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# *Photobacterium damselae* ssp. *piscicida*: detection by direct amplification of 16S rRNA gene sequences and genotypic variation as determined by amplified fragment length polymorphism (AFLP)

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ABSTRACT: A PCR protocol for the rapid diagnosis of fish 'pasteurellosis' based on 16S rRNA gene sequences was developed. The procedure combines low annealing temperature that detects low titers of *Photobacterium damselae* but also related species, and high annealing temperature for the specific identification of *P. damselae* directly from infected fish. The PCR protocol was validated on 19 piscine isolates of *P. damselae* ssp. *piscicida* from different geographic regions (Japan, Italy, Spain, Greece and Israel), on spontaneously infected sea bream *Sparus aurata* and sea bass *Dicentrarchus labrax*, and on closely related American Type Culture Collection (ATCC) reference strains. PCR using high annealing temperature (64°C) discriminated between *P. damselae* and closely related reference strains, including *P. histaminum*. Sixteen isolates of *P. damselae* ssp. *piscicida*, 2 *P. damselae* ssp. *piscicida* reference strains and 1 *P. damselae* ssp. *damselae* reference strain were subjected to Amplified Fragment Length Polymorphism (AFLP) analysis, and a similarity matrix was produced. Accordingly, the Japanese isolates of *P. damselae* ssp. *piscicida* were distinguished from the Mediterranean/European isolates at a cut-off value of 83% similarity. A further subclustering at a cut-off value of 97% allowed discrimination between the Israeli *P. damselae* ssp. *piscicida* isolates and the other Mediterranean/European isolates. The combination of PCR direct amplification and AFLP provides a 2-step procedure, where *P. damselae* is rapidly identified at genus level on the basis of its 16S rRNA gene sequence and then grouped into distinct clusters on the basis of AFLP polymorphisms. The first step of direct amplification is highly sensitive and has immediate practical consequences, offering fish farmers a rapid diagnosis, while the AFLP is more specific and detects intraspecific variation which, in our study, also reflected geographic correspondence. Because of its superior discriminative properties, AFLP can be an important tool for epidemiological and taxonomic studies of this highly homogeneous genus.

KEY WORDS: AFLP · *Dicentrarchus labrax* · PCR · *Photobacterium damselae* · 16S rRNA gene · *Sparus aurata*



## INTRODUCTION

*Photobacterium damselae* ssp. *piscicida* is the etiological agent of one of the most devastating bacterial diseases of fish in warm and temperate mariculture. Sea bream *Sparus aurata* and sea bass *Dicentrarchus labrax* farms in particular have consequently suffered

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severe economic losses in several countries around the Mediterranean basin. The disease has been reported in Spain (Toranzo et al. 1991), France (Baudin-Laurencin et al. 1991), Italy (Ceschia et al. 1991), Greece (Ghittino et al. 1993), Turkey (Candan et al. 1996), Malta (Bakopoulos et al. 1997b) and Israel (A. Colorni unpubl. data). In Israel the pathogen was first isolated in our laboratory in Eilat, in 1993 from sea bream *S. aurata* cultured on the Mediterranean coast. The infection was subsequently detected in striped bass *Morone saxatilis* and sea bass *D. labrax*, and outbreaks still occur to date (A. Colorni unpubl. data), threatening the mariculture industries on both Mediterranean and Red Sea coasts of the country.

The disease was originally diagnosed in white perch and striped bass from Chesapeake Bay (Maryland, USA) and termed 'pasteurellosis' due to certain morphological and physiological similarities to the genus *Pasteurella* (Snieszko et al. 1964). The bacterium was consequently named *Pasteurella piscicida* (Janssen & Surgalla 1968). Since the disease pathology also presents some similarity to fish mycobacteriosis, Japanese authors named the condition 'pseudotuberculosis' (Kusuda & Yamaoka 1972). However misleading, both terms have often been retained for historical and reference reasons.

'*Pasteurella*' *piscicida* was later placed in the genus *Photobacterium* as *Photobacterium damsela* (later corrected to *damselae* by Trüper & de Clari 1997) ssp. *piscicida* by Gauthier et al. (1995) on the basis of its 16S rRNA gene sequence. More recent biochemical, physiological and genetic analyses (Thyssen et al. 1998, 2000, Osorio et al. 1999) have supported this classification.

In recent years, it has become clear that 16S rRNA sequences alone are often insufficient to detect variation within bacterial species (Goebel & Stackebrandt 1994, Palys et al. 1997); therefore, other methods for detecting genetic variation in closely related bacteria have been employed. The Amplified Fragment Length Polymorphism (AFLP) technique (Zabeau & Vos 1993, Vos et al. 1995) has provided a new level of precision in genotyping (Blears et al. 1998), and in fact is becoming a useful genetic tool for detecting variation in intraspecific strains (Janssen et al. 1996).

The combination in our study of PCR direct amplification and AFLP was thus expected to provide a 2-step procedure, where *Photobacterium damselae* isolates would be rapidly identified at genus level on the basis of their 16S rRNA gene sequences, and grouped in discrete clusters on the basis of their genotypic variation. While the first step was designed to have more immediate practical consequences, offering the fish farmers a rapid presumptive diagnosis, the AFLP technique was tested in view of its highly discriminative properties.

## MATERIALS AND METHODS

All general chemicals were of analytical grade. Taq DNA polymerase (5 U $\mu l^{-1}$, Promega) was used for all amplification procedures. T4 polynucleotide kinase, T4 ligase and restriction endonucleases were supplied by New England Biolabs. As a molecular weight marker, AmpliSize™ molecular ruler (Bio-Rad) was used.

**Cultures.** Nineteen *Photobacterium damselae* ssp. *piscicida* isolates were used in this study. Their sources of isolation and geographic origins are listed in Table 1. The isolates from Stirling were obtained preserved in DMSO solution according to Seutin et al. (1991). All reference strains were obtained directly from the American Type Culture Collection (ATCC), Manassas, VA, USA (Table 2). All the strains were re-hydrated in Tryptic Soy Broth (Difco) and sub-cultured on Tryptic

Table 1. *Photobacterium damselae* ssp. *piscicida* isolates utilized in the study

| Isolate code | Host | | Origin and code as they appear in Fig. 3 |
|---|---|---|---|
| MS011194[a] | Mullet | *Mugil* (prob. *cephalus*) | Israel (Red Sea), IS1 |
| SA071194[a,c] | Sea bream | *Sparus aurata* | Israel (Red Sea), IS2 |
| SA060295[a,c] | Sea bream | *Sparus aurata* | Israel (Red Sea), ISh |
| SA300695[a] | Sea bream | *Sparus aurata* | Israel (Red Sea), IS7 |
| DL130795[a] | Sea bass | *Dicentrarchus labrax* | Israel (Red Sea), IS5 |
| SA040296[a] | Sea bream | *Sparus aurata* | Israel (Red Sea), IS8 |
| SA020596[a] | Sea bream | *Sparus aurata* | Israel (Red Sea), IS10 |
| DL260795 [a,c] | Sea bass | *Dicentrarchus labrax* | Israel (Red Sea), ISu |
| HSB 0696[a] | Hybrid striped bass | ♀*D. labrax* × ♂*Morone saxatilis* | Israel (Mediterranean), IS11 |
| SA010500[a] | Sea bream | *Sparus aurata* | Israel (Mediterranean), IS12 |
| I736[b] | Sea bass | *Dicentrarchus labrax* | Italy, I1 |
| 386/97[b] | Sea bass | *Dicentrarchus labrax* | Italy, I2 |
| I746[b] | Sea bass | *Dicentrarchus labrax* | Italy, I8 |
| I362-2-97[b] | Sea bass | *Dicentrarchus labrax* | Italy, I10 |
| OT97290[b] | Yellowtail | *Seriola quinqueradiata* | Japan, J4 |
| OT97299[b] | Yellowtail | *Seriola quinqueradiata* | Japan, J5 |
| SPP97127-15[b] | Sea bream | *Sparus aurata* | Spain, S6 |
| SPP97127-16[b] | Sea bream | *Sparus aurata* | Spain, S7 |
| PN2[b] | Sea bass | *Dicentrarchus labrax* | Greece, G9 |

[a]Israel Oceanographic & Limnological Research Ltd., National Center for Mariculture, Eilat, Israel (last 2 digits indicate the year of isolation)
[b]Obtained from Dr. A. Adams, Institute of Aquaculture, University of Stirling, Scotland
[c]Not included in the AFLP study

Table 2. ATCC (American Type Culture Collection) reference strains used for 16S rRNA gene sequence comparison and primer validation. NA: not available

| Reference strain | ATCC no. | GenBank accession no. |
|---|---|---|
| *Photobacterium damselae* ssp. *piscicida* [a] | 51736 | X78105 |
| *Photobacterium damselae* ssp. *piscicida* [a] | 17911 | NA |
| *Photobacterium damselae* ssp. *damselae* [a] | 33539 | X74700 |
| *Photobacterium histaminum* | 51805 | D25308 |
| *Photobacterium leiognathi* | 25521 | X74686 |
| *Vibrio hollisae* | 33564 | X74707 |
| *Photobacterium phosphoreum* | 11040 | X74687 |
| *Vibrio natriegens* | 14048 | X74714 |

[a]Included in the AFLP study

Soy Agar (Difco) prepared with 25% filtered aged seawater. Identification was confirmed by agglutination test with Mono-Pp test kit (Bionor A/S).

**DNA extraction.** Five mg tissue (spleen or whole fish) or pure fresh bacterial cultures were ground gently on ice with 300 µl of grinding buffer (100 mM Tris-HCl pH 9, 100 mM EDTA, 1% SDS). The homogenate was incubated at 70°C for 30 min. 42 µl of 8 M potassium acetate were then added and the tube was gently mixed for 1 min. The sample was placed on ice for 30 min, then centrifuged at 4°C, 12 000 rpm (15 300 × *g*) for 15 min. The supernatant was immediately transferred to a fresh tube and again centrifuged at 4°C, 12 000 rpm for 5 min. The centrifugation step was repeated until no traces of pellet were visible. DNA was precipitated with 1 volume of isopropanol for 15 min at room temperature. Pelleted DNA was washed in 70% ethanol and resuspended in 40 µl double distilled (dd) $H_2O$. DNA quality was assessed by electrophoresis in 0.7% agarose gel and ethidium bromide staining. DNA quantity and purity (optical density ratio $OD_{260}$:$OD_{280}$) were also estimated in a RNA/DNA Calculator (Gene Quant *pro*).

**Primers.** The primers utilized in this study are listed in Table 3. Specific primers for detection of *Photobacterium damselae* ssp. *piscicida* and *P. damselae* ssp. *damselae* as well as universal primers were designed by comparing the 16S rRNA gene sequences of *P. damselae* ssp. *piscicida* and *P. damselae* ssp. *damselae* (accession no. X78105 and X74700, respectively) with *Escherichia coli* 16S rRNA gene sequence (accession no. J01859) as well as with closely related *Photobacterium* and *Vibrio* species according to Gauthier et al. (1995) (accession no. D25308, X74686, X74707, X74687, and X74714, Table 2). The 16S rRNA gene sequences were retrieved from the GenBank database and compared using program On Line CLUSTAL W Multiple Sequence Alignment Tool (Thompson et al. 1994) (http://www.mbshortcuts.com/mbsalign/index.htm).

Four regions of the 16S rRNA gene were chosen as variable regions within the family *Vibrionaceae*. The primers (specific and universal) were planned accordingly. Primer set P3-P4 was designed to detect *Photobacterium damselae* (both subspecies) and related species. Primer set P1-P2 was designed to distinguish *P. damselae* from *P. histaminum* and related species. Primer 246 was designed by Böddinghaus et al. (1990), while the other universal primers Uni1, Uni2 and Uni3 were designed by us. All were utilized to sequence the 1300 bp 16S rRNA gene of *P. damselae* from pure cultures.

**PCR and sequencing.** PCRs were performed with Programmable Thermal Controller (PTC-100™, MJ Research). A typical 50 µl reaction mixture contained 50 to 100 ng of DNA, 1 U of Taq DNA polymerase, 0.25 µM of each primer, 0.2 mM dNTP's, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 0.1% (v/v) Triton X-100. Typical cycling parameters were: 1 min denaturation (94°C), 1 min annealing (64 or 53°C) and 1.5 min extension (72°C) for 30 cycles. The reaction was started by a denaturation step (3 min at 94°C) and ended by a 5 min extension step at 72°C. For the sequencing reaction, double stranded PCR templates were purified using PCR purification kit (QIAGEN); 50 ng of DNA template were used for each reaction. Both strands of each product were sequenced to confirm reading accuracy. Templates were sequenced by Automated DNA Sequencer (Perkin Elmer, model 377) using the Big Dye Terminator from ABI Cycle Sequencing Kit.

**Validation of the primers.** The specificity of primer sets P1-P2 and P3-P4 was tested on ATCC reference strains (see Table 2), isolates from different geographic

Table 3. PCR primers utilized in the study. Numbers in brackets represent primer position according to GenBank accession no. X78105. F: forward; R: reverse

| Primer name and position | Sequence |
|---|---|
| 246 [a] | F 5′-AGAGTTTGATCCTGGCTCAG-3′ |
| P1 (444 to 452) | F 5′-TAGTGTAGTTAACACCTGCAC-3′ |
| Uni1 (496 to 513) | R 5′-GTATTACCGCGGCTGCTG-3′ |
| Uni2 (767 to 786) | F 5′-AGATACCCTGGTAGTCCACG-3′ |
| P2 (982 to 1001) | R 5′-ACACTCGAATCTCTTCAAGT-3′ |
| P3 (980 TO 1000) | F 5′-AAGCTTGAAGAGATTCGAGTG-3′ |
| P4 (1231 TO 1250) | R 5′-CCACCTCGCGGTCTTGCTGC-3′ |
| Uni3 (1372 TO 1390) | R 5′-ATGGTGTGACGGGCGGTGT-3′ |

[a]Böddinghaus et al. (1990)

regions (see Table 1) and on DNA extracted from infected and non-infected fish (both *Sparus aurata* and *Dicentrarchus labrax*). Fish health status was confirmed by histological means and isolation of the pathogen from infected individuals. The specificity of the primer sets was tested using different annealing temperatures, ranging from 53 to 64°C (data not shown) and these 2 temperatures were selected as most suitable.

**Limits of detection.** When using primer set P3-P4 on clinically healthy fish (annealing temperature 53°C) non-specific PCR products were produced. Since the non-specific products may create competitive PCR conditions interfering with specific amplification of *Photobacterium damselae* sequences, the limit of *P. damselae* detection was measured. To this purpose, primer set P3-P4 was tested for amplification with 10-fold dilutions of purified DNA from *P. damselae* ssp. *piscicida* ATCC strain #51736 containing from 35 ng to 0.035 fg. For comparison, these primers were tested on the same 10-fold dilutions with a fixed amount (200 ng) of DNA extracted from clinically healthy *Sparus aurata* added to each sample. The same procedure was carried out with clinically healthy *Dicentrarchus labrax*.

Primer set P1-P2 was tested for amplification on the same serial dilutions of purified DNA from *Photobacterium damselae* ssp. *piscicida* ATCC strain #51736 as described for primer set P3-P4. Also, both primer sets were tested for amplification at an annealing temperature of 64°C. The limit of detection was based on the calculations of Osorio et al. (1999).

**AFLP.** Restriction enzyme digests, adapter annealing, and amplification procedures were performed as described by Vos et al. (1995) with the following modifications: 500 ng of genomic DNA were digested with 20 U of *Eco*RI and 5 U of *Mse*I restriction endonuclease for 3 h at 37°C. Preamplification was performed with the AFLP adapters:

*Eco*RI-adapter: 5′-CTCGTAGACTGCGTACC (E1)

*Mse*I-adapter: 5′-GACGATGAGTCCTGAG (M1)

The cycling parameters were: denaturation step (2 min at 94°C) followed by 19 cycles of 30 s denaturation (94°C), 30 s annealing (50°C) and 1 min extension (72°C). AFLP reactions employed 2 oligonucleotide primers; M1 corresponding to the *Mse*I ends and a radioactively labeled *Eco*RI primer (5-GACTGCG-TACCAATTN-3) corresponding to the *Eco*RI ends. The *Eco*RI primers had one selective nucleotide extension (N = G,A,T or C). In each reaction one selective *Eco*RI primer was employed. Four primer combinations were used to detect AFLP variation between isolates. The cycling parameters were as described for the preamplification with the annealing time extended to 1 min. Four µl of each sample were loaded into 6% denaturing polyacrylamide gels (8 M urea) and electrophoresed for 1.5 h at 2000 W. The gels were then transferred onto chromatography paper, dried and a FUJI Medical X-Ray Film was exposed to them overnight (14 to 18 h) at room temperature.

Sixteen isolates of *Photobacterium damselae* ssp. *piscicida*, 2 *P. damselae* ssp. *piscicida* reference strains (ATCC #51736 and ATCC #17911) and 1 *P. damselae* ssp. *damselae* reference strain (ATCC #33539) were subjected to AFLP analysis (see Tables 1 & 2). AFLPs were scored as presence (1) or absence (0) of a fragment in the polyacrylamide gels (see Fig. 1). The total number of amplified fragments was scored for both polymorphic and monomorphic fragments. The banding patterns of the 4 primer sets were combined and the pairwise similarities calculated using Jaccard's coefficient. Cluster analysis was performed on the resultant similarity matrix using the Unweighted-Pair Group Method using Average linkage (UPGMA) (Sneath & Sokal 1973).

## RESULTS

### 16S rRNA gene sequence analysis

The 16S rRNA gene sequences of the 19 isolates and of *Photobacterium damselae* ssp. *piscicida* ATCC strain #51736 (GenBank accession no. X78105) exhibited 100% homology.

### Detection

Using low annealing temperature (53°C), both primer sets (P1-P2 and P3-P4) produced a unique product of the expected size (570 and 270 bp, respectively) in spontaneously infected fish, *Photobacterium damselae* (both subspecies) and *P. histaminum*, but not in clinically healthy fish. Also, a typical pattern of non-specific products (approximately 1200, 800, 650 and 500 bp for *Sparus aurata* and 1600, 1100 and 500 bp for *Dicentrarchus labrax*) was produced with primer set P3-P4 (Fig. 2A). Subsequently, presence of the non-specific PCR products characteristic of *S. aurata* and *D. labrax* was used as an internal positive control when testing for presence/absence of *P. damselae* PCR products. PCR products, although considerably less conspicuous, were produced with both primer sets in *Vibrio hollisae* and *P. leiognathi*. Conversely, *P. phosphoreum* and *V. natriegens*, which were used as negative controls in both PCR reactions, did not yield any amplification product (Fig. 2A lanes 10 and 11; results for primer set P1-P2 at annealing temperature of 53°C are not shown). However, when using primer set P1-P2 at annealing temperature of 64°C the unique product

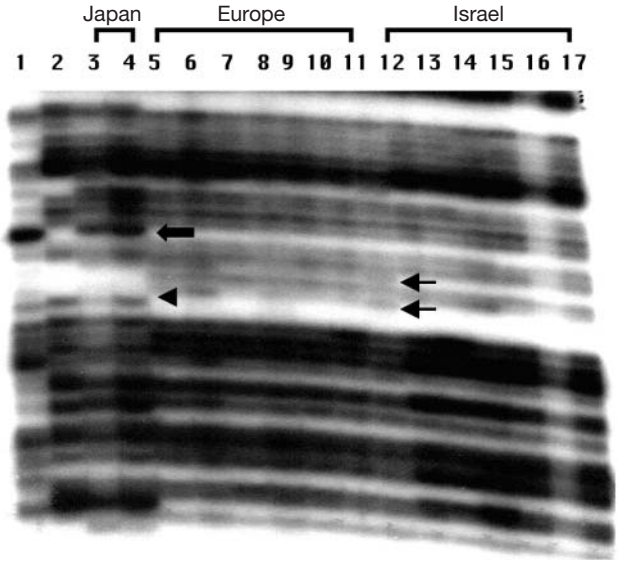


Fig. 1. Partial AFLP patterns. Lanes 1, *Photobacterium damselae* ssp. *damselae* ATCC #33539; 2 to 17, *P. damselae* ssp. *piscicida*; 2, ATCC #51736; 3 and 4, isolates from Japan; 5 to 11, isolates from Europe; 12 to 17, isolates from Israel. Polymorphic bands are arrowed: (⬅) lanes 1, 3 and 4; (⬅) lanes 5 to 11; (◀) lanes 2 to 4

(570 bp) was produced only in spontaneously infected fish, *P. damselae* ssp. *piscicida* and *P. damselae* ssp. *damselae*. *P. histaminum* and the non-*P. damselae* strains, used as negative controls, did not yield any amplification product (Fig. 2B). At this temperature, primer set P3-P4 produced a unique product of the expected size (270 bp) in spontaneously infected fish, *P. damselae* ssp. *piscicida*, *P. damselae* ssp. *damselae* and *P. histaminum*. No non-specific PCR products were observed in DNA samples from clinically healthy fish (results not shown). The identity of the 270 and 570 bp PCR products from infected fish and *P. damselae* (both subspecies) was confirmed by sequencing.

### Limit of detection

Using primer set P3-P4, presence of *Photobacterium damselae* ssp. *piscicida* could be detected down to 0.35 pg at both annealing temperatures (which corresponds to a limit of detection of approximately 40 bacterial cells). Limit of detection (at annealing temperature of 53°C) was not altered when DNA extracted from clinically healthy *Sparus aurata* was added. The same results were obtained when DNA from clinically healthy *Dicentrarchus labrax* was added (results not shown). Using primer set P1-P2, presence of *P. damselae* ssp. *piscicida* could similarly be detected down to 0.35 pg; however, at annealing temperature of 64°C the limit of detection rose to 3.5 pg (which corresponds to approximately 350 bacterial cells).

### Genetic diversity as revealed by AFLPs

In the isolates subjected to AFLP analysis (see Tables 1 & 2), the number of polymorphic bands differed according to the different primer combinations used. The total number of AFLP bands per primer set ranged from 147 to 179. Summing the bands of the 4 primer combinations, 388 polymorphic and 255 monomorphic distinct bands (60% polymorphism) were revealed. A similarity matrix was produced using all 643 bands and a dendrogram constructed (Fig. 3). All *Photobacterium damselae* ssp. *piscicida* isolates and reference strains were grouped in 1 cluster (cluster *I*), whereas *P. damselae* ssp. *damselae* ATCC #33539 fell outside this cluster at 52% similarity. Cluster *I* was further divided in 2 discrete subclusters, *Ia* and *Ib*, at 83% similarity. Subcluster *Ia* was composed of isolates from Japan and ATCC #51736 which was isolated in yellowtail *Seriola quinqueradiata*, also from Japan. Subcluster *Ib* was composed of isolates from Israel, Spain, Italy, Greece

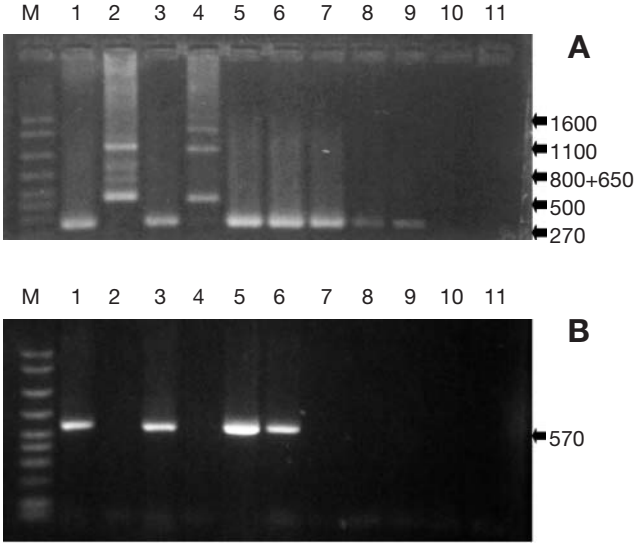


Fig. 2. (A) Low annealing temperature PCR (53°C) using primer set P3-P4 and (B) high annealing temperature PCR (64°C) using primer set P1-P2. Lanes: M, molecular weight marker; 1, *Sparus aurata* spontaneously infected with *Photobacterium damselae* ssp. *piscicida*; 2, clinically healthy *S. aurata*; 3, *Dicentrarchus labrax* spontaneously infected with *P. damselae* ssp. *piscicida*; 4, clinically healthy *D. labrax*; 5, *P. damselae* ssp. *piscicida* ATCC #51736; 6, *P. damselae* ssp. *damselae* ATCC #33539; 7, *P. histaminum* ATCC #51805; 8, *Vibrio hollisae* ATCC #33564; 9, *P. leiognathi* ATCC #25521; 10, *P. phosphoreum* ATCC #11040; 11, *Vibrio natriegens* ATCC #14048. The product of the expected length of 570 or 270 bp, as well as the non-specific products in lanes (A2) and (A4) ranging from ~1600 to 500 bp, are indicated on the right

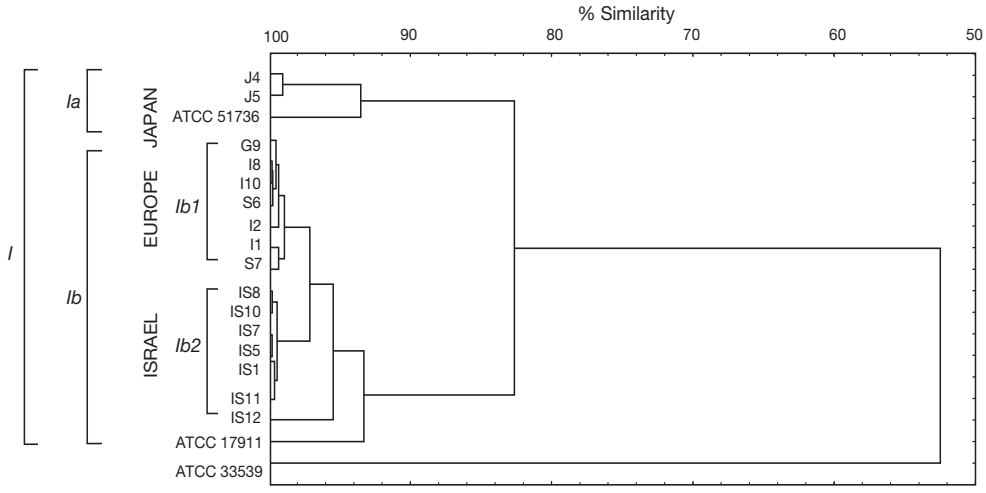


Fig. 3. Dendrogram derived from an UPGMA cluster analysis of the AFLP polymorphism of all strains used. Numbers and letters on the left in bold italics indicate clusters (*I* at 52%, *Ia* and *Ib* at 83%, *Ib1* and *Ib2* at 97% similarity), whereas numbers and letters in upper case indicate the sources of isolates and code (J: Japan, G: Greece, I: Italy, S: Spain, IS: Israel, ATCC: American Type Culture Collection; see also Tables 1 & 2)

(Mediterranean isolates) and ATCC #17911 which was isolated in white perch *Roccus americanus* from USA. At 97% similarity, subcluster *Ib* was further divided in 2 distinct subclusters: subcluster *Ib1*, containing the isolates from Spain, Italy and Greece, and subcluster *Ib2*, containing the isolates from Israel, with the exception of isolate IS12, which fell outside both subclusters *Ib1* and *Ib2* at 95% similarity.

## DISCUSSION

The development of direct PCR for detection and identification of fish pathogens based on 16S rRNA gene sequences has drawn much interest in recent years. This technique was established successfully in our laboratory in Eilat for *Mycobacterium marinum* (Knibb et al. 1993) and its use has become established for other bacterial fish pathogens (Hiney & Smith 1998, Zlotkin et al. 1998). Direct detection of *Photobacterium damselae* ssp. *piscicida* using various other molecular tools has been reported. However, these methods often proved unsuitable for positive identification of this species (see review by Osorio et al. 2000). Studies of 16S rRNA gene sequences of *P. damselae* ssp. *piscicida* and *P. damselae* ssp. *damselae* showed high homogeneity of the gene in these subspecies (Gauthier et al. 1995, Osorio et al. 1999), even though reports have often been discordant: Osorio et al. (1999) report 100% sequence homology for isolates of *P. damselae* ssp. *damselae* and *P. damselae* ssp. *piscicida*; Gauthier et al. (1995) report 1 nucleotide difference between the 2 sub-species, whereas GenBank submission (accession no. X74700) shows a 2-nucleotide difference between the 2 sub-species (A at position 255, C at position 452; position according to accession no. X78105). In our study, 16S rRNA gene sequences obtained for isolates of *P. damselae* ssp. *piscicida* from different regions and host sources (Table 1) exhibited 100% sequence homology to *P. damselae* ssp. *piscicida* ATCC #51736 (= NCIMB $2058^T$ according to Gauthier et al. 1995) regardless of the geographic origins and host sources of the isolates. In this respect, our results are consistent with the work of Osorio et al. (1999).

In the present study, the combination of low annealing temperature PCR that detects low titers of *Photobacterium damselae* as well as related species, and high annealing temperature PCR for identification of *P. damselae* directly from infected fish should provide a rapid, simple and accurate diagnosis of fish 'pasteurellosis'.

Primer combination P1-P2 and P3-P4 at low annealing temperature PCR (53°C) successfully detected *Photobacterium damselae* in spontaneously infected *Sparus aurata* and *Dicentrarchus labrax*. The non-specific products evidently resulting from amplification of fish DNA did not interfere with sensitivity of the PCR. As negative result (no PCR product) indicates that the pathogen is either absent or below limit of detection, and since several factors can affect PCR quality, the non-specific products characteristic of *S. aurata* and *D. labrax* were used as an internal positive control for PCR efficiency. The second step (high annealing temperature PCR) using primer set P1-P2 was applied only to the positive samples. The limit of bacterial detection (approximately 40 bacterial cells) is similar to that reported by Osorio et al. (1999) and was not influenced by an amount of fish DNA as high as 200 ng. Since *P. histaminum* and *P. leiognathi* are not pathogenic, and *Vibrio hollisae* is not common to the fish environment, the fact that the primers at low annealing temperature PCR (53°C) can identify these closely related species should not impose any diagnostic problem. At any rate, high annealing temperature PCR (64°C) using primer

set P1-P2 can successfully discriminate between *P. damselae* (both subspecies) and the non-*P. damselae* strains, including *P. histaminum*. High annealing temperature PCR did not affect the limit of bacterial detection for primer set P3-P4, whereas for primer set P1-P2, the limit of bacterial detection was raised from approximately 40 to 350 bacterial cells.

Primer P1 was designed to distinguish *Photobacterium damselae* from *P. histaminum* and related species by identifying base number 452 (reported as C in *P. damselae* ssp. *piscicida*, GenBank accession no. X78105 and as T in both *P. damselae* ssp. *damselae*, GenBank accession no. X74700 and in *P. histaminum*, GenBank accession no. D25308). Although P1 distinguished *P. damselae* ssp. *piscicida* and *P. damselae* ssp. *damselae* from *P. histaminum*, (despite the fact that no such distinction between *P. damselae* ssp. *damselae* and *P. histaminum* should exist according to Kimura et al. 2000), it failed to distinguish *P. damselae* ssp. *piscicida* from *P. damselae* ssp. *damselae*.

Recently, a nested method for the detection of both subspecies based on 16S rRNA gene sequences was reported (Osorio et al. 1999). In it, the nested primer Nestcar1 is based on position 1225 (according to accession no. X78105) reported as G in *Photobacterium histaminum* and as C in *P. damselae* (both subspecies). However, no such distinction should exist according to Kimura et al. (2000), pointing at position 1225 as unsuitable for discriminating between *P. histaminum* and *P. damselae*. Moreover, Osorio et al. (1999) based their limit of detection on tissues of salmonids mixed with cultured bacteria, although salmonids are not known to be sensitive to the infection. Validation for one host cannot reliably be applied to a different one, as interaction of the primers with unpredictable DNA sequences can occur. In the present report, the tests were carried out on spontaneously infected fish, thus targeting the pathogen in its natural environment. *P. damselae* ssp. *piscicida* carries plasmids (Magariños et al. 1992) and these may be integrated (Aoki et al. 1997) or, similarly to what is known in other bacteria, lost during growth *in vitro*. It is hence important to establish validation of PCR not only on the pathogen and its natural host in 1 reaction, but also on the 2 combined in a spontaneous infection, thus eliminating those factors linked to possible plasmid loss and competitive PCR conditions.

An innovative approach reported by Osorio et al. (2000) can differentiate between the 2 subspecies at least in culture. Accordingly, *Photobacterium damselae* ssp. *damselae* harbors the *ureC* (urease) gene, whereas *P. damselae* ssp. *piscicida* does not. Unfortunately, this technique was not applied to ATCC #51736 (= NCIMB 2058), the strain on which Gauthier et al. (1995) assigned the taxonomic status of *P. damselae* ssp. *piscicida*. Moreover, neither the limit of bacterial detection nor direct detection from fish tissue was reported; thus, the practical consequences of the method of Osorio et al. (2000) are unclear.

Genetic variation among isolates of *Photobacterium damselae* ssp. *piscicida* has been detected by different methods (Magariños et al. 1992, 1997, 1999, 2000, Bakopoulos et al. 1995, 1997b, Thyssen et al. 2000). With some of these methods the Japanese isolates were found to differ from the Mediterranean/European isolates (Bakopoulos et al. 1997a, Magariños et al. 1997, 2000, Thyssen et al. 2000). Magariños et al. (1997) distinguished one Israeli isolate from the Mediterranean/European isolates, whereas Thyssen et al. (2000) could not detect any genetic differentiation among the Mediterranean/European isolates. However, it is unclear whether Israeli isolates were included in their analysis.

According to our AFLP analysis, 2 main groups could be clustered at 83% similarity: one cluster with the isolates from Japan, including ATCC strain #51736, which was isolated in yellowtail, also from Japan, and a second cluster with the Mediterranean/European isolates (see Fig. 3, *Ia* and *Ib*). However, in our study a further subclustering at 97% similarity was clearly possible, discriminating between the Israeli and European isolates (Fig. 3, *Ib1* and *Ib2*). The European isolates formed a relatively heterogeneous group that could not be clustered according to either their geographic origin or their host. The Israeli isolates, on the other hand, formed a relatively homogeneous group with the exception of IS12, isolated from Mediterranean fish in the year 2000, whereas all the others were isolated in the years 1994–1996. This diversity may be explained by the fact that in recent years an extensive movement of fingerling stocks between Israel and other neighboring Mediterranean countries has occurred, exposing the local fisheries to pathogens from different geographic sources.

Despite the high homogeneity of *Photobacterium damselae* ssp. *piscicida* from diverse hosts and geographic origins (Magariños et al. 1992, Romalde et al. 1995, Thyssen et al. 2000), AFLP could detect intraspecific variations, discriminating between isolates at similarity as high as 97%, indicating that AFLP has indeed a superior discriminative ability over other molecular methods, and can therefore be an important tool for epidemiological and taxonomic studies of this highly homogeneous genus.

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