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Existence of two geographically-linked clonal lineages in the bacterial fish pathogen *Photobacterium damselae* subsp. *piscicida* evidenced by random amplified polymorphic DNA analysis

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SUMMARY

In this work, we applied the random amplified polymorphic DNA (RAPD) technique to evaluate the genetic diversity in *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), an important pathogen for different marine fish. Regardless of the oligonucleotide primer employed, the 29 isolates of *Ph. damselae* subsp. *piscicida* tested were separated into two groups, the RAPD-PCR analysis differentiated the European strains from the Japanese strains. The similarity between both groups estimated on the basis of the Dice coefficient was 75–80%. These results show that European and Japanese isolates of *Ph. damselae* subsp. *piscicida*, regardless of their host fish species, belong to two different clonal lineages. Our findings also indicate that RAPD profiling constitutes a useful tool for epidemiological studies of this fish pathogen.

INTRODUCTION

Pasteurellosis, currently described also as photobacteriosis, caused by the halophilic bacterium *Photobacterium damselae* subsp. *piscicida* is an important fish disease which has produced significant losses in marine aquaculture. Originally isolated from natural populations of white perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 in the Chesapeake Bay [1], this pathogen now occurs in most countries, affecting mainly cultured yellowtail (*Seriola quinqueradiata*), gilthead seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) [2–4]. Recently, Kawakami and colleagues [5] reported also a mass mortality in wild largescale blackfish (*Girella punctata*) in Japan.

Accurate methods of strain identification are fundamental in the epidemiological studies of bacterial fish diseases in order to understand the spread of the disease and to evaluate the control measures. Traditional phenotypic and serological methods for typing *Ph. damselae* subsp. *piscicida* have been used without success because of the very homogeneous nature of the subspecies [6]. Moreover, genotypic methods such as restriction fragment length polymorphism analysis and 16S rRNA gene sequencing showed genetic homogeneity for this pathogen [6, 7]. Only plasmid profiling and ribotyping procedures yielded minor variation among isolates [6, 8].

In recent years, a randomly amplified polymorphic DNA (RAPD) approach which can also be referred to as arbitrarily-primed polymerase chain reaction (AP–PCR) has been described as a very powerful technique for intra-species identification [9–11]. This method has the advantage of being independent of gene expression and allows the study of DNA polymorphisms between organisms without the prerequisite knowledge of their molecular biology.

The aim of this work was to use RAPD to establish DNA fingerprints for *Ph. damselae* subsp. *piscicida* strains from diverse host and geographical areas of the world and, therefore, to analyse the possible genetic diversity within this pathogen and to de-

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Table 1. *Origin of* Photobacterium damselae *subsp.* piscicida *strains*

Strain	Fish	Country	Donor*
DI 21	*Sparus aurata*	Spain	A. E. Toranzo
DI 71	*Sparus aurata*	Spain	A. E. Toranzo
DI 91	*Sparus aurata*	Spain	A. E. Toranzo
B3.1	*Sparus aurata*	Spain	J. J. Borrego
R-46	*Sparus aurata*	Spain	J. J. Borrego
B-180	*Sparus aurata*	Spain	J. J. Borrego
B5.1	*Dicentrarchus labrax*	Spain	J. J. Borrego
Pp 5	*Solea senegalensis*	Spain	J. J. Borrego
DS 1.1	*Sparus aurata*	Spain	CULMASUR
DS 2.1	*Sparus aurata*	Spain	CULMASUR
C1	*Sparus aurata*	Spain	F. Real
C2	*Sparus aurata*	Spain	F. Real
10831	*Dicentrarchus labrax*	France	F. Baudin-Laurencin
IT-1	*Sparus aurata*	Italy	G. Giorgetti
IT-2	*Sparus aurata*	Italy	G. Giorgetti
B3	*Sparus aurata*	Portugal	T. Baptista
619.1	*Sparus aurata*	Portugal	T. Baptista
666.1	*Dicentrarchus labrax*	Portugal	T. Baptista
MP-7801	*Seriola quinqueradiata*	Japan	T. Kitao
MZS-8001	*Seriola quinqueradiata*	Japan	T. Kitao
EPOY-8803-II	*Epinephelus akaara*	Japan	K. Muroga
P3333	*Seriola aureovittata*	Japan	R. Kusuda
P3334	*Seriola quinqueradiata*	Japan	R. Kusuda
P3335	*Seriola quinqueradiata*	Japan	R. Kusuda
069A	*Sparus aurata*	Greece	H. Nousias
069E	*Sparus aurata*	Greece	H. Nousias
069F	*Sparus aurata*	Greece	H. Nousias
ATLIT 2	*Morone* sp	Israel	A. Colorni
ATCC 29690	*Seriola quinqueradiata*	Japan	T. Kitao

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termine the applicability of this technique in epidemiological studies.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

A total of 29 *Ph. damselae* subsp. *piscicida* strains were included in this study. The sources of isolation and geographic origin of these strains are listed in Table 1. Strains were routinely grown aerobically on Brain Heart Infusion Agar (Difco) supplemented with 1% NaCl for 24–48 h at 22–25 °C. Stock cultures were maintained frozen at −70 °C in Tryptic Soy Broth (Difco) with 15% added glycerol.

DNA was isolated using the InstaGene Matrix (BioRad) following the manufacturer's recommendations. The InstaGene DNA preparations were stored at −20 °C until they were used for PCR reactions. One μl of each DNA solution was used in the respective amplification reaction.

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Oligonucleotide primers

A randomly designed 10-mer oligonucleotide set, comprising six distinct primers, was obtained from Pharmacia Biotech. The oligonucleotide primer sequences have been described by Romalde and colleagues [11].

RAPD analysis

The PCR reactions were carried out as previously described [11] in a final volume of 25 μl using Ready-To-Go RAPD analysis Beads (Pharmacia Biotech). RAPD reaction mixture contained 1·5 units of *Taq* polymerase, 10 mM Tris-HCL (pH 9·0), 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM of each dNTP, 25 pmoles of respective primer and, as described before, 1 μl of each DNA solution.

Reactions were performed using a Master Cycler Personal (Eppendorf). The programme (30 cycles) consisted of denaturation at 95 °C for 1 min, annealing at 35 °C for 1 min and elongation at 72 °C for 2 min. The RAPD products were electrophoresed on a 1·5% agarose gel, stained with ethidium bromide and photographed as previously described [11]. A 50–2000 bp ladder (Sigma, St. Louis) was used as a molecular marker.

All the gels were scanned and the images were captured by a Gel Doc-2000 gel documentation system (BioRad). The data analysis was performed by using the Diversity Database software (BioRad) and the computed similarities among isolates were estimated by means of the Dice coefficient [12]. Dendrograms were produced on the basis of the unweighted average pair group method (UPGMA).

In order to determine significant differences in the patterns, reproducibility of results was assessed by repetition in at least three independent RAPD assays.

RESULTS

The RAPD technique was applied to 29 strains of *Ph. damselae* subsp. *piscicida* in an attempt to detect strain-specific DNA profiles. Initially, the RAPDs of one strain of *Ph. damselae* subsp. *piscicida* (DI 21) were obtained using all the primers provided in the kit. Three of the six primers tested, oligonucleotides P4, P5 and P6, gave several reproducible bands and generated an appropriate pattern of amplified fragments.

The patterns obtained with primers 4 and 6 allowed us to differentiate two groups within *Ph. damselae* subsp. *piscicida*, with a similarity level of approximately 80% (Figs 1, 2), that could be related to the geographical origin of the isolates. Therefore, group 1 contained all the strains isolated in Europe including Spain, France, Italy, Portugal, Israel and Greece regardless of their host fish species and year of isolation. Group 2 included the Japanese *Ph. damselae* subsp. *piscicida* isolates obtained from yellowtail (*S. quinqueradiata*) and red grouper (*Epinephelus aakara*). When primer 4 was employed, a profile with 9 major bands was detected for all the group 1 strains, while group 2 isolates showed a pattern with 8 bands. The similarity within each group was 100%. Five different bands with similar size were shared by both clusters, yielding a similarity of 78% between them when the Dice coefficient was applied. A similar clustering of the isolates was obtained with primer 6 (data not shown), showing approximately the same Dice coefficient values within and between groups.

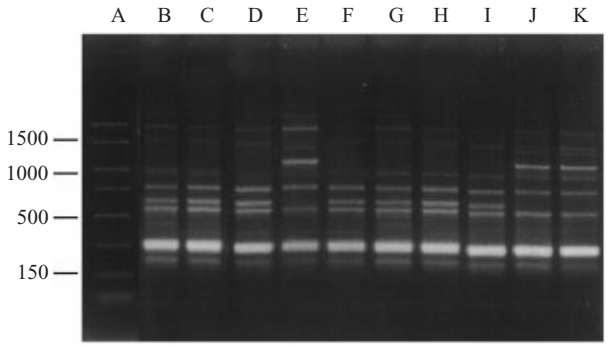

Fig. 1. RAPD fingerprints obtained for the *Ph. damselae* subsp. *piscicida* isolates employing the primer 4. Lanes: A, AmpliSize™ Molecular Ruler (50–2000 bp ladder; Bio-Rad); B, DI 21; C, IT-1; D, B3; E, ATCC 29690; F, Pp 5; G, 666·1; H, 10831; I, 069A; J, MZS-8001; K, EPOY-8803-II. The molecular sizes (in kb) are indicated on the left.

With primer 5, we could not only separate *Ph. damselae* subsp. *piscicida* European strains from the Japanese isolates but also discriminate within each group (Figs 3, 4). Then, two subgroups could be differentiated within the European cluster, one comprised the isolates from Spain, Greece and Israel and the other corresponded to the Portuguese and Italian strains. The level of similarity between both subgroups was approximately 92%. On the other hand, within the Japanese cluster, the reference strain ATCC 29690, isolated from yellowtail, yielded a RAPD profile different to those obtained for the other isolates included in this cluster being possible to distinguish also both subgroups.

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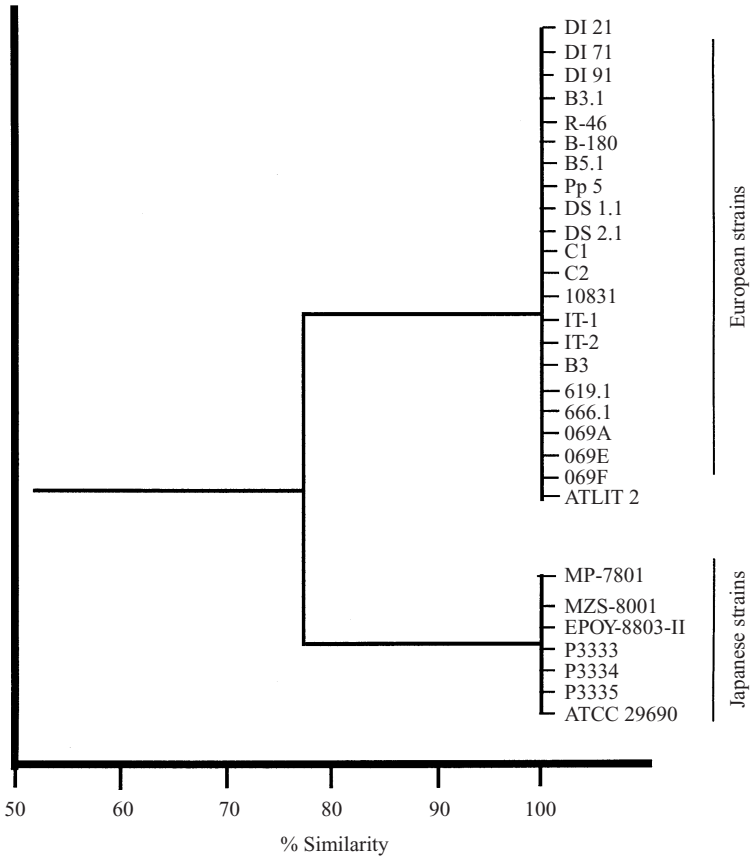

Fig. 2. Dendrogram obtained from the analysis of the patterns obtained after RAPD with primer 4 for *Photobacterium damselae* subsp. *piscicida* strains using the Dice coefficient as a measure of similarity and the unweighted average pair group method (UPGMA).

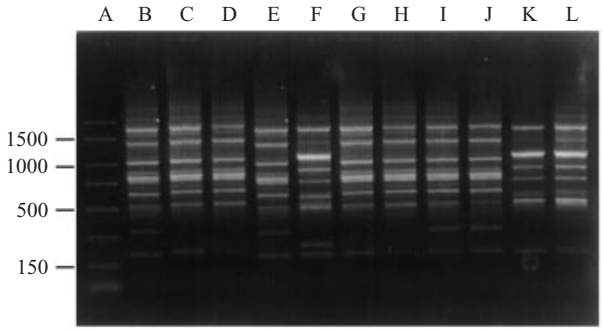

Fig. 3. RAPD fingerprints obtained for the *Ph. damselae* subsp. *piscicida* isolates employing the primer 5. Lanes: A, AmpliSize™ Molecular Ruler (50–2000 bp ladder; Bio-Rad); B, DI 21; C, IT-1; D, B3; E, B5·1; F, ATCC 29690; G, IT-2; H, 666·1; I, 10831; J, 069A; K, MZS-8001; L, EPOY-8803-II. The molecular sizes (in kb) are indicated on the left.

The RAPD assays were repeated at least three times for each primer tested. Apart from some variations in the band intensity, no significant differences were observed between the profiles obtained and demonstrated the reproducibility of the RAPD method.

DISCUSSION

Photobacterium damselae subsp. *piscicida* has been considered an homogeneous taxon on the basis of different studies using classical phenotypical procedures [1–3, 6]. Although Thyssen and colleagues [13] reported some heterogeneity in some biochemical characters, their numerical taxonomy study has some points of concern: i) the arbitrary reading of weak positive reactions obtained in some tests as negative and, ii) the utilization of miniaturized systems with long incubation periods because they usually yield a variety of false positive and/or negative reactions [14]. These criticisms clearly rule out the existence of such biochemical variability. Therefore, the use of sensitive molecular methods is necessary to assess heterogeneity within this fish pathogen. In this work, the RAPD analysis was applied to obtain genetic fingerprints of *Ph. damselae* subsp. *piscicida* strains, and to evaluate its discriminatory power to be employed as useful genotyping method for epidemiological purposes.

The RAPD assay is a simple process which is based on the amplification of genomic DNA with single

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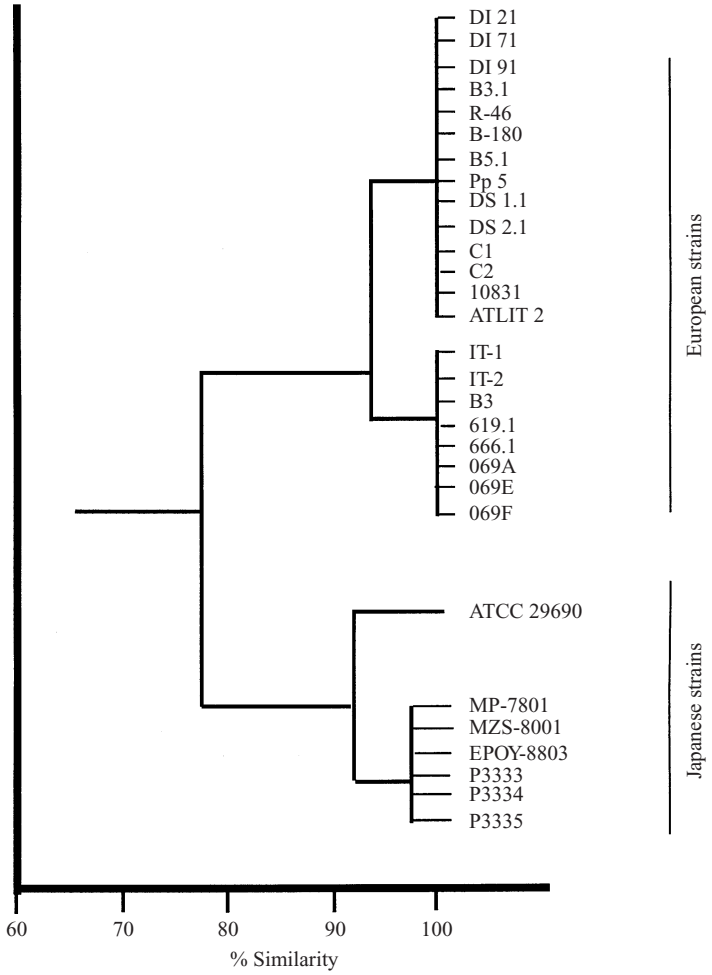

Fig. 4. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and the UPGMA on the basis of the RAPD profiles from *Photobacterium damselae* subsp. *piscicida* strains with the oligonucleotide primer 5.

primers which detect polymorphisms in the absence of specific nucleotide sequence information. In addition, it is not reliant on the actual transcription and translation of proteins and, since this procedure analyses the whole genome, it is potentially highly discriminatory. This technique has proved to be a useful method in the study of intra- and interspecific relationships in a wide range of microorganisms including bacterial and yeast species [10, 11, 15, 16].

The results obtained demonstrate the existence of genetic heterogeneity among the *Ph. damselae* subsp. *piscicida* isolates used in this study. The RAPD patterns obtained with the selected primers provided the basis for distinguishing two major groups strongly correlated with the geographical origin of the strains but not with the host fish species. One includes all the isolates from Japan and the other comprises the European strains. The differentiation into these two major groups has previously been reported by several authors. In fact, Bakopoulos and colleagues [17] in a study on the serological relationships within *Ph. damselae* subsp. *piscicida* using monoclonal antibodies described significant differences between the strains isolated from Japan and the isolates obtained from Europe. Similarly, Magariños and colleagues [8] and Thyssen and colleagues [18] using ribotyping and amplified fragment length polymorphisms procedures respectively, suggested the existence of two clonal lineages within *Ph. damselae* subsp. *piscicida* which were highly correlated with the geographic origin of the strains. However, RAPD technique possesses distinct advantages over those methods such as high sensitivity, rapid performance, clarity in the profiles obtained and, therefore, a simpler interpretation of the results.

In the present study, primer 5 allowed, in addition, the discrimination of strains within each group. Thus, the *Ph. damselae* subsp. *piscicida* Japanese strain

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isolated from yellowtail (ATCC 29690) was separated from the others included in this group. Within the European cluster, although all the strains showed a similar pattern, two subgroups could also be established. Although, the use of RAPD with this primer yielded a higher discrimination power than other genotyping procedures employed until now, the choice of longer primers and more stringent temperatures could allow the achievement of a concrete profile for each strain.

Variability in the RAPD profiles has been reported for several bacterial groups [19–21], probably due to the low stringency conditions used in the amplification. In this work, we have found reproducible profiles for all the strains tested in at least three independent RAPD–PCR reactions.

The results obtained in this work strongly corroborate the existence of genetic heterogeneity within this microorganism. Moreover, the knowledge of the geographical distribution of different genetic groups of this fish pathogen can be very helpful in design of preventive measures for effective control of pasteurellosis, such as vaccine formulation. Finally, on the basis of the findings reported here, we believe that RAPD analysis has potential as a valuable tool for epidemiological studies of *Ph. damselae* subsp. *piscicida*.

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