–	–	–	–	1.000	0.992	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
10. SCO-98C	–	–	–	–	–	–	–	–	–	1.000	0.988	0.992	0.984	0.996	0.996	0.759	0.764	0.931	0.920
11. SCO-02A	–	–	–	–	–	–	–	–	–	–	1.000	0.988	0.996	0.992	0.992	0.755	0.761	0.927	0.916
12. SCO-02D	–	–	–	–	–	–	–	–	–	–	–	1.000	0.984	0.996	0.996	0.763	0.768	0.931	0.920
13. SCO-02E	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.988	0.988	0.751	0.757	0.923	0.912
14. SCO-02F	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	0.763	0.768	0.935	0.923
15. SCO-02G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.763	0.768	0.935	0.923
16. IRE-91A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.966	0.765	0.753
17. IRE-98A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.770	0.759
18. IRE-99C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.988
19. IRE-99D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000

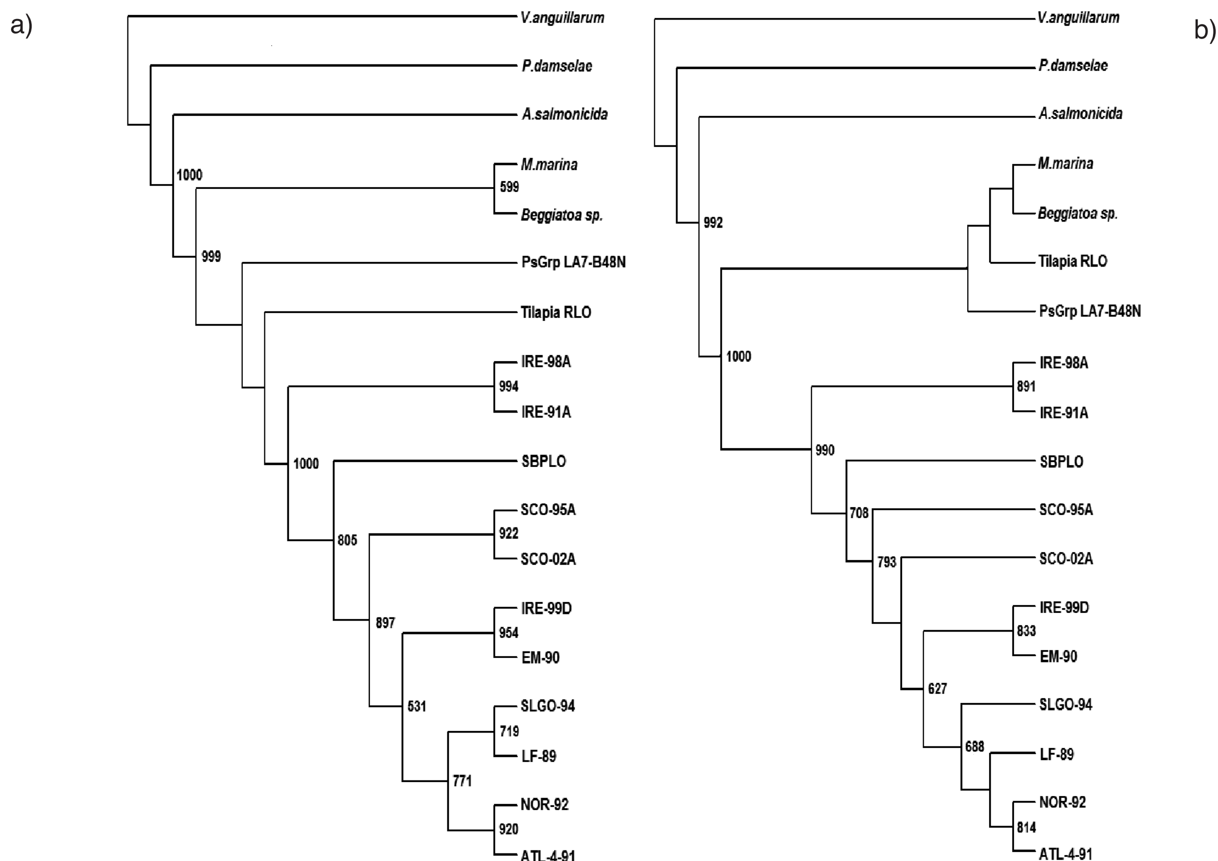

Fig. 3. Phylogenetic relationships of partial 16S rDNA sequences between the SBPLO, *Piscirickettsia salmonis* strains and members of the Gammaproteobacteria (Table 2) inferred from the (a) neighbour-joining and (b) maximum-likelihood methods using *Vibrio anguillarum* (GenBank accession no. X16895) as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 1132 positions, between nucleotide positions 226 and 1350 of *P. salmonis* LF-89. See Table 2 for full species names

and representatives of the Gammaproteobacteria 16S rDNA sequences by neighbour-joining, parsimony (not shown) and maximum likelihood methods produced similar trees (Fig. 3). The analysis places the SBPLO strain within the *P. salmonis* group (bootstrap support of 80.5 and 70.8% by the neighbour-joining and maximum-likelihood methods, respectively) and less distantly associated with the *P. salmonis* type-strain than the divergent Irish strains, IRE-91A and IRE-98A.

ITS region

Nested PCR of the sea bass RLO ITS region using primer pair PS16SA/PS23SB in the first round and primer pair PS16SH/PS23SC in the second round amplifications was successful on only 1 occasion and produced readable sequence from an amplified product of approximately 520 bp. Subsequent direct or nested PCR amplifications using primers ITSUF and ITSUR produced 2 fragments, a major product of approximately 750 bp and a faint, secondary product of approximately 1050 bp (Fig. 4). When sequenced, the major SBPLO product (ITS_0) confirmed our initial sequence obtained using primers PS16SA/PS23SB and PS16SH/PS23SC. This sequence also corresponded overall with the LF-89 ITS sequence (GenBank accession number U36943) found by Mauel et al. (1999) and with the LF-89 ITS B sequence reported by Casanova et al. (2001). The SBPLO ITS_0 differed at 32 positions from LF-89, mainly as the result of a 17-base gap in the SBPLO sequence between positions 223 and 239 (numbering calculated from the 5′ end of the LF-89 ITS sequence) (Fig. 5). Comparison of the complete ITS_0 DNA sequence (between positions 1 and 292 and including gaps) reveals a similarity of 87.7 % with the corresponding LF-89 sequence (Table 4). If the 17-base gap region is excluded from the analysis for all strains, the SBPLO/LF-89 sequence similarity is increased to 93.8% but the position of the SBPLO within the range of similarities is not significantly altered (data not shown). The sequence identity matrix shows that the

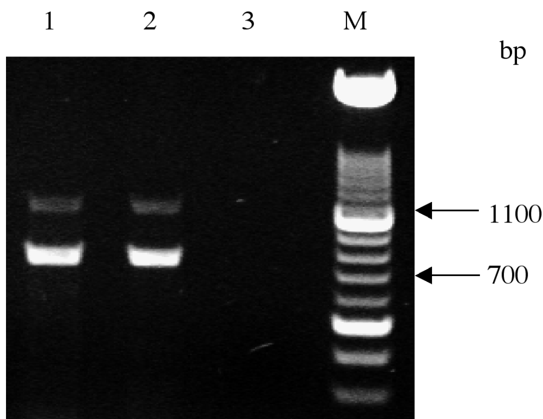

Fig. 4. *Piscirickettsia salmonis*. Products of direct PCR, using primer pair ITSUF/ITSUR, for amplification of ITS DNA. Lanes 1 and 2: DNA isolated from SBPLO-infected sea bass; Lane 3: negative control; M: molecular weight marker

SBPLO ITS_0 sequence exhibits a higher sequence identity with other members of the *Piscirickettsia salmonis* group than the Irish isolates IRE-91A and IRE-98A (76.8 and 77.4%, respectively). The Scottish isolates and the remaining Irish isolates, IRE-99C and IRE-99D, show a sequence divergence from the type-strain of less than 10%. Dendrograms inferred from the SBPLO ITS_0 and *P. salmonis* ITS sequences (nucleotide positions 49 to 310), obtained with either distance or parsimony tree-building methods, produced similar trees (Fig. 6). This analysis again placed the SBPLO strain within the *P. salmonis* group and, while this strain is divergent from most of the group, it is more closely related to the type-strain than the Irish isolates, IRE-91A and IRE-98A. Bootstrap support for this position was 100 and 99.1% for neighbour-joining and parsimony methods, respectively.

```
                                                                                70
SBPLO ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGT-C ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
LF-89 ITStRNA    ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATATTT AGTTAATGAA GAACGATTTG
SBPLO ITSo       ATTTATAGAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA
LF-89 ITS        ATTTAY-GAC TTGAAGTTGC TTAAGTGTTC ACACAAATTG CTTGATGATT TTATTGTTTA GTGAGAATGA

                                                                               140
SBPLO ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
LF-89 ITStRNA    AAGGCCTGTA GCTCAGCTGG TTAGAGCGCA CCCCTGATAA GGGTGAGGTC GGTGGTTCAA GTCCACTCAG
SBPLO ITSo       TA-------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        TA-------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                               210
SBPLO ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
LF-89 ITStRNA    GCCTACCAGT TTTGGTAGAT AGATCATGGG GCTATAGCTC AGCTGGGAGA GCGCCTGCTT TGCACGCAGG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                               280
SBPLO ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
LF-89 ITStRNA    AGGTCTGCGG TTCGATCCCG CATAGCTCCA CCATATCTTC ACTCTAAACG ATATTTTTAT AAGATTTTAG
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- ----------
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                               350
SBPLO ITStRNA    AATGCCGTGA AATGATTATT AG--ATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
LF-89 ITStRNA    AATGCCGTGA AATGATTATT TATAATGATT ATTTCACGTT GTTTTGACTT GGTTAAAATA ATGTATTTTT
SBPLO ITSo       ---------- ---------- ---------- ---------- ---------- ---------- -------TTT
LF-89 ITS        ---------- ---------- ---------- ---------- ---------- ---------- -------TTT

                                                                               420
SBPLO ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITStRNA    GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG
SBPLO ITSo       GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTTG
LF-89 ITS        GTTCTTTAAC AATGTGGTAA AAAGTATAAG TAAAGATTCC TTGATTAATT TAGGGTTATT TTTAGTTTAG

                                                                               490
SBPLO ITStRNA    ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITStRNA    GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
SBPLO ITSo       ATTAAGATGT ATTTTTATAT CTTGATTGAT AATTGGGAAT AATTTTTAGT TTATTTAATT AACGAGTCTT
LF-89 ITS        GTTGAGATGT ATTTTTATGT CTTGATTGAT TATTAGAAAT AATTTTTAGT TTATTTAATT AACGAGTCTT

                                                                               560
SBPLO ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITStRNA    GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG
SBPLO ITSo       GGTAATTTTT GAAAACCGGT GTTGAGATAT AATGTTGATT TGTTTTATTT AAGA------ ----------
LF-89 ITS        GGTAATTTTT GAAAACCGGT GTTGAGATAT AGTTTGATT GGTATTAGTT AATAGATTTT AGATTTATTG

                                      580
SBPLO ITStRNA    ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITStRNA    ATATAAGACT TCTTGGGGTT ATATGA
SBPLO ITSo       ---TAAGACT TTTTGGGGTT ATATGA
LF-89 ITS        ATATAAGACT TTNTGGGGTT ATATGA
```

Fig. 5. *Piscirickettsia salmonis*. Sequences of the complete SBPLO ITS_0 and ITS_{tRNA} aligned with the corresponding sequences from the *P. salmonis* type-strain, LF-89. Sequences corresponding to the $tRNA^{ile}$ and $tRNA^{ala}$ genes are underlined. Dashes indicate gaps

Table 4. *Piscirickettsia salmonis*. Sequence similarities of partial 16S rDNA sequences between 10 isolates of *P. salmonis* and the SBPLO isolate. Comparison was made using 1297 nucleotide positions, including gaps

Isolates	1	2	3	4	5	6	7	8	9	10	11
1. LF-89	1.000	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
2. SLGO-94	–	1.000	0.987	0.989	0.971	0.982	0.993	0.993	0.971	0.979	0.980
3. ATL-4-91	–	–	1.000	0.989	0.971	0.976	0.988	0.988	0.966	0.973	0.976
4. NOR-92	–	–	–	1.000	0.972	0.977	0.989	0.989	0.966	0.974	0.976
5. EM-90	–	–	–	–	1.000	0.960	0.972	0.972	0.959	0.959	0.975
6. SBPLO	–	–	–	–	–	1.000	0.988	0.988	0.976	0.983	0.974
7. SCO-95A	–	–	–	–	–	–	1.000	1.000	0.977	0.985	0.986
8. SCO-02A	–	–	–	–	–	–	–	1.000	0.977	0.985	0.986
9. IRE-91A	–	–	–	–	–	–	–	–	1.000	0.991	0.972
10. IRE-98A	–	–	–	–	–	–	–	–	–	1.000	0.972
11. IRE-99D	–	–	–	–	–	–	–	–	–	–	1.000

Following excision from the gel, re-amplification and sequencing, the secondary SBPLO product (ITS_{tRNA}) was found to contain genes coding for $tRNA^{ile}$ and $tRNA^{ala}$, and to correspond essentially with the LF-89 ITS A sequence described by Casanova et al. (2001). Both SBPLO ITS sequences (ITS_0 GenBank accession number AY607584; ITS_{tRNA} GenBank accession number AY607585) are shown in Fig. 5.

DISCUSSION

Histological analysis of tissues from European sea bass revealed the presence of RLOs in macrophages in the brain, which was the organ most consistently affected in diseased fish. Necrotic lesions in the anterior medulla oblongata and the presence of 1 µm basophilic organisms in cytoplasmic vacuoles have previously been described for RLO infection of sea bass (Comps et al. 1996). The morphological similarity between organisms detected in the sea bass medulla oblongata and *Piscirickettsia salmonis* LF-89 cell culture smears by IFAT suggested that the sea bass pathogen was related to *P. salmonis*. Furthermore, the association of DAB deposition with areas of necrosis

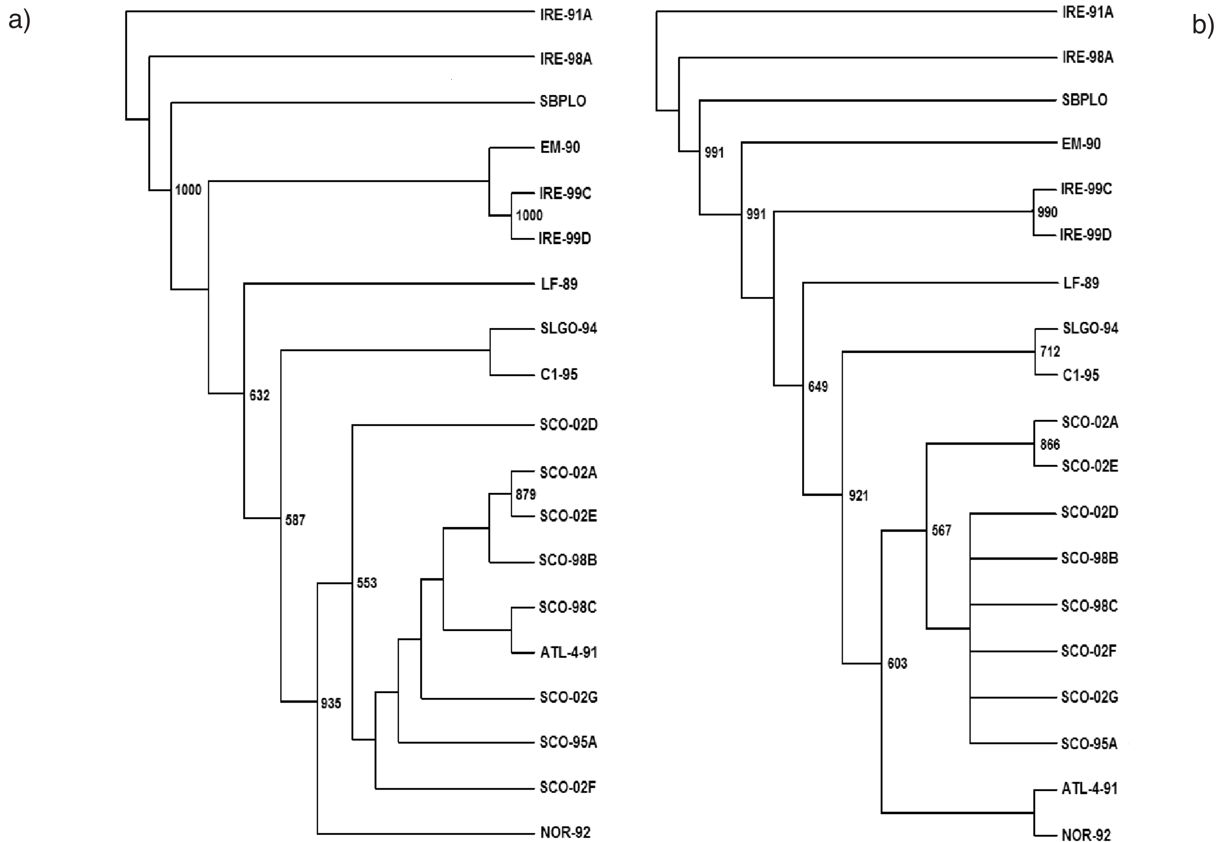

Fig. 6. *Piscirickettsia salmonis.* Phylogenetic relationships of partial ITS sequences between the SBPLO and *P. salmonis* strains inferred from the (a) neighbour-joining and (b) parsimony methods, using strain IRE-91A as outgroup. Bootstrap values from 1000 replicates appear at the nodes when >50%. Dendrograms were inferred from 260 positions, between nucleotides 50 and 310 from the 5′ end of the *P. salmonis* LF-89 ITS sequence

seen in IHC strengthened the argument that a *P. salmonis*-related organism was responsible for the pathology found in the sea bass. While initial, on-farm diagnosis suggested that *Photobacterium damselae* subsp. *piscicida* might have played a role in the disease, evidence for involvement of *P. damselae* in the sea bass pathology was not found, as IHC using anti-*P. damselae* MAb was negative.

In salmonids, *Piscirickettsia salmonis* produces a systemic disease and, while pathological changes are found in heart, brain, intestine, ovary and gill, the most characteristic pathology is severe inflammation and necrotic lesions of the kidney, liver and spleen (Fryer et al. 1990, Rodger & Drinan 1993, Palmer et al. 1996, Olsen et al. 1997). Likewise, in diseases of white seabass (M. F. Chen et al. 2000) and grouper (S. C. Chen et al. 2000), both associated with PLOs, and in diseases of tilapia (Chen et al. 1994, Chern & Chao 1994) which were associated with RLOs, the kidney, spleen and liver also exhibited the most marked pathology, although lesions were commonly found in all tissues, including the brain. In the European sea bass, however, the most significant pathology has been associated with the brain (Comps et al. 1996 and this study) and, while antigenic similarities between the sea bass RLO and *Piscirickettsia salmonis* have been intimated by cross-reaction with anti-*P. salmonis* antiserum in IHC (Steiropoulos et al. 2002), the different pathology suggested that the organisms might not be related. Of possible interest in this regard is a RLO which was associated with brain lesions and encephalitis in Scottish farmed Atlantic salmon, but which did not react in a latex agglutination test for *P. salmonis* (Grant et al. 1996).

Since the development of molecular genetic techniques, several methods have been used to differentiate between genera, species and strains of bacteria (Grayson et al. 1999, Romalde et al. 1999, Gürtler & Mayall 2001, Houpikian & Raoult 2001). For phylogenetic studies, where differentiation between strains is used to determine their inter-relatedness and how they may have evolved from a common ancestor, methods such as DNA-DNA hybridisation or pulse-field gel electrophoresis (PFGE) allow very fine discrimination between isolates (Leclerc et al. 2000, Houpikian & Raoult 2001, Le Roux et al. 2004). However, as these methods are not practical for routine use and may prove too sensitive, their application might be most useful in differentiating between strains where a close relationship has already been demonstrated (Leclerc et al. 2000, Gürtler & Mayall 2001, Le Roux et al. 2004). Another method widely used for phylogeny is the comparison of aligned nucleotide sequences of conserved genes, which have been termed 'molecular chronometers' (Fournier et al. 1998). The rRNA genes are highly conserved across the bacterial kingdom and the 16S rRNA gene within the *rrn* operon contains highly conserved regions as well as regions that vary according to species, genera and family. Therefore, comparison of 16S rDNA sequence has become widely used for the classification of organisms (Ruimy et al. 1994, Spröer et al. 1999, Gürtler & Mayall 2001). However, in some cases, the level of conservation between strains is such that differentiation between them on the basis of 16S rDNA sequence analysis is not possible. This is the case, for example, with mammalian rickettsiae (Stothard et al. 1994) and, in these situations, workers have looked to sequencing other, less-highly conserved, genes (Roux et al. 1996, Roux et al. 1997, Fournier et al. 1998). Another alternative is the ITS region DNA found between the 16S and 23S rRNA genes in the *rrn* operon (García-Martínez et al. 1999, Leclerc et al. 2000, Houpikian & Raoult 2001, Hamid et al. 2002). Bacteria frequently possess several copies of the *rrn* operon in their genome (Gürtler & Stanisich 1996, Crosby & Criddle 2003) although there are exceptions such as the *Mycobacteria* and *Mycoplasma*, where, as a rule, only 1 or 2 copies are present (Gürtler & Stanisich 1996). It has been found that there can be considerable variation in the length and sequence of the copies of the 16S-23S ITS region even within a single genome and, therefore, the scope for comparison between strains using ITS sequences is increased (Gürtler & Stanisich 1996, Gürtler & Mayall 2001, Hamid et al. 2002). The main source of variation is the number and type of tRNA genes found in the region between the 16S and 23S rRNA genes (Gürtler & Stanisich 1996). The majority of Gram-positive bacteria do not possess tRNA genes within the ITS region but either $tRNA^{ala}$ or $tRNA^{ile}$ or both genes may be present. In Gram-negative bacteria, however, it is common to find genes for both $tRNA^{ile}$ and $tRNA^{ala}$, or only $tRNA^{glu}$ (Gürtler & Stanisich 1996, Christensen et al. 2000). A further major source of variation between the ITS regions of different strains is in the form of insertion/deletions believed to have arisen from recombination events (Pérez-Luz et al. 1998, Andersson et al. 1999, Garcia-Martinez et al. 1999, Gürtler & Mayall 2001). Because considerable variation in ITS sequence can occur not only between different strains of a genus but also between the operons of a single organism (intercistronic variation), the strength of ITS analysis may lie in fine discrimination between strains of bacteria where 16S rDNA analysis has already suggested a close relationship exists (Ruimy et al. 1994, Pérez-Luz 1998, García-Martínez et al. 1999). Furthermore, the limited number of published ITS sequences compared with the number of 16S rDNA sequences means that initial phylogenetic placement of an organism will, most likely, be based on analysis of its 16S rRNA.

In the study of fish pathogens, differences in 16S rDNA sequences have been used for classifying isolates (Romalde et al. 1999, Reid et al. 2003). Using 16S rDNA sequences, Mauel et al. (1999) demonstrated a monophyletic relationship between strains of *Piscirickettsia salmonis* from Chile, Canada and Norway, with similarities ranging from 99.7 to 98.5%. These workers also examined the DNA sequence of the ITS and of the 23S rRNA and found that, while these also showed low levels of divergence between isolates, the greater variability of these regions was reflected in the wider range of similarities; 95.2 to 99.7% and 97.9 to 99.8% respectively. Nevertheless, the phylogenetic trees derived from all 3 data sources, 16S rDNA, ITS and 23S rDNA, were in close agreement. Recently, Reid & Birkbeck (2003) compared 16S rDNA and ITS sequences from Scottish and Irish isolates and found that the Scottish isolates formed a homogenous group which was closely related to the Chilean isolates, LF-89 and SLGO-94. Irish isolates from 1999 clustered with EM-90, hitherto the most distantly related strain, while Irish isolates from 1991, 1995 and 1998 formed a new divergent group. However, while these groups found only a single *rrn* operon, Casanova et al. (2001), using different PCR primers, identified 2 ITS sequences in the type-strain, LF-89 and in EM-90, by polyacrylamide gel electrophoresis of PCR amplified ITS region DNA. One ITS sequence was the same as that described by Mauel et al. (1999), while the second ITS contained genes for $tRNA^{ile}$ and $tRNA^{ala}$. Based on this observation, it was proposed that more than one *rrn* operon might exist in these Chilean isolates.

In this study, and in work by Steiropoulos et al. (2002), serological analysis had established the likelihood of a relationship between the organism found in European sea bass and those organisms identified as *Piscirickettsia salmonis* from Chilean, Canadian and Norwegian salmonids. However, detailed analysis of genetic similarities has not previously been undertaken. It has been confirmed, through phylogenetic analysis of 16S rDNA and ITS sequences, that the organism found in European sea bass is a member of the *P. salmonis* genus which clusters with other members of this genus for which sequence information is available. Sequence similarity between the sea bass 16S rRNA gene and the *P. salmonis* type-strain is 98.2%, which compares well with sequence similarities of the Canadian and Norwegian strains. The greater variability of the 16S-23S spacer region is reflected in the lower sequence similarity of the ITS_0 sequence, but this does not affect the overall placement of the SBPLO isolate within the range of ITS sequence similarities exhibited among the currently available *P. salmonis* sequences. Although it is more divergent than the Scottish isolates and Irish isolates from 1999, both sequence similarity and dendrograms for 16S rDNA and ITS sequences suggest that the SBPLO isolate is more closely related to the majority of *P. salmonis* strains than the Irish isolates from 1991 and 1998.

The present study was conducted on a single isolate from one site in Greece. A more robust phylogenetic analysis will require the availability of further isolates from sea bass in the Mediterranean to determine whether or not these form a separate cluster within the *Piscirickettsia salmonis* group. Detailed comparisons of the tRNA-containing ITS sequence between *P. salmonis* strains may yield useful information about the geographical spread of the organism, while sequence comparisons of other genes coding for shared antigens, evident from serological analysis, may help to further elucidate relationships among the group. Ultimately, DNA-DNA hybridisation studies may suggest the requirement for creation of separate species.

The finding of RLOs in widely differing fish species, from numerous locations world-wide, suggests that these are ubiquitous parasites adapted to utilise whatever hosts are available locally. As with other pathogens, it will be important to determine whether it is environmental changes or the availability of naïve hosts which result in reduced host immunity or increased pathogenicity (host susceptibility), leading to the establishment of conditions which encourage disease outbreaks. Given the rapidly expanding aquaculture industry, with the frequent introduction of new species for farming, it seems likely that PLOs may, in the future, pose disease problems. As antibiotic treatment of disease caused by this intracellular pathogen is both costly and relatively inefficient (Fryer & Hedrick 2003), successful combatting of this disease will rely largely upon the availability of vaccines. Therefore, it will be crucial to understand the mechanisms the organism uses to evade the host immune system and, using this knowledge, develop more effective vaccines.

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