

Evaluation of different DNA-based fingerprinting methods for typing *Photobacterium damsela* ssp. *piscicida*

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ABSTRACT

This study evaluates the effectiveness of three different molecular techniques, repetitive extragenic palindromic PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and the random amplified polymorphic DNA (RAPD-PCR) for rapid typing of *Photobacterium damsela* ssp. *piscicida* strains isolated from different species of marine fish and geographic areas. The results obtained by the three methods showed that RAPD and ERIC-PCR were more discriminative for suitable rapid typing of *Ph. damsela* ssp. *piscicida* than REP-PCR. The analysis of DNA banding patterns generated by both molecular methods (RAPD and ERIC-PCR) clearly separated the strains into two main groups that strongly correlated with their geographic origin. Moreover, the REP-PCR analysis was less reproducible than the RAPD and ERIC-PCR methods and does not allow the establishment of genetic groups. RAPD and ERIC-PCR constitute valuable tools for molecular typing of *Ph. damsela* ssp. *piscicida* strains, which can be used in epidemiological studies of photobacteriosis infections.

Key terms: *Photobacterium damsela* ssp. *piscicida*, RAPD, ERIC-PCR, REP-PCR.

INTRODUCTION

Photobacterium damsela ssp. *piscicida*, previously known as *Pasteurella piscicida*, is the etiological agent of fish photobacteriosis or pasteurellosis, which is one of the most important diseases in Japan, affecting mainly yellowtail (*Seriola quinqueradiata*). From 1990 it has caused economic losses in the marine culture of gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) and sole (*Solea solea* and *Solea senegalensis*) in the Mediterranean European countries and hybrid striped bass (*Morone saxatilis* x *M. chrysops*) in USA (Toranzo et al., 2005).

Although this pathogen is biochemically and serologically homogeneous regardless of the geographic origin and source of isolation (Magariños et al., 1996;

Bakopoulos et al., 1997), DNA fingerprinting methods such as rRNA gene restriction analysis (ribotyping) (Magariños et al., 1997), amplified fragment length polymorphism (AFLP) (Thyssen et al., 2000; Kvitt et al., 2002) and random amplified polymorphic DNA (RAPD) (Magariños et al., 2000; 2003; Hawke et al., 2003; Dalla Valle et al., 2002) have been described as powerful tools to discriminate European strains from Japanese and USA isolates.

In recent years, the development of new molecular techniques has progressed rapidly. Therefore, PCR amplification with primers specific to the repetitive genetic element REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) have been frequently used for genomic fingerprinting

of Gram-negative bacteria, since they are fast and simple. These repetitive elements, located in the intergenic regions of many bacteria genomes, are considered to be highly conserved (Stern et al., 1984; Hulton et al., 1991; Martin et al., 1992) and, due to this, they are useful for elucidating relationships within and among bacterial species.

The main aim of this study was to compare three PCR-based techniques for the analysis of genetic variability within *Ph. damsela* ssp. *piscicida* strains isolated from different host and geographical regions of the world, and therefore, to establish the DNA fingerprinting of this pathogen as well as to evaluate the applicability of these techniques in epidemiological studies.

MATERIALS AND METHODS

1. Bacterial strains

A total of 26 *Ph. damsela* ssp. *piscicida* strains were included in this study. Their sources of isolation and geographic origins are listed in Table 1. The identity of each isolate was confirmed as *Ph. damsela* ssp. *piscicida* with standard phenotypical procedures (Magariños et al., 1992), and using the PCR-based analysis described by Osorio et al., (1999). For all experiments, the strains were routinely grown aerobically on Tryptic Soy Agar (TSA; Oxoid Ltd) supplemented with 1% (wt/vol) NaCl and incubated at 24°C for 24-48 h. Stock cultures were maintained frozen at -80°C in Criobille tubes (AES Lab).

2. DNA extraction

Chromosomal DNA was extracted using InstaGene Matrix (Bio-Rad) following the manufacturer's recommendations. Briefly, *Ph. damsela* ssp. *piscicida* colonies were scraped off agar plates, suspended in 1 ml of sterile water and centrifuged at 12,000 xg for 1 min. After the supernatants were removed, the pellets were resuspended in 200 µl of InstaGene Matrix and incubated at 56°C for 30 min. Then, the suspensions

were mixed at high speed for 10 s and boiled in a water bath for 8 min. The lysates were mixed again at high speed and spun at 12,000 xg for 3 min. The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm and adjusted to a concentration of 20-30 ng µl⁻¹. All DNAs were maintained at -20°C until they were used for PCR reactions. One microliter of each DNA solution was used in the respective amplification reaction.

3. ERIC-PCR typing

The ERIC-PCR amplifications were performed with the commercial kit Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech), which included all the reagents needed for the PCR reactions (buffer, nucleotides and *Taq* DNA polymerase), with the exception of the specific primers and DNA template. A pair of 22-mer primers (Sigma): ERIC 1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used as previously reported by Versalovich et al. (1991). Reactions were carried out simultaneously in a T Gradient Thermocycler (Biometra) and Mastercycler personal (Eppendorf) equipment using an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation (92°C for 45 s), annealing (52°C for 1 min), and extension (70°C for 10 min), with a final extension step at 70°C for 20 min. Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each batch of PCR reaction.

4. REP-PCR typing

The same Ready-To-Go™ PCR beads were employed for REP-PCR amplifications using the following 18-mer primers (Sigma): REP 1D (5'-NNN RCG YCG NCA TCM GGC-3') and REP 2D (5'-RCG YCT TAT CMG GCC TAC-3'), where M is A or C, R is A or G, Y is C or T and N is any nucleotide (Stern et al. 1984). PCRs were performed in 25 µl reaction mixture containing the same components as the ERIC-PCR mixture

except that here REP primers were used. Reactions were performed using simultaneously a T Gradient Thermocycler and Mastercycler personal instrument. The programme consisted in an initial denaturation at 95°C for 7 min followed by

35 cycles of denaturation at 92°C for 45 s, annealing at 40°C for 1 min and elongation at 72°C for 8 min with a final extension step at 72°C for 15 min. As reported above, negative controls were included in each PCR-reaction.

TABLE 1

Origin of *Photobacterium damsela* ssp. *piscicida* strains used in this study

Strain	Host species	Origin	Donor*
404/2	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
418/1	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
499	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
524	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
557	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
349	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
355	<i>Dicentrarchus labrax</i>	Italy	A. Manfrin
387	<i>Sparus aurata</i>	Italy	A. Manfrin
L7C	<i>Dicentrarchus labrax</i>	Italy	M.Mancuso
IT -2	<i>Sparus aurata</i>	Italy	G. Giorgetti
DI 21	<i>Sparus aurata</i>	Spain	A.E. Toranzo
B5.1	<i>Dicentrarchus labrax</i>	Spain	J. J. Borrego
R-46	<i>Sparus aurata</i>	Spain	J. J. Borrego
DS 1.1	<i>Sparus aurata</i>	Spain	CULMASUR
10831	<i>Dicentrarchus labrax</i>	France	F. Baudin-Laurencin
619.1	<i>Sparus aurata</i>	Portugal	T. Baptista
666.1	<i>Dicentrarchus labrax</i>	Portugal	T. Baptista
ATLIT 2	<i>Morone sp.</i>	Israel	A.Colorni
069 A	<i>Sparus aurata</i>	Greece	H. Nousias
069 E	<i>Sparus aurata</i>	Greece	H. Nousias
ATCC 29690	<i>Seriola quinqueradiata</i>	Japan	T. Kitao
MZS 8001	<i>Seriola quinqueradiata</i>	Japan	T. Kitao
MP-7801	<i>Seriola quinqueradiata</i>	Japan	T. Kitao
EPOY-8803-II	<i>Epinephelus akaara</i>	Japan	K. Muroga
P-3333	<i>Seriola aureovittata</i>	Japan	R. Kusuda
ATCC 17911	<i>Roccus americanus</i>	U.S.A.	ATCC

* ATCC, American Type Culture Collection; T. Baptista, Unidade de Ciencias e Tecnologias dos Recursos Aquáticos, Universidade do Algarve, Portugal; F. Baudin-Laurencin, Laboratoire National de Pathologie des Animaux Aquatiques, Brest, France; J. J. Borrego, Departamento de Microbiología, Universidad de Málaga, Spain; A. Colorni, National Center for Mariculture, Eilat, Israel; CULMASUR, Cultivos Marinos del Sur S. A., Huelva, Spain; G. Giorgetti, Laboratorio di Ittiopatologia, Venice, Italy; T. Kitao, Department of Fisheries, Miyazaki University, Japan; R. Kusuda, Fish Disease Laboratory, Faculty of Agriculture, Kochi University, Japan; M. Mancuso, I.A.M.C. CNR-Sezione di Messina; A. Manfrin, Istituto Zooprofilattico delle Venezie, Italy; K. Muroga, Faculty of Applied Biological Science, Hiroshima University, Japan; H. Nousias, Thalassa S. A., Hiliadou Doridos, Greece; A. E. Toranzo, Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Spain.

5. RAPD-PCR typing

The RAPD-PCR amplifications were performed using Ready-To-Go™ RAPD analysis beads (Amersham Pharmacia Biotech) as previously described (Magariños et al., 2000). These commercial beads have been optimised for PCR reactions and contain buffer, nucleotides and *Taq* DNA polymerase. The only reagents which must be added are template DNA and primers, also supplied in the kit. Two distinct random 10-mer primers (Amersham Pharmacia Biotech) were included in this study: P5 5'-d[AACGCGCAAC]-3' and P6 d[CCCGTCAGCA]-3'. Each RAPD bead was resuspended in 19 µl of sterile water and 1 µl of purified bacterial DNA and 25 pmol of respective primer were added. The amplification protocol for RAPD analysis started with denaturation (95°C for 5 min) followed by 30 cycles at 95°C for 1 min (denaturation), 35°C for 1 min (annealing) and 72°C for 2 min (DNA chain extension) simultaneously in a T Gradient Thermocycler and Mastercycler personal instrument. As negative controls, water instead of bacterial DNA was included in each reaction.

6. Gel electrophoresis

The RAPD, ERIC and REP-PCR products were separated using horizontal electrophoresis on a 1.5% agarose gel in TAE 1x (0.04 M Tris, 0.0001M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml⁻¹ of ethidium bromide (Bio-Rad) and photographed under UV light and computer digitised (Gel Doc 100, Bio-Rad).

In order to determine significant differences in the patterns, reproducibility of results was assessed by the repetition of RAPD, ERIC and REP-PCR assays at least three times, in which DNAs obtained in two different extractions were employed. A 50-2000-pb ladder (Sigma) was used as a molecular mass marker. A set of reproducible bands produced for a particular primer was defined as a pattern profile.

7. Computer-assisted analysis of genomic fingerprints

The genomic fingerprints obtained were compared for similarity by visual observation of the band patterns according to their presence or absence in each isolate. For the cluster analysis and comparison from all RAPD, ERIC, REP-PCR patterns, a data analysis was performed by the use of the Diversity Database software (Bio-Rad). The computed similarities among isolates were estimated by means of the Dice coefficient (S_d) (Dice 1945) as a measure of homology, according to the equation: $S_d = [2A / (2A + B + C)] \times 100$, where A is the number of the matching band and B and C are the numbers of bands present in one strain but not in the other. Dendrograms were produced on the basis of the unweighted average pair group method (UPGMA).

RESULTS AND DISCUSSION

PCR-based typing is an effective approach in the epidemiological study of various Gram-negative bacteria. From a biochemical and serological point of view, *Photobacterium damsela* ssp. *piscicida* have been demonstrated to be identical, but genetic studies have supported the existence of genetic variability among the isolates which was associated with the geographical origin (Magariños et al. 1997; Thyssen et al. 2000).

In this paper we compared three different techniques, ERIC-PCR, REP-PCR and RAPD, frequently used as molecular tools for rapid typing of microorganisms. These assays have been reported as fast and simple to use, and the information supplied could be used in epidemiological studies of the photobacterioses.

1. ERIC and REP fingerprinting

Repetitive element PCR is a group of techniques that generate DNA fingerprints which can be utilized for the discrimination of bacterial species and/or strains (Versalovic et al., 1991). In fact, REP-PCR

and ERIC-PCR methodologies have been successfully used for typing *V. cholerae* (Rivera et al., 1995), *Vibrio parahaemolyticus* (Maluping et al. 2005) and members of the family Enterobacteriaceae (Bachellier et al., 1999). The results obtained in this work revealed that all the strains were typeable by ERIC-PCR. The fingerprinting of *Ph. damsela* ssp. *piscicida* isolates consisted of five to seven amplification bands ranging in size from 50 to 1200 bp (Fig. 1). The analysis of bands allowed us to identify two genomic clusters. The first group (98.5% similarity) compiled all the Italian isolates together with these from Spain, Portugal, Greece, France, Israel and the ATCC 17911 reference strain. The second group (92% similarity) included the Japanese strains. When the Dice coefficient was applied the similarity between the two clusters was of 82.3% (Fig. 2). The same results were obtained when ERIC-PCR was repeated at least three times demonstrating the reproducibility of the technique.

The analysis of the isolates by REP-PCR showed a similar profile among all the strains with amplification bands ranging in size from 50 to 2000 bp and, therefore, a unique group could be established. Moreover, when this technique was repeated in different times, some of the

minor light amplification bands were inconsistent making their analysis more difficult.

2. RAPD fingerprinting

Similarly to that reported previously by Magariños et al. (1997) the two primers evaluated (P5 and P6) gave reproducible bands and generated patterns of amplified fragments suitable for accurate analysis. Then, they were selected for further studies with all strains. The assays were repeated at least three times for each primer tested and did not show significant differences in the profiles obtained, demonstrating that the method was reproducible.

The patterns obtained with primer 5 allowed to differentiate two genetic groups within *Ph. damsela* ssp. *piscicida* (I and II) with a similarity level of approximately 42.8% that seem to be related to the host origin of the isolates. The first group (group I) compiled all the European isolates that showed a profile of six major bands ranging from 500 to 1500 bp in size. The other group (group II), contained the strains isolated in Japan showing a pattern with five bands ranging from 500 to 1200 bp in size (Fig. 3 and Fig. 4). The similarity within each group was 100%.

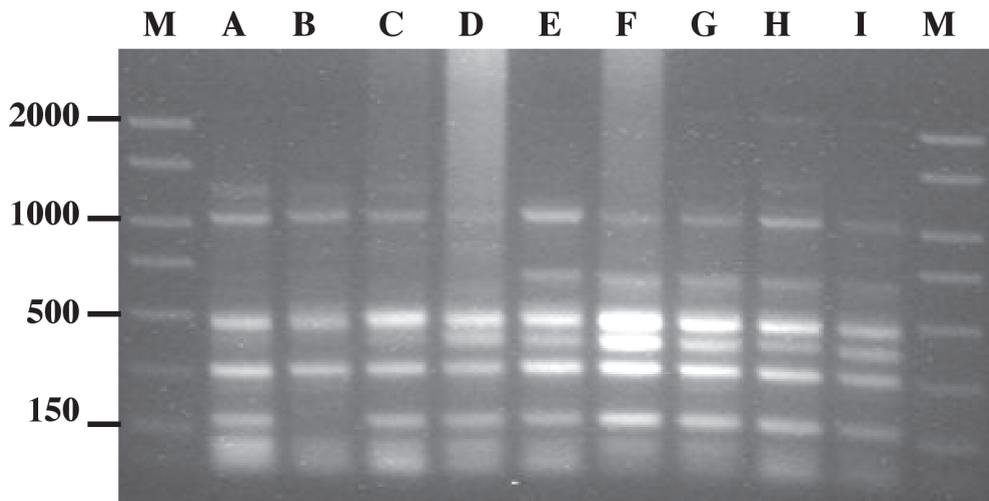


Figure 1: ERIC fingerprinting obtained for representative strains of *Ph. damsela* ssp. *piscicid*. Lanes: M, AmpliSize Molecular Ruler (50-2000 bp; Sigma); A: ATCC- 29690; B: MP-7801; C: MZS-800; D: P-3333; E: 387; F: 1/43; G: 404/2; H: B5.1; I: 069.A.

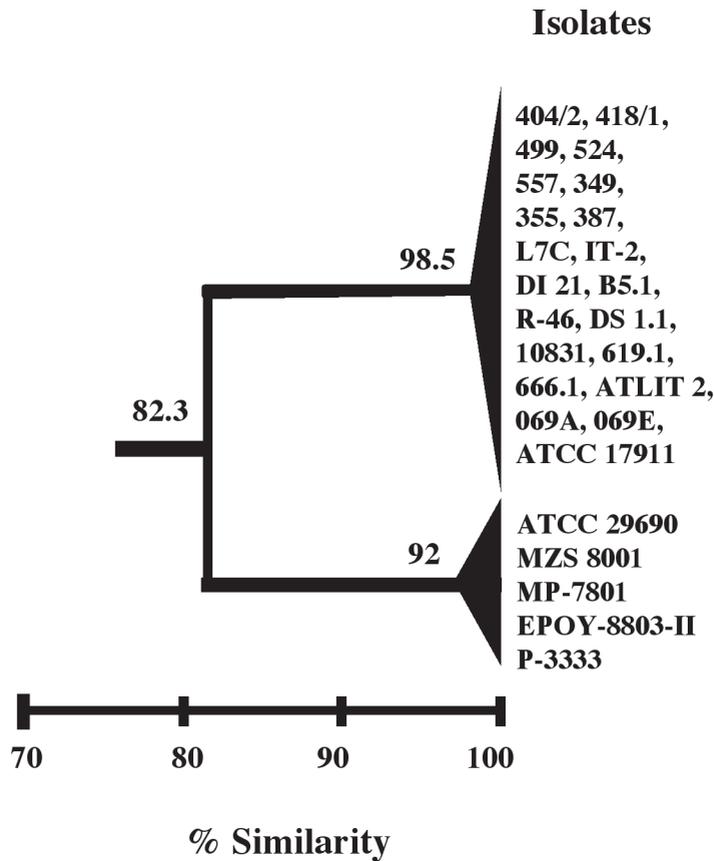


Figure 2: Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profiles of *Ph. damsela ssp. piscicida* strains.

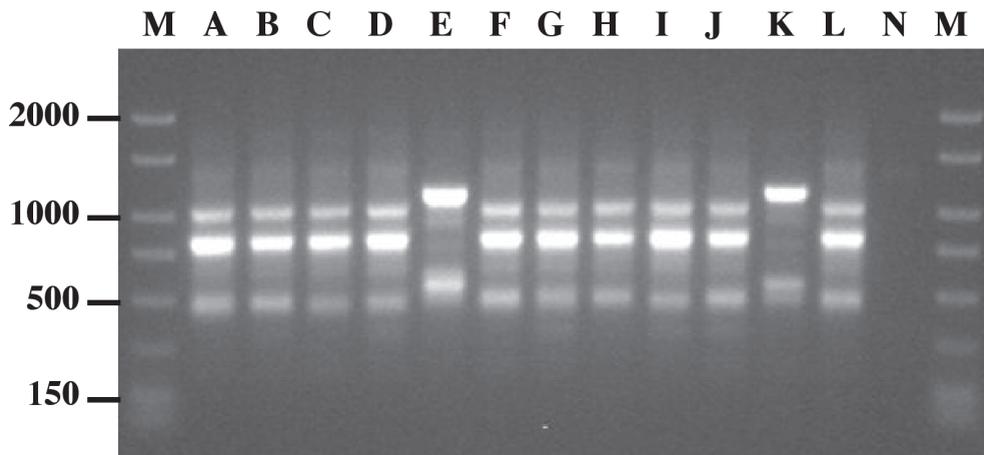


Figure 3: RAPD fingerprinting obtained for representative strains of *Ph. damsela ssp. piscicida* with P5. Lanes: M, AmpliSize Molecular Ruler (50-2000 bp; Sigma); A: ATCC- 17911; B: 387; C: 355; D: 404/2; E: MP-7801; F: 349; G: 557; H: 619-1; I: 540; J: 499; K: MZS-8001; L: B5.1; N: DW. Numbers on the left indicate the position of molecular size marker in bp.

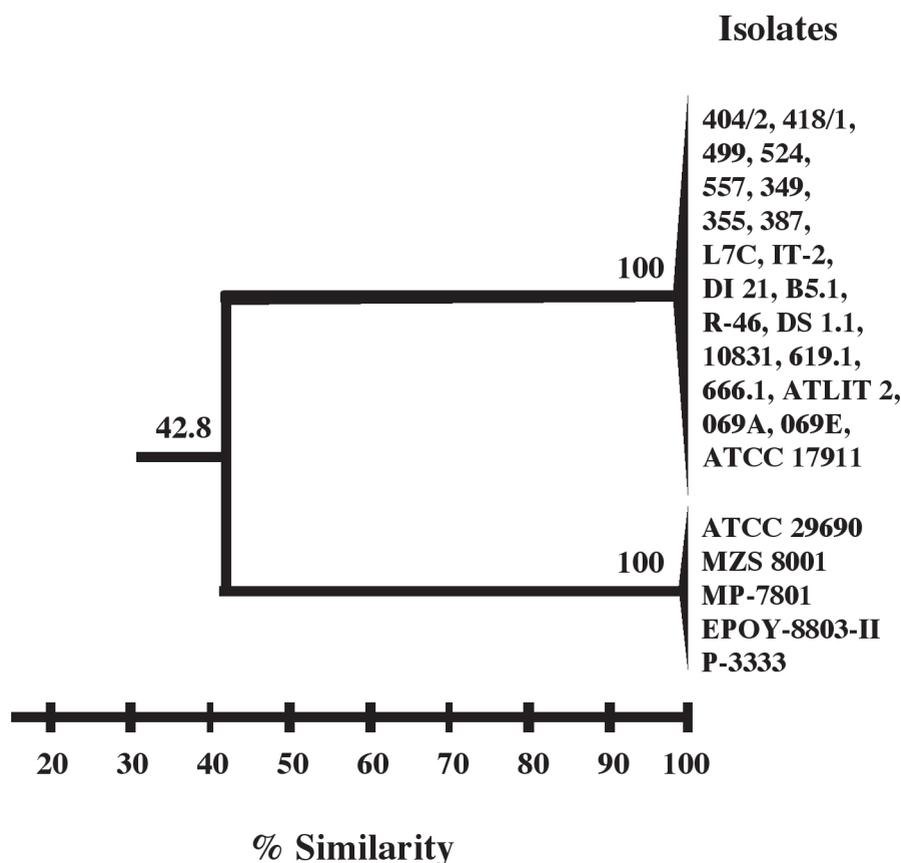


Figure 4: Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *Ph. damsela* ssp. *piscicida* strains obtained with primer 5.

With primer 6 a similar clustering of the isolates was obtained (data not shown), showing approximately the same Dice coefficient values within and between groups.

These results are in accordance with those previously reported by Magariños et al. (2000) in which two genetic groups were described, the European and the Japanese, and confirm the existence of two clonal lineages within this bacterial fish pathogen. However, although in that report using primer 5 two subgroups within each cluster could be differentiated, in the present study such discrimination between strains was not possible. This discrepancy could be explained because those subgroups were established based on minor amplification bands which were not

amplified now or not visualized due to their very low intensity.

Although some authors reported that with the RAPD technique variability in the fingerprinting profiles can be obtained (Ellesworth et al. 1993; Oakey et al. 1996), in the present study we have found reproducible profiles for all the strains tested in the three independent RAPD-PCR reactions.

In conclusion, comparing the results obtained by the three methods it was found that, as reported by Maluping et al. (2005) for *V. parahaemolyticus*, RAPD and ERIC-PCR methods were more discriminative for rapid typing for *Ph. damsela* ssp. *piscicida* than REP-PCR. Therefore, both techniques constitute valuable tools for epidemiological studies of photobacteriosis infections.

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