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Direct identification of *Photobacterium damselae* subspecies *piscicida* by PCR-RFLP analysis

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ABSTRACT: Fish pasteurellosis is an infectious disease that affects several teleost species living in temperate marine waters. The pathogen responsible, *Photobacterium damselae* subspecies *piscicida*, shows high genetic similarity with *P. damselae* subsp. *damselae*, making subspecies discrimination extremely laborious. Here we report for the first time a PCR-RFLP method for the identification of *P. damselae* subsp. *piscicida* without prior isolation in pure culture. Genomic sequence information was obtained through cloning and sequencing of RAPD products. Two *P. damselae*-specific primer pairs were developed and tested on 17 strains of *P. damselae* subsp. *piscicida*, 10 strains of *P. damselae* subsp. *damselae*, and 6 closely related control species. High sensitivity was achieved in PCR amplification on serially diluted samples (<180 fg of pure bacterial DNA or <10 fg, depending on the amplified fragment). Restriction analysis of PCR products showed a unique digestion profile for all *P. damselae* subsp. *piscicida* strains. The same PCR-RFLP method was implemented on total DNA samples extracted from experimentally infected sea bream and sea bass. Positive results were obtained on fish with clear signs of the disease as well as on challenged, but asymptomatic, fish. The method presented here might provide a useful tool for both prevention and rapid diagnosis of fish pasteurellosis.

KEY WORDS: *Photobacterium damselae* subsp. *piscicida* · Fish pasteurellosis · PCR-RFLP · Diagnostic tool

INTRODUCTION

Fish pasteurellosis is an infectious disease that affects several teleost species living in temperate marine waters (Magariños et al. 1996). The responsible pathogen, *Photobacterium damselae* subspecies *piscicida*, was firstly isolated in North America in 1963 (Snieszko 1964). Since then it has been reported to cause severe outbreaks in farmed marine fish in Japan and the Mediterranean area. The etiological agent of the disease has been assigned to the genus *Photobacterium* (Family Vibrionaceae) as a separate subspecies (subsp. *piscicida*) than *P. damselae* subsp. *damselae.* High genetic similarity was found between the 2 subspecies by DNA-DNA hybridization and 16S rRNA gene sequencing (Gauthier et al. 1995, Osorio et al. 1999), despite the fact that they show distinctive biochemical and morphological characteristics (Magariños et al. 1992, Thyssen et al. 1998). Both subspecies are considered pathogenic for marine fish. However, only subspecies *piscicida* causes acute infections with high mortality rates. The role of *P. damselae* subsp. *damselae* as a fish pathogen is not clear because it is often isolated in healthy fish (Botella et al. 2002, Pujalte et al. 2003). Therefore, the ability to detect very low quantities of the micro-organism directly in fish tissues and in water or sediment samples, as well as to discriminate between the 2 subspecies, is necessary for

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a better prevention of the disease through control of the presence of the pathogen in the water and/or carrier fishes. Thus far, a broad variety of methods has been evaluated for highly sensitive, species-(subspecies) identification of the pathogen without prior isolation as pure culture. Tested methods include enzyme-linked immunosorbent assays (ELISA) and enzyme immuno-assay (EIA) techniques (Bakopoulos et al. 1997, Romalde et al. 1999), plasmid DNA PCR (Aoki et al. 1997), 16S rRNA gene sequencing (Osorio et al. 1999), and primers designed following random amplified polymorphic DNA (RAPD) (Dalla Valle et al. 2002). All these methods, however, could not reliably discriminate between the 2 subspecies. A method based on duplex PCR of *ureC* and 16S rRNA genes was proposed by Osorio et al. (2000), due to the lack of the *ureC* gene in the subspecies *piscicida*, but subspecies discrimination was based on indirect evidence (lack of amplification of *ureC*). Lately, a combined approach of PCR technique and plating method has been proposed (Rajan et al. 2003). Other methods such as ribotyping (Magariños et al. 1997), RAPD (Random Amplified Polymorphic DNA) (Magariños et al. 2000), and amplified fragment length polymorphism (AFLP) (Thyssen et al. 2000, Kvitt et al. 2002, L. Bargelloni unpubl. data) showed the ability to differentiate the 2 subspecies as well as to identify clusters of *P. damselae* subsp. *piscicida* strains. However, these methods require DNA extraction from pure cultures and reference profiles are needed for strain identification.

In the present study, we describe a highly sensitive test based on PCR-RFLP analysis that allows culture-independent discrimination between the 2 subspecies.

MATERIALS AND METHODS

Bacterial cultures and DNA extraction. A total of 17 strains of *Photobacterium damselae* subsp. *piscicida* and 10 strains of *P. damselae* subsp. *damselae* were examined in the present study, including type and reference strains for both subspecies (Table 1). Among the 10 strains of *P. damselae* subsp. *damselae*, 3 strains formerly classified as *P. histaminum* were included. Based on the results

Table 1. Bacterial strains included in the study of *Photobacterium damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. NR: host species not reported

Strain	Origin	Source
P. damselae* subsp. *piscicida		
NCIMB 2058^T	Japan	Yellowtail *Seriola quinqueradiata*
ATCC 17911	USA	White perch *Roccus americanus*
I736	Italy	Sea bass *Dicentrarchus labrax*
K12	Italy	Sea bass *Dicentrarchus labrax*
OO4	Italy	Sea bass *Dicentrarchus labrax*
PD010099	Italy	Sea bass *Dicentrarchus labrax*
PD010701	Italy	Sea bream *Sparus aurata*
PN510	Greece	Sea bream *Sparus aurata*
K5	Greece	Sea bass *Dicentrarchus labrax*
B3	Greece	Sea bass *Dicentrarchus labrax*
B7	Greece	Sea bass *Dicentrarchus labrax*
SU7	Greece	Sea bass *Dicentrarchus labrax*
M28561	Japan	Yellowtail *Seriola quinqueradiata*
TFP88012	Japan	Yellowtail *Seriola quinqueradiata*
SP98037	Japan	Yellowtail *Seriola quinqueradiata*
SA071194	Israel	Sea bream *Sparus aurata*
XXX000696	Israel	Hybrid striped bass
249/Ittio99	Italy	Sea bass *Dicentrarchus labrax*
332/Ittio01	Italy	Sea bass *Dicentrarchus labrax*
349/Ittio00	Italy	Sea bass *Dicentrarchus labrax*
Dl21	Portugal	Sea bream *Sparus aurata*
P. damselae* subsp. *damselae		
ATCC 33539^T	USA	Damselfish *Chromis punctipinnis*
ATCC 35083	USA	Brown shark *Carcharhinus plumbeus*
NCIMB 2181	USA	Sea water
NCIMB 2182	USA	Human leg wound
NCIMB 2183	USA	Human leg wound
JCM 8967[a]	Japan	Sea water
JCM 8969[a]	Japan	Sea water
NCTC 11646	UK	Human leg wound
NCTC 11648	UK	NR
NCIMB 13351[a]	Japan	Sea water
External controls		
Photobacterium leiognathi (CCUG 16229^T)	Malaysia	Teleost fish *Leiognathus equula*
P. phosphoreum (NCIMB 1282^T)	NR	NR
P. fischeri (ATCC 7744^T)	USA	NR
Vibrio hollisae (NCTC 11640^T)	USA	Human faeces
V. natriegens (ATCC 14048^T)	USA	Marine sediment
V. carchariae (CCUG 19116)	USA	Brown shark *Carcharhinus plumbeus*
V. anguillarum 5894/83	UK	NR

[a]Formerly *P. histaminum*

of Kimura et al. (2000), *P. histaminum* has been now reclassified as *P. damselae* subsp. *damselae*. Negative controls were other *Photobacterium* species as well as selected marine *Vibrio* species, as listed in Table 1. Standard microbiological procedures were applied to obtain pure cultures for each strain. Total bacterial DNA was extracted from pure cultures using a DNeasy tissue kit (Qiagen) following the manufacturer's instructions. Extracted DNA was run on 1% agarose gel to check for quality, and concentration was estimated using a UV spectrophotometer. A similar procedure was used to extract total genomic DNA from 5 to 15 mg of fish tissue. These samples were collected in the course of a challenge experiment (see below).

Experimental infections. European sea bass (60 individuals) of 10 g body weight and gilthead sea bream (150 individuals) of 0.5 g body weight were used in 2 separate experiments of controlled infection. All fishes were obtained from the fish farm 'Valle Ca' Zuliani' (Pila di Porto Tolle, Rovigo, Italy). Fishes were transferred to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSV, Legnaro, Italy) and kept in aerated and recirculated seawater at 20°C for 1 wk for acclimation. Ten individuals for each species were sacrificed before starting the challenge. Gills, kidney, spleen, and liver were dissected from each specimen and stored in 80% ethanol at 4°C for subsequent analysis.

Sea bass were divided in 5 groups of 10 individuals each. All individuals were subjected to intraperitoneal injection with 0.1 ml of a suspension of *Photobacterium damselae* subsp. *piscicida* (10^7 bacteria l^{-1}). For each group a different strain was used: Strain NCIMB 2058^T (Group Ba1), Strain 249/Ittio99 (Group Ba2), Strain 349/ Ittio00 (Group Ba3), Strain 332/Ittio01 (Group Ba4) (strains are listed in Table 1). The latter 3 strains were isolated and characterized at the IZSV from infection outbreaks in different fish farms. A fifth group (Ba5) was injected with 0.1 ml of sterile water, and kept in the same tank with individuals of Group Ba4, separated by a metal grid (cohabitation group). Mortality was monitored 5 d after challenge, dead fish were removed and tissue samples collected as described above. Starting from Day 2 after challenge, 2 live individuals per group were removed every day if no mortality was observed. Tissue samples from these fish were collected and stored as described previously.

Sea bream were divided in 3 groups. Individuals of 2 groups (Br1 and Br2) were challenged by immersion for 30 min in 10 l of aerated seawater with *Photobacterium damselae* subsp. *piscicida* (3×10^6 bacteria l^{-1}). Group Br1 was challenged with Strain Dl21 (Osorio et al. 1999), Group Br2 with Strain 249/Ittio99 (see above). A third group (Br3) was mock-infected by immersion for 30 min in 10 l of clean aerated seawater. Mortality was monitored for 15 d after challenge, dead fish were removed and tissue samples collected as described above. Starting from Day 2 after challenge, 2 to 5 live individuals per group were also removed every day if no mortality was observed. Tissue samples from these fish were collected and stored as described.

Amplification and cloning of RAPD PCR-products. RAPD was carried out on 8 *Photobacterium damselae* subsp. *piscicida* strains (ATCC17911; I736; PN510; M28561; K5; SA040296; SA071194; TFP88012) and 2 *P. damselae* subsp. *damselae* strains (ATCC35083; NCIMB2184). Two 10-mer primers (Operon 10-mer Kit A, Operon [now Qiagen]) were used: OPA-04 (AATCGGGCTG) and OPA-07 (GAAACGGGTG). The PCR reaction was carried out following a protocol modified by Grayson et al. (1999). A final volume of 25 µl RAPD reaction mixture contained 0.5 units of *Taq* DNA polymerase (Promega), 1× thermophilic DNA polymerase buffer, magnesium free, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 12 pmol (picomol) of primer and approximately 50 to 100 ng of DNA. The thermal profile, carried out in a GeneAmp 9700 (Applied Biosystems), included a pre-denaturation step (2 min at 94°C), plus 40 cycles consisting of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C. All the amplified products were analyzed on 1.8% agarose gel, visualized with ethidium bromide under UV light. Gel pictures were obtained with an Image Master® VDS, Fujifilm Thermal Imaging System FTI 500.

RAPD products were cloned into a plasmid vector (pCR-II, Invitrogen) using a commercial kit (TOPO-TA Cloning, Invitrogen), following the manufacturer's instructions. In each ligation reaction, 2 µl of amplified DNA was used for each of the following RAPD amplifications: Strains I736 and ATCC35083 with Primer OPA-04; Strains I736 and M28561 with Primer OPA-07. Insert size of recombinant clones (35 distinct colonies) was assessed by PCR using vector primers. Plasmids containing each of 6 inserts differing in size were prepared using a commercial kit (QIAprep Spin Miniprep Kit, Qiagen) and cycle-sequenced on both strands with vector universal primers in a 3700 ABI sequencer.

The nucleotide sequences obtained were used as a query to search the GenBank section of complete bacterial genomes (www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) using the program BLAST with default settings, either with the BlastN or the TblastX option. Specific primers for 2 genomic regions (Table 2) were developed using the program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

***Photobacterium damselae*-specific PCR amplification.** PCR amplifications using the primers listed in Table 2 were carried out under the following conditions: a final volume of 20 µl PCR mixture contained 0.5 U of *Taq* DNA Polymerase (Promega), 1× thermo-

Table 2. Primers used in the present study. Ta: annealing temperature

Primer name	5′-3′ sequence	Ta (°C)
P.dam-5a_F	CAACCCTGCAACATTTCTACCAAG	55
P.dam-5a_R	GGAGTGCATGCCGAACAAGC	55
P.dam-1a_F	CTTAACGCTACGTGGTGACAGTT	55
P.dam-1a_R	AGACGATCGCCTGCAATAAC	55

philic DNA polymerase buffer, magnesium free, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer and approximately 50 to 100 ng of bacterial DNA. The thermal profile included a predenaturation step (2 min at 94°C), plus 30 cycles consisting of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C. DNA extracted from all strains listed in Table 1 were subjected to PCR amplification with primer pairs P.dam-5a_F-R and P.dam-1a_F-R. Undiluted DNA extracted from fish tissues was amplified using similar PCR conditions, except for the number of cycles which was increased to 40. PCR products were analyzed by gel electrophoresis as previously described.

The sensitivity of both PCR amplifications was assessed as follows: 1:10 serial dilutions of bacterial DNA were prepared from DNA stock solutions adjusted to 180 ng DNA μl^{-1} (amplification with primer pair P.dam-5a_F-R) or 50 ng μl^{-1} (amplification with primer pair P.dam-1a_F-R) of *Photobacterium damselae* subsp. *piscicida* Strain NCIMB 2058^T and *P. damselae* subsp. *damselae* Strain ATCC 33539^T. The concentration of amplified DNA ranged from 0.9 µg DNA ml^{-1} to 0.9 fg DNA μl^{-1} (primer pair P.dam-5a_F-R) or from 0.5 ng DNA μl^{-1} to 0.5 fg DNA μl^{-1} (primer pair P.dam-1a_F-R). Two µl of each dilution was used for PCR amplification. The amplification protocol was the same as described above, except for the number of cycles, which was increased to 45.

Restriction fragment length polymorphism (RFLP) analysis. RFLP was carried out on DNA fragments amplified with primer pairs P.dam-5a_F-R and P.dam-1a_F-R. All restriction enzymes and buffers were purchased from New England Biolabs (Beverly). Fragment P.dam-5a_F-R was serially digested with 4 restriction enzymes. Five to 10 µl of each amplified DNA, depending on intensity of PCR products, were used for restriction. The reaction mixture had a final volume of 30 µl containing 1.8 U of *Hha I* and 2 U of *EcoR V*, 1× NEBuffer 3 and 100 µg bovine serum albumin ml^{-1}. The reaction mixture was incubated at 37°C for 90 min. Subsequently, to each sample was added 5 µl of a solution containing 1 U of *BsmA I* and 1× NEBuffer 3. The reaction was then incubated for 60 min at 55°C. Finally, 5 µl of a solution containing 2 U of *Bsr I* and 1× NEBuffer 3 was added, and the reaction was incubated at 65°C for 60 min. Fragment P.dam-1a_F-R was digested with a single restriction enzyme under the following conditions: as above, 5 to 10 µl of amplified DNA was used in a 30 µl reaction mixture containing 3 U of *BstU I* and 1× NEBuffer 2. The reaction was incubated at 60°C for 90 min.

Restriction fragments were separated by electrophoresis on 4% agarose obtained mixing 3 parts of NuSieve® agarose (FMC Bioproducts [now Cambrex]) with 1 part of normal agarose. Restriction fragments were visualized and documented as described previously.

RESULTS

Six RAPD fragments were cloned and sequenced (GenBank AY191120, AY191121, AY332620–AY332623). Similarity searches against bacterial nucleotide sequence databases identified 5 out of these 6 selected regions as putative homologues of genomic sequences from *Vibrio cholerae* (Strain N16961). On the basis of the alignment with these sequences, specific primers (P.dam-5a_F-R, P.dam-1a_F-R, Table 2) were designed on non-conserved sites of 2 genomic regions (GenBank AY191120, AY191121). The expected size of the amplification products, P.dam-5a_F-R and P.dam-1a_F-R, were 713 and 201 base pairs (bp), respectively. The use of these newly designed primers in PCR amplification produced a visible product of correct size for all strains of *Photobacterium damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*, including the 3 strains (JCM8967, JCM8969, NCIMB13351) formerly classified as *P. histaminum*. No amplification product was obtained from external controls using either primer pair. External controls included closely related species such as *P. leiognathi*, *P. fisheri*, and *P. phosphoreum*. These results indicate that amplifications using either primer pair are highly specific to *P. damselae*.

The sensitivity of amplification was assessed on 1:10 serial dilutions of pure bacterial DNA. PCR amplification with primer pair P.dam-5a_F-R showed that the minimum detectable amount of *Photobacterium damselae* DNA was between 1.8 pg and 180 fg (Fig. 1A). Higher sensitivity was achieved with primer pair P.dam-1a_F-R. In this case the lowest amount of DNA producing a visible product was 1 to 10 fg (Fig. 1B). Neither amplification with primer pair P.dam-5a_F-R nor with P.dam-1a_F-R, however, could discriminate between *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. To achieve subspecies identification, a restriction analysis was carried out on both amplified fragments. Four restriction enzymes (*Hha I*, *Bsr I*, *EcoR V*, *BsmA I*) were used for multiple

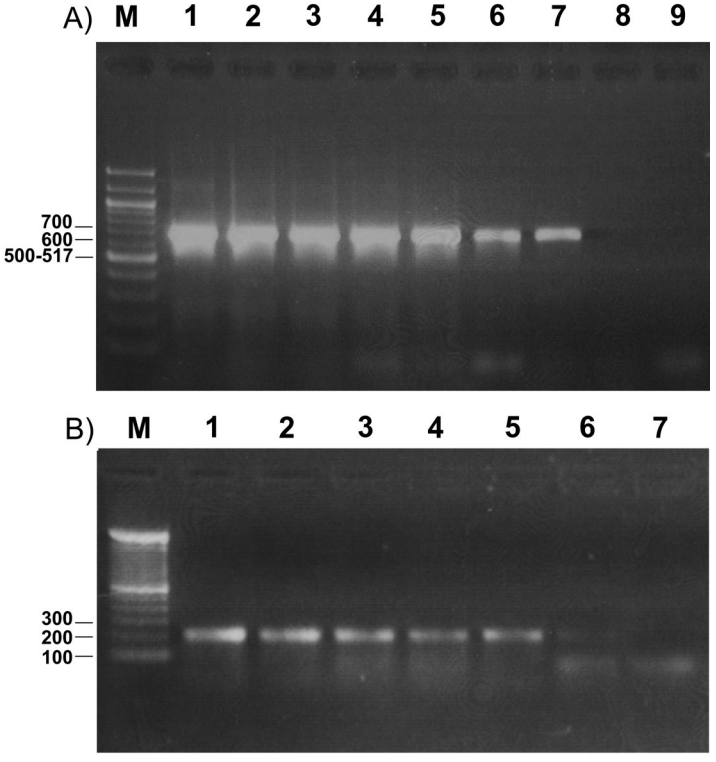

Fig. 1. Sensitivity of PCR amplification with *Photobacterium damselae*-specific primers. (A) Agarose gel electrophoresis of PCR products using primer set P.dam-5a_F-R. Lane M: size-marker 100 bp DNA ladder (New England Biolabs); Lanes 1 to 9 are serial dilutions (1:10) of purified DNA of *P. damselae* subsp. *piscicida* strain ATCC 17911 used as template: (1) 1.8 µg, (2) 180 ng, (3) 18 ng, (4) 1.8 ng, (5) 180 pg, (6) 18 pg, (7) 1.8 pg, (8) 180 fg, (9) 18 fg. (B) Agarose gel electrophoresis of PCR products using primer set P.dam-1a_F-R. Lane M: size-marker 100 bp DNA ladder (Invitrogen); Lanes 1 to 7 are serial dilutions (1:10) of purified DNA of *P. damselae* subsp. *piscicida* strain NCIMB 2058^T used as template: (1) 1 ng, (2) 100 pg (3) 10 pg, (4) 1 pg, (5) 100 fg, (6) 10 fg, (7) 1 fg

Table 3. Restriction analysis of fragment P.dam-5a_F-R

Strain(s)	Enzymes	Expected fragments (size in bp)
All *Photobacterium damselae* subsp. *piscicida* strains	*EcoR V*, *BsmA I*	459, 158, 63, 33
P. damselae subsp. *damselae* strains ATCC33539^T, NCIMB2181, JCM8967, NCTC11646, NCIMB13351	*Hha I*, *BsmA I*	310, 182, 158, 63
P. damselae subsp. *damselae* strain JCM8969	*EcoR V*, *Bsr I*	351, 221, 108, 33
P. damselae subsp. *damselae* strains ATCC35083, NCIMB2182, NCIMB2183, NCTC11648	*Hha I*, *Bsr I*, *EcoR V*, *BsmA I*	310, 158, 108, 63, 41, 33

digestion of fragment P.dam-5a_F-R. Four different restriction profiles were obtained depending on the strain analyzed (Table 3). A single digestion pattern was observed for all 17 strains of *P. damselae* subsp. *piscicida* (Fig. 2, Lane 1). Three additional profiles characterized the examined strains of *P. damselae* subsp. *damselae* (Fig. 2, Lanes 2 to 4). Each amplified fragment was cut by at least 2 enzymes. The restriction pattern of all *P. damselae* subsp. *piscicida* strains could be clearly distinguished by the presence of a fragment larger than 400 bp and the absence of a fragment of ca. 310 to 350 bp. In the restriction analysis of the amplified fragment P.dam-1a_F-R, a single enzyme (*BstU I*) was used. Only 1 *BstU I* restriction site is present in DNA amplified from all the strains of *P. damselae* subsp. *piscicida*, whereas 2 restriction sites are found for all *P. damselae* subsp. *damselae* strains (Fig. 3, Table 4). The only exception is *P. damselae* subsp. *damselae* strain NCIMB2181, where only 1 restriction site is present, namely the one distinguishing all *P. damselae* subsp. *damselae* strains from all *P. damselae* subsp. *piscicida* (Fig. 3, Lane 2).

The PCR-RFLP method described above was then tested on DNA extracted from fish tissues that were collected during a challenge experiment on sea bream and sea bass (see 'Materials and methods'). After experimental infection, 3 sea bass groups (Ba2, Ba3 and Ba4) showed high mortality. Group Ba1, which was infected with Reference Strain NCIMB2058^T, and Group Ba5, which was maintained in cohabitation with individuals of Group Ba4, displayed no mortality after 1 wk. Therefore, for Groups Ba1 and Ba5, tissue samples were collected only from fish that were still alive and subsequently sacrificed. For Groups Ba2, Ba3 and Ba4, deceased fish were also analyzed. Similarly, in the sea bream experiment, only Group Br2, which had been infected with *Photobacterium damselae* subsp. *piscicida* Strain 249/Ittio99, showed high mortality after 4 d post-challenge. No mortality was recorded after 15 d for Group Br1, which was challenged with the repeatedly-transferred Strain Dl21 (see 'Materials and methods'), nor for Group Br3 that included only mock-infected individuals. As in the sea bass experiment, only sacrificed animals were therefore analyzed for Groups Br1 and Br3. Spleen tissue samples of 2 or 3 randomly selected individuals per group were processed to extract total genomic DNA. Extracted DNA was subsequently used for PCR-amplification and restriction analysis of both fragments P.dam-5a_F-R and P.dam-1a_F-R. Two

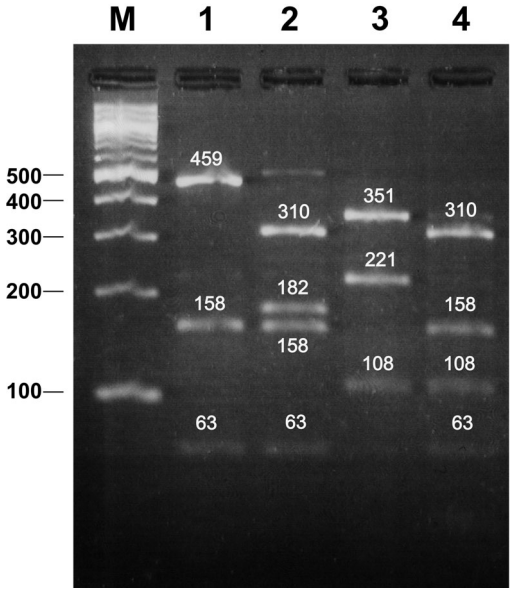

Fig. 2. RFLP analysis of *Photobacterium damselae*-specific PCR product P.dam-5a_F-R. Electrophoresis on 3:1 (Nusieve: normal) agarose gel of fragment P.dam-5a_F-R amplified on *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* strains and digested with *Hha I*, *Bsr I*, *EcoR V*, and *BsmA I*. Lane M: size-marker 100 bp DNA ladder (Invitrogen); Lane 1: *P. damselae* subsp. *piscicida* Strain NCIMB 2058^T; Lanes 2 to 4: *P. damselae* subsp. *damselae* Strains JCM 8967, JCM 8969, and NCTC 11648, respectively. Size of each restriction fragment is indicated

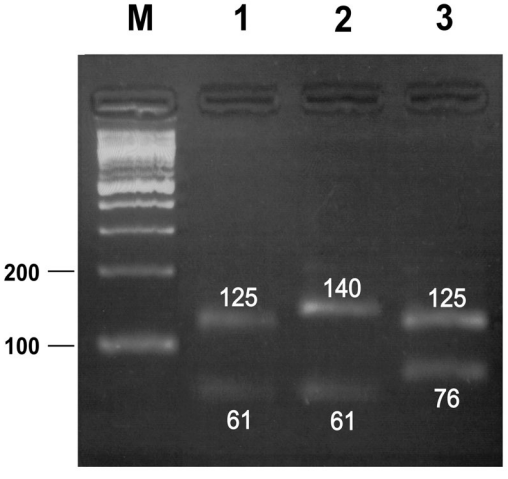

Fig. 3. RFLP analysis of *Photobacterium damselae*-specific PCR fragment P.dam-1a_F-R. Electrophoresis on 3:1 (Nusieve:normal) agarose gel of fragment P.dam-1a_F-R amplified on *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* strains and digested with restriction enzyme *BstU I*. Lane M: size-marker 100 bp DNA ladder (Invitrogen); Lanes 1 to 3: *P. damselae* subsp. *damselae* Strains NCTC 11648, NCIMB 2181, and *P. damselae* subsp. *piscicida* Strain NCIMB 2058^T, respectively. Size of each restriction fragment is indicated

sea bream and 2 sea bass individuals belonging to the group of fishes sacrificed before starting the challenge experiment (negative controls) were included in the analysis as well. Results of amplification from fish tissues are summarized in Table 5. An example of amplification products is presented in Fig. 4. A visible product was obtained from all individuals of sea bass Groups Ba2, Ba3 and Ba4 and of sea bream Group Br2, when analyzing fragment P.dam-1a_F-R. All these groups presented high mortality after infection. Similar results were obtained using the primer pair P.dam-5a_F-R, except for 1 individual of sea bass Group Ba2 that was negative. The same individual showed a weak product for the shorter amplified fragment (Table 5). For sea bass Groups Ba1 and Ba5, and for sea bream Group Br1 only amplification with primer pair P.dam-1a_F-R yielded a faint, but detectable PCR product (Fig. 4). No amplified fragments were observed for sea bream Group Br3 (mock-infected animals) and for sea bass and sea bream negative controls. Amplified DNA from positive individuals was subsequently digested with the appropriate restriction enzyme(s). A single digestion pattern was obtained for each fragment, confirming the presence of *P. damselae* subsp. *piscicida* in the tissue samples examined. An example of the profiles obtained is presented in Fig. 5.

DISCUSSION

As already mentioned, in the last few years, several different molecular techniques have been evaluated in order to develop a highly sensitive test for discriminating the 2 highly genetically related subspecies of *Photobacterium damselae*. In the present study, we used cloning and sequencing of RAPD amplified fragments as a source of genomic sequence information. In fact, this approach provided a good starting point to develop specific primers. Dalla Valle et al. (2002) used a similar method to design primers that specifically amplified strains of both subspecies, although no discrimination between subspecies was achieved. In the present study, 2 pairs of specific primers were developed allowing amplification of all strains from both subspecies, without cross-amplification of external controls. Among the outgroup strains, the most closely related species to *P. damselae* and other common *Vibrio* species were included. This evidence indicates that amplification of both DNA fragments occurs with high specificity and sensitivity. In controlled conditions it was possible to detect very low amounts of bacterial DNA, with a limit of 1.8 pg DNA for fragment P.dam-5a_F-R, and 10 fg for fragment P.dam-1a_F-R. The sensitivity achieved in this work for fragment P. dam-5a_F-R was generally comparable to that obtained in previous studies for direct PCR detection of *P. damse-*

Table 4. Restriction analysis of fragment P.dam-1a_F-R

Strain(s)	Restriction site(s) for *BstU I*	Expected fragments (size in bp)
All *Photobacterium damselae* subsp. *piscicida* strains	124–128	125, 76
All *P. damselae* subsp. *damselae* strains except for NCIMB2181	124–128 139–142	125, 61, 15
P. damselae subsp. *damselae* strain NCIMB2181	139–142	140, 61

Table 5. PCR-RFLP analysis of fish tissues from challenge experiments. Under 'Sample' column, initial letters denote species: Dl represents *Dicentrarchus labrax*; Sa represents *Sparus aurata*. S and D in parentheses after sample code denote sacrificed and deceased individuals, respectively

Experimental group	Sample	Primers	
		P.dam-1a_F-R	P.dam-5a_F-R
Sea bass *Dicentrarchus labrax*			
Ba1 (Strain NCIMB 2058^T)	Dl14a (S)	–	–
Ba1 (Strain NCIMB 2058^T)	Dl31a (S)	+/–	–
Ba1 (Strain NCIMB 2058^T)	Dl39a (S)	+/–	–
Ba2 (Strain 249/Ittio99)	Dl12b (S)	+	+
Ba2 (Strain 249/Ittio99)	Dl29b (D)	+	+
Ba2 (Strain 249/Ittio99)	Dl41b (D)	+/–	–
Ba3 (Strain 349/Ittio00)	Dl11c (S)	+	+
Ba3 (Strain 349/Ittio00)	Dl32c (D)	+	+
Ba3 (Strain 349/Ittio00)	Dl33c (D)	+	+
Ba4 (Strain 332/Ittio01)	Dl10d (S)	+	+
Ba4 (Strain 332/Ittio01)	Dl36d (D)	+	+
Ba4 (Strain 332/Ittio01)	Dl37d (D)	+	+
Ba5 (cohabitation)	Dl7e (S)	+/–	–
Ba5 (cohabitation)	Dl23e (S)	+/–	–
Negative control	Dl3f (S)	–	–
Negative control	Dl5f (S)	–	–
Sea bream *Sparus aurata*			
Br1 (Strain Dl21)	Sa109 (S)	+/–	–
Br1 (Strain Dl21)	Sa111 (S)	+/–	–
Br2 (Strain 249/Ittio99)	Sa99 (S)	+	+
Br2 (Strain 249/Ittio99)	Sa130 (D)	+	+
Br2 (Strain 249/Ittio99)	Sa131 (D)	+	+
Br3 (negative control)	Sa41 (S)	–	–
Br3 (negative control)	Sa45 (S)	–	–

lae (1 pg, Osorio et al. 1999; 350 fg, Kvitt et al. 2002), whereas the sensitivity for the amplification of fragment P.dam-1a_F-R is similar or slightly higher than the lowest detection limit reported to date (20 fg, Dalla Valle et al. 2002). The higher sensitivity observed for fragment P.dam-1a_F-R is likely due to the smaller size of the amplified product.

Different estimates of the DNA content of single bacterial cells have been reported for *P. damselae* (0.5 fg per cell, Osorio et al. 1999; less than 10 fg per cell, Kvitt et al. 2002). The DNA content in related species ranges between 4.5 and 7 fg DNA per cell based on their genome size (*V. cholerae* 4.03 Mbp, *V. vulnificus* 5.26 Mbp, *P. profundum* 6.4 Mbp). The DNA content of *P. damselae* is likely comparable to that of the above-mentioned species, and similar to the value proposed by Kvitt et al. (2002; <10 fg per cell). If this holds true, the PCR method presented here allows the detection of less than 10 bacterial cells. This sensitivity is 3 orders of magnitude higher than the most sensitive immunological test that is presently in use for routine diagnosis of fish pasteurellosis (10^4 cells, Romalde et al. 1999).

The most important feature of the method proposed in the present work, however, is the ability to discriminate between *Photobacterium damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. A unique restriction pattern for both amplified fragments was observed for all the *P. damselae* subsp. *piscicida* strains examined. Digestion profiles of all the *P. damselae* subsp. *damselae* strains could always be distinguished from the RFLP profile of *P. damselae* subsp. *piscicida*. None of the previously reported culture-independent methods was suitable for subspecies identification. A partial exception is the 16S-*ureC* method proposed by Osorio et al. (2000), where lack of amplification of the *ureC* gene indicates the presence of *P. damselae* subsp. *piscicida*. Compared to the 16S-*ureC* method, the PCR-RFLP analysis proposed here represents a substantial improvement in terms of sensitivity and specificity. For instance, a distinctive RFLP pattern for *P. damselae* subsp. *piscicida* allows the detection of this subspecies even when *P. damselae* subsp. *damselae* is also present (data not shown). This cannot be obtained with the method proposed by Osorio et al. (2000), because lack of *ureC* amplification for *P. damselae* subsp. *piscicida* would be obscured by the presence of a PCR product from *P. damselae* subsp. *damselae.* This is an important issue because active and dormant states of the microorganism have been found to be present for both subspecies in the same environment, and in similar fish species (Fouz et al. 1998, Fouz et al. 2000, Botella et al. 2002). High sensitivity is obviously required to detect very low concentrations of the micro-organism, but subspecies discrimination is necessary as well, because of the biological differences between the 2 subspecies,

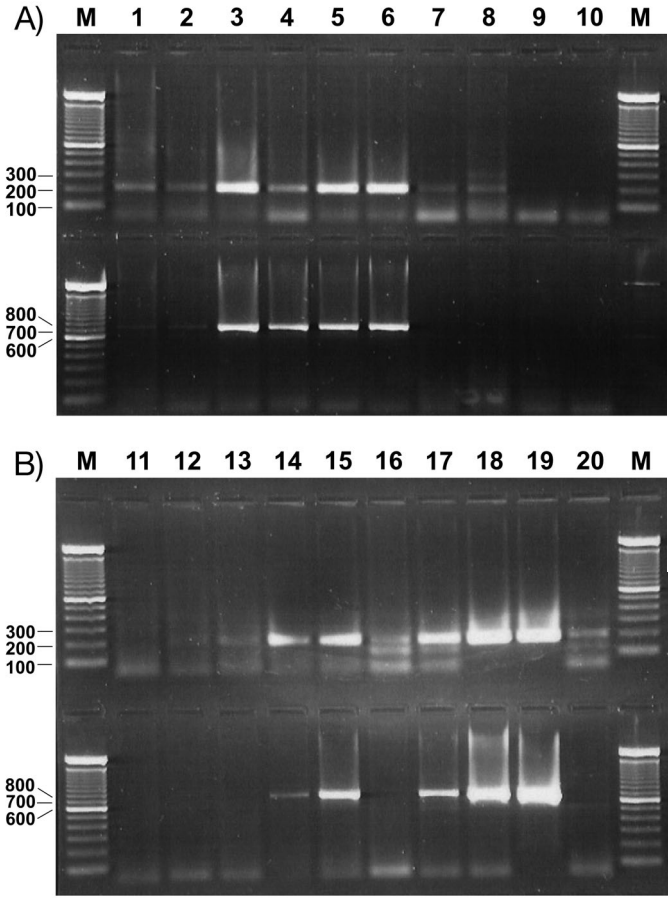

Fig. 4. PCR detection of *Photobacterium damselae* in tissues from experimentally infected fishes. Agarose gel electrophoresis of PCR products from DNA extracted from tissues using primer set P.dam-1a_F-R (A) and P. dam-5a_F-R (B). Lane M (A, B): size-marker 100 bp DNA ladder (Invitrogen); Lanes 1 to 20: Dl11c, Dl32c, Dl33c, Dl10d, Dl36d, Dl37d, Dl7e, Dl23e, Dl5f, Sa41, Dl14a, Dl31a, Dl39a, Dl12b, Dl29b, Dl41b, Sa99, Sa130, Sa131, and Sa109. See Table 5 for sample names

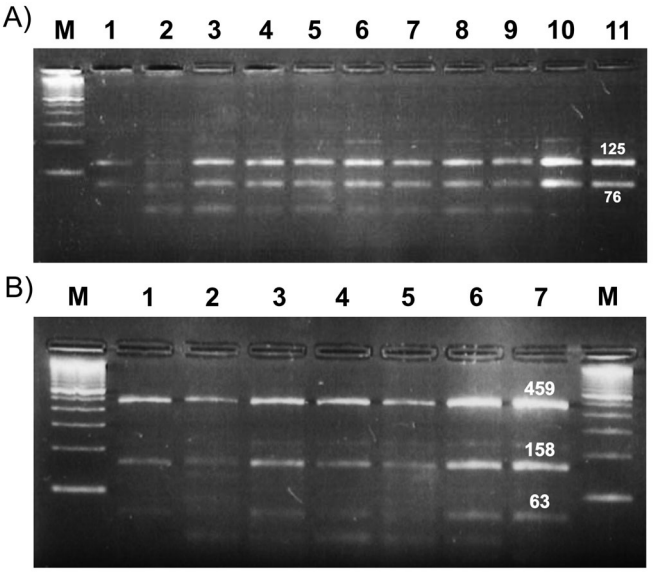

Fig. 5. RFLP analysis of *Photobacterium damselae*-specific PCR products from experimentally infected fishes. (A) *BstU I* digestion of fragment P.dam-1a_F-R. Lane M: size-marker 100 bp DNA Ladder (Invitrogen); Lanes 1 to 11: positive control (*P. damselae* subsp. *piscicida* strain NCIMB 2058^T), Dl39a, Dl12b, Dl29b, Dl11c, Dl33c, Dl10d, Dl36d, Sa99, Sa130, and Sa131. (B) *EcoR V* and *BsmA I* digestion of fragment P.dam-5a_F-R. Lane M: size-marker 100 bp DNA ladder (Invitrogen); Lanes 1 to 7: positive control (*P. damselae* subsp. *piscicida* strain NCIMB 2058^T), Dl12b, Dl33c, Dl36d, Sa99d, Sa130d, and Sa131d

especially the diverse potential for pathogenicity. An additional feature of a rapid test should be the possibility of identifying the microorganism from fish tissue samples. To evaluate this aspect, the PCR-RFLP method was applied to tissue samples collected from fishes experimentally infected with different strains of *P. damselae* subsp. *piscicida*. Positive amplification of fragment P.dam-5a_FR was obtained only from those experimental groups of fish showing high mortality, where pathogen titre was likely very high. The use of primer pair P.dam-1a_F-R, however, allowed the amplification of bacterial-specific DNA fragments even from individuals that belonged to experimental groups showing no mortality. These groups had been either infected with low virulence strains or left in cohabitation with infected fish without direct challenge. Restriction analysis subsequently demonstrated the presence of *P. damselae* subsp. *piscicida*. This evidence suggests that the pathogen might be identified at low titre from fishes not showing clinical signs of the disease.

Compared to routine cultural methods, the PCR-RFLP approach proposed here requires the knowledge of a few simple molecular techniques. However, the reagents used are not expensive, and the experimental protocols are neither complicated nor excessively time-consuming. The time needed to extract DNA from tissue samples, to carry out a PCR reaction, to perform a digestion with a single restriction enzyme, and to run samples in a normal agarose gel is less than 1 working day in total. Routine culture methods take days to provide a reliable answer. This makes a difference when trying to prevent an outbreak of fish pasteurellosis. The total cost for each sample is negligible, and the entire analytical process can be easily performed in parallel for tens of samples. For the above reasons, the method proposed here can be used in the routine screening for the presence of the pathogen.

Routine implementation of the PCR-RFLP tests presented in this paper, and especially the amplification and restriction of fragment P.dam-1a_F-R, on healthy fish might contribute toward an improved efficacy of prevention programs against fish pasteurellosis.

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LITERATURE CITED

Aoki T, Ikeda D, Katagiri T, Hirono I (1997) Rapid detection of the fish-pathogenic bacterium *Pasteurella piscicida* by Polymerase Chain Reaction targeting nucleotide sequences of the species-specific plasmid pZP1. Fish Pathol 32:143–151

Bakopoulos V, Volpatti D, Papanagiotou E, Richards R, Galeotti M, Adams A (1997) Development of an ELISA to detect *Pasteurella piscicida* in culture and in 'spiked' fish tissue. Aquaculture 156:359–366

Botella S, Pujalte MJ, Macián MC, Ferrús MA, Hernández J, Garay E (2002) Amplified fragment length polymorphism (AFLP) and biochemical typing of *Photobacterium damselae* subsp. *damselae*. J Appl Microbiol l93:681–688

Dalla Valle L, Zanella L, Belvedere P, Colombo L (2002) Use of random amplification to develop a PCR detection method for the causative agent of fish pasteurellosis, *Photobacterium damselae* subsp *piscicida* (*Vibrionaceae*). Aquaculture 207:187–202

Fouz B, Toranzo AE, Marco-Noales E, Amaro C (1998) Survival of fish-virulent strains of *Photobacterium damselae* subsp. *damselae* in seawater under starvation conditions. FEMS Microbiol Lett 168:181–186

Fouz B, Toranzo AE, Milan M, Amaro C (2000) Evidence that water transmits the disease caused by the fish pathogen *Photobacterium damselae* subsp *damselae*. J Appl Microbiol 88:531–535

Gauthier G, Lafay B, Ruimy R, Breittmayer V, Nicolas JL, Gauthier M, Christen R (1995) Small-subunit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Snieszko et al.). Jansen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. Int J Syst Bacteriol 45:139–144

Grayson TH, Cooper LF, Atienzar FA, Knowles MR, Gilpin ML (1999) Molecular differentiation of *Renibacterium salmoninarum* from worldwide locations. Appl Environ Microbiol 65:961–968

Kimura B, Hokimoto S, Takahashi H, Fujii T (2000) *Photobacterium histaminum* Okuzumi et al. 1994 is a later subjective synonym of *Photobacterium damselae* subsp. *damselae* (Love et al. 1981) Smith et al. 1991. Int J Syst Evol Microbiol 50:1339–1342

Kvitt H, Ucko M, Colorni A, Batargias C, Zlotkin A, Knibb W (2002) *Photobacterium damselae* ssp. *piscicida*: detection by direct amplification of 16S rRNA gene sequences and genotypic variation as determined by amplified fragment length polymorphism (AFLP). Dis Aquat Org 48:187–195

Magariños B, Romalde JL, Bandìn I, Fouz B, Toranzo AE (1992) Phenotypic, antigenic and molecular characterization of *Pasteurella piscicida* strains isolated from fish. Appl Environ Microbiol 58:3316–3322

Magariños B, Toranzo AE, Romalde JL (1996) Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. Annu Rev Fish Dis 6:41–64

Magariños B, Osorio CR, Toranzo AE, Romalde JL (1997) Applicability of ribotyping for intraspecific classification and epidemiological studies of *Photobacterium damselae* subspecies *piscicida*. Syst Appl Microbiol 20:634–639

Magariños B, Toranzo AE, Barja JL, Romalde JL (2000) Existence of two geographically-linked clonal lineages in the bacterial fish pathogen *Photobacterium damselae* subsp. *piscicida* evidenced by random amplified polymorphic DNA analysis. Epidemiol Infect 125:213–219

Osorio CR, Collins MD, Toranzo AE, Barja JL, Romalde JL (1999) 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl Environ Microbiol 65:2942–2946

Osorio CR, Toranzo AE, Romalde JL, Barja JL (2000) Multiplex PCR assay for *ureC* and 16S rRNA genes clearly discriminates between both subspecies of *Photobacterium damselae*. Dis Aquat Org 40:177–183

Pujalte MJ, Sitjà-Bobadilla A, Álvarez-Pellitero P, Garay E (2003) Carriage of potentially fish-pathogenic bacteria in *Sparus aurata* cultured in Mediterranean fish farms. Dis Aquat Org 54:119–126

Rajan PR, Lin JHY, Ho MS, Yang HL (2003) Simple and rapid detection of *Photobacterium damselae* ssp. *piscicida* by a PCR technique and plating method. J Appl Microbiol 95: 1375–1380

Romalde JL, Magariños B, Lores F, Osorio CR, Toranzo AE (1999) Assessment of a magnetic bead-EIA based kit for rapid diagnosis of fish pasteurellosis. J Microbiol Methods 37:147–154

Snieszko SF, Bullock GL, Hollis E, Boone JG (1964) *Pasteurella* sp. from an epizootic of white perch (*Roccus americanus*) in Chesapeake Bay tidewater areas. J Bacteriol 88:1814–1815

Thyssen A, Grisez L, van Houdt R, Ollevier F (1998) Phenotypic characterization of the marine pathogen *Photobacterium damselae* subsp. *piscicida*. Int J Syst Bacteriol 48: 1145–1151

Thyssen A, Van Eygen S, Hauben L, Goris J, Swings J, Ollevier F (2000) Application of AFLP for taxonomic and epidemiological studies of *Photobacterium damselae* subsp. *piscicida*. Int J Syst Evol Microbiol 50:1013–1019

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