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Multiplex PCR assay for *ureC* and 16S rRNA genes clearly discriminates between both subspecies of *Photobacterium damselae*

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ABSTRACT: A multiplex-PCR approach, employing 2 primer pairs directed to internal regions of the 16S rRNA and *ureC* genes, was utilized to analyze a collection of *Photobacterium damselae* strains, including 25 isolates of subspecies *piscicida* and 15 isolates of subspecies *damselae*. With this procedure, all the *P. damselae* subsp. *damselae* strains yielded 2 amplification products, one of 267 bp and the other of 448 bp, corresponding to internal fragments of the 16S rRNA and *ureC* genes, respectively. However, *P. damselae* subsp. *piscicida* isolates only showed the PCR product of 267 bp (16S rRNA fragment), indicating the absence of the urease gene in its genome. We have constructed a DNA probe directed to an internal region of the *ureC* gene, and corroborated by dot blot hybridization that the *P. damselae* subsp. *piscicida* lacks this gene, whereas it is present in the subspecies *damselae*. This constitutes the first successful discrimination between both subspecies using a PCR procedure, which could become a useful tool for diagnosis of pasteurellosis in the field. In addition, since these 2 subspecies have been shown to share nearly the same *rrn* operon sequence, our results provided evidence that one of the steps in the *P. damselae* speciation proccess included gain/loss events associated with the *ure* operon.

KEY WORDS: Multiplex-PCR · *Photobacterium damselae* · *ureC* gene · 16S rRNA gene · Subspecies discrimination

INTRODUCTION

Pasteurellosis or pseudotuberculosis is a fish disease that causes enormous losses in fish aquaculture production worldwide (Kusuda & Salati 1993, Magariños et al. 1996, Romalde & Magariños 1997). The causative agent of fish pasteurellosis is the Gram-negative bacterium *Photobacterium damselae* subsp. *piscicida*, formerly named *Pasteurella piscicida* (Snieszko et al. 1964). Though the taxonomic position of this fish pathogen remained unclear for decades, preliminary phylogenetic studies (Gauthier et al. 1995) based on 16S rRNA sequencing conducted in 2 strains of *Pasteurella piscicida* showed that this species is closely related to members of the genus *Photobacterium*, and that it shares its species epithet with *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*), known as a pathogen for poikilotherms and mammals. More recent studies on this ribosomal gene, from an extensive number of strains from different geographical and host origin, clearly confirmed that both bacteria share the same sequence in their 16S rRNA gene and that they definitively belong to the genus *Photobacterium* (Osorio 1998, Osorio et al. 1999), *Photobacterium histaminum* being the most closely related species with a 98.1% similarity with all the *P. damselae* isolates analyzed.

Initial attempts to detect the causative agent of fish pasteurellosis using molecular tools were based upon a short DNA sequence obtained from a *Photobacterium damselae* subsp. *piscicida* genomic library which served as a DNA probe for the detection of the pathogen (Aoki et al. 1995, Zhao & Aoki 1989). Subsequently, the same DNA fragment was employed as the basis for the design of oligonucleotide primers for the PCR-based

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detection of *P. damselae* subsp. *piscicida*. Nevertheless, these approaches proved to be inconvenient for the diagnosis of fish pasteurellosis since it has been demonstrated that this short target DNA sequence is also present in some *P. damselae* subsp. *damselae* isolates (Osorio 1998). Moreover, these described molecular tools were assayed with only Japanese isolates, and no European strains were included.

In addition, some attempts have been made to detect *Photobacterium damselae* subsp. *piscicida* by PCR using plasmid sequences as a target (Aoki et al. 1997). Unfortunately, these plasmid-based primers were only tested with Japanese and American isolates, but not with any European isolate. In this regard, this approach would not be suitable for detection of most of the European isolates, since the plasmid content has proved to be very different depending on the geographical origin of subsp. *piscicida* strains (Magariños et al. 1992, 1996).

Although the information obtained with the 16S rRNA gene sequencing of a great number of worldwide isolates from both subspecies of *Photobacterium damselae* allowed the design of a species-specific PCR-based detection method (Osorio et al. 1999), the design of a subspecies-specific molecular detection method has been hampered by the high degree of overall genomic homology between both subspecies. Therefore, identification of differential gene occurrence between the 2 subspecies of *P. damselae* was required. For this reason, we focused on the main phenotypical traits in which the subspecies differ, such as motility, nitrate reduction, urease and haemolysin production (Fouz et al. 1992, Magariños et al. 1992). This strategy has proved to be useful for taxonomical and discriminative purposes in other bacterial groups (Dasen et al. 1998, Lucchini et al. 1998).

Since the ability to hydrolyze urea seems to be a common feature in all the *Photobacterium damselae* subsp. *damselae* strains, but it is not found in any subsp. *piscicida* isolate (Magariños et al. 1996), in this study we developed a multiplex PCR assay using *P. damselae*-specific 16S rRNA gene-directed primers in conjunction with others designed on the basis of the partial *ureC* gene sequence. This constitutes the first successful approach to discriminating between subsp *damselae* and subsp. *piscicida* by means of PCR, accounting for one of the few insights into the genetic divergence between these taxa achieved so far.

MATERIAL AND METHODS

Bacterial strains. A total of 25 strains of *Photobacterium damselae* subsp. *piscicida* isolated from different fish species in Europe, Japan and USA were used in this study. Since *P. damselae* subsp. *damselae* is a common pathogen for both poikilotherm and homoiotherm animals, the source of the 16 strains used comprised fish, shellfish, mammals (including humans) and seawater (Table 1). For comparative purposes, the *P. histaminum* strain JCM 8968 was also included in the present study (Okuzumi et al. 1994).

All the bacterial strains were routinely cultured on BHA (Brain Heart Infusion Agar) (Pronadisa, Madrid, Spain) supplemented with 1% NaCl at 25°C for 24 to 48 h. The taxonomical position of the isolates was confirmed following conventional plate and tube tests procedures described elsewhere (Fouz et al. 1992, Magariños et al. 1992), as well as by API-20E systems, obtaining the typical profile numbers 2005004 (*Photobacterium damselae* subsp. *piscicida*) and 2015004 (*P. damselae* subsp. *damselae*).

DNA extraction. Genomic DNA was extracted following a modification to the method described by Lawson et al. (1989). Briefly, bacterial cells were resuspended in 500 µl of TES buffer, and incubated at 37°C for 30 min after addition of 5 µl of lysozyme (10 mg ml^{-1}). Then, 5 µl each of Proteinase K (Sigma, Madrid, Spain) (10 mg ml^{-1}) and RNase (Sigma) (10 mg ml^{-1}) were added and the solution was incubated for 1 h at 65°C. After addition of 50 µl of 20% SDS, tubes were returned immediately to the 65°C water-bath for a further 10 min. After cell lysis, a phenol/chloroform (Amresco, Barcelona, Spain) extraction was carried out. DNA was precipitated with cold ethanol (–20°C), centrifuged (15000 × *g*) and the DNA pellet air-dried and resuspended in 50 to 100 µl sterile water. DNA quality and concentration was measured by loading a 5 µl sample onto a 1% agarose gel or, alternatively, by spectrophotometrical measurement at 260 nm.

Oligonucleotide design for *ureC* gene amplification. Nucleotide sequence of partial *ureC* gene from *Photobacterium damselae* subsp. *damselae* was retrieved from GenBank database with accession number U40071. A forward primer, Ure-5' (20-mer 5'-TCCGGAATAGGTAAAGCGGG-3'), and a reverse primer, Ure-3' (22-mer 5'-CTTGAATATCCATCTCATCTGC-3'), were designed flanking a 448 bp-long stretch of the *ureC* gene, as it is shown in Fig. 1.

A forward primer, Car1 (18-mer 5'-GCTTGAAGAGATTCGAGT-3') (positions 1016 to 1033 in *Escherichia coli* 16S rRNA gene), and a reverse primer, Car2 (18-mer 5'-CACCTCGCGGTCTTGCTG-3') (positions 1266 to 1283), flanking a 267 bp fragment of the 16S gene of strain ATCC 29690 of *Photobacterium damselae* subsp. *piscicida* (GenBank accession number Y18496) (Osorio et al. 1999) were used in conjunction with Ure-5' and Ure-3' in a multiplex PCR reaction. PCR amplifications were performed in a DNA thermal cycler (Eppendorf, Hamburg, Germany). A typical

Table 1. *Photobacterium* strains used in this study

Strain[a]	Origin	Country	Urease activity[b]	Presence of *ureC* gene[c]
P. damselae* subsp. *piscicida				
DI 21	Gilthead seabream	Spain	–	–
DI 91	Gilthead seabream	Spain	–	–
C1	Gilthead seabream	Spain	–	–
C2	Gilthead seabream	Spain	–	–
R 46	Gilthead seabream	Spain	–	–
DS 11	Gilthead seabream	Spain	–	–
B 21	Seabass	Spain	–	–
B 51	Seabass	Spain	–	–
619.1	Gilthead seabream	Portugal	–	–
693.2	Gilthead seabream	Portugal	–	–
666.1	Seabass	Portugal	–	–
10831	Seabass	France	–	–
IT-1	Gilthead seabream	Italy	–	–
IT-2	Gilthead seabream	Italy	–	–
O69 A	Gilthead seabream	Greece	–	–
O69 E	Gilthead seabream	Greece	–	–
ATLIT 2	Seabass hybrid	Israel	–	–
2101	Seabass hybrid	Israel	–	–
MP-7801	Yellowtail	Japan	–	–
EPOY-8803-II	Red grouper	Japan	–	–
P3333	Yellowtail	Japan	–	–
MZS 8001	Yellowtail	Japan	–	–
P3335	Yellowtail	Japan	–	–
ATCC 29690	Yellowtail	Japan	–	–
ATCC 17911	White perch	USA	–	–
P. damselae* subsp. *damselae				
RG-91	Turbot	Spain	+	+
RG-153	Turbot	Spain	+	+
RG-214	Turbot	Spain	+	+
RM-71	Turbot	Spain	+	+
LD-07	Gilthead seabream	Spain	+	+
340	Seawater	Spain	+	+
309	Mussel	Spain	+	+
158	European eel	Belgium	+	+
162	European eel	Belgium	+	+
PG 801	Shrimp	Taiwan	+	+
J3G 801	Shrimp	Taiwan	+	+
192	Dolphin	USA	+	+
238	Dolphin	USA	+	+
ATCC 33539	Damselfish	USA	+	+
ATCC 35083	Brown shark	USA	+	+
CDC 2227-81	Human	USA	+	+
P. histaminum				
JCM 8968	Labracoglossid fish	Japan	+	+

[a]ATCC, American Type Culture Collection; CDC, Center for Diseases Control; JCM, Japan Collection of Microorganisms
[b]Urease activity determined by tube and API-20E tests
[c]Presence of *ureC* gene demonstrated by multiplex PCR (see Fig. 2)

reaction mixture (100 µl) consisted of 160 pmol of each primer, 2 U of *Taq* polymerase (Perkin Elmer, Madrid, Spain), 1 × *Taq* polymerase buffer (Perkin Elmer), 2 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, and 100 ng of template DNA. The amplification conditions were: 95°C for 4 min followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 40 s. A final extension step of 5 min at 72°C was carried out. Amplification products were analysed on 1% (w/v) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA) electrophoresis buffer, and were visualized on a UV transilluminator after staining with ethidium bromide. A 100 bp DNA ladder (Bio-Rad, Madrid, Spain) was included as a molecular weight marker.

***ureC* DNA probe synthesis and labelling.** The DNA probe for the partial *ureC* gene was synthesized by PCR amplification of the 448 bp *ureC* fragment flanked by primers Ure-5' and Ure-3' (Fig. 1). PCR conditions were as described above for PCR detection of the *ureC* gene. The DNA probe was digoxigenin-labelled using a nucleotide mix from the DIG DNA labelling and detection kit (Boehringer Mannheim, Barcelona, Spain) containing 1 mM each of dATP, dGTP and dCTP, 0.65 mM dTTP and 0.35 mM DIG-labelled dUTP. For a final 100 µl PCR reaction 20 µl of this nucleotide mix were added, so that the final concentration was 200 µM of each dNTP.

After PCR synthesis, probe DNA was purified as follows: to 100 µl of probe solution, 12.5 µl of 4 M LiCl and 375 µl absolute ethanol (–20°C) were added, and samples were incubated at –70°C for 30 min. Then, tubes were centrifuged at 13 000 rpm (15 000 × *g*) for 15 min and subsequently washed with 200 µl of 70% ethanol. Samples were dried using a Speedvac apparatus (Savant, New York, USA). The DNA pellet was resuspended in approximately 50 µl distilled water and its concentration was spectrophotometrically measured at 260 nm.

'Dot-blot' hybridization with *ureC* gene DNA probe. The *ureC* DNA probe was used to carry out a hybridization directly onto purified *Photobacterium damselae* genomic DNA. DNA samples were diluted in a final volume of 50 µl in TE buffer so that each sample contained approximately 200 to 400 ng DNA. Diluted samples were boiled for 5 min to denature double-stranded chromosomal DNA and immediately transferred onto a nylon membrane (0.45 µm pore size, Boehringer Mannheim), previously activated with 2× SSC, using a Minifold II apparatus (Schleicher & Schuell, Dassel, Germany). Transferred DNA was fixed to the nylon with UV light for 2 min in a Ultraviolet Crosslinker

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  1  CGTATATCCC  GAATAGGTAA  AGCGGCTAAC  CCAGACGTCC  AGCCTAATGT
 51  TGATATTGTT  ATCGGTCCCG  GTACAGAAGT  TGTGGCAGGC  GAAGGCAAGA
101  TCGTAACAGC  TGGAGGAATC  GATACTCACA  TTCATTTTAT  CTGTCCTCAA
151  CAAGCCGAAG  AAGGATTATG  TTCTGGCTTA  ACAACCTTTA  TCGGTGGCGG
201  AACCGGTCCA  GTGGCGGGTT  CCAATGCAAC  AACCGTGACA  CCAGGGGTCT
251  GGAATATGTC  ACGAATGCTG  GAAGCCGTTG  ATGACTTACC  TATTAATGTG
301  GGTTTATTTG  GTAAAGGTTG  TGTCAGTAAA  CCAGAAGCAT  TACGAGAGCA
351  AATTGAAGCT  GGAGCTGTTG  GTTTAAAACT  GCATGAAGAT  TGGGGTGCAA
401  CGCCCGCTGC  TATTAATAAC  TGTATGAATG  TGGCAGATGA  GATGGATATT
451  CAAGTTGCTA  TC
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Fig. 1. Partial *ureC* gene sequence of *Photobacterium damselae* subsp. *damselae* (GenBank, accession number U40071). Target sites for Ure-5' and Ure-3' primers are highlighted. *ureC*-targeted probe was constructed by PCR amplification using these primers and digoxigenin-labelled dUTP

(Amersham Life Science, Barcelona, Spain). After being dried at 60°C in a oven, the membrane was kept at 4°C until hybridization was carried out.

Prehybridization (68°C for 1 h) and hybridization (56°C for 14 h) were performed in hybridization solution containing 5× SSC, blocking reagent (1% w/v, Boehringer Mannheim), 0.1% (w/v) sarcosyl, and 0.02% (w/v) SDS. For the hybridization, 1 µg of the labelled DNA probe was added to the hybridization buffer. After hybridization, nylon membranes were washed twice at room temperature in 2×SSC, 0.1% (w/v) SDS and twice in 1×SSC, 0.1% SDS at 68°C. Then, membranes were blocked and incubated with the anti-digoxigenin-alkaline phosphatase conjugate, and positive hybridizations were visualized colorimetrically using the DIG DNA detection kit (Boehringer Mannheim), following the manufacturer's recommendations.

RESULTS

A pair of 16S rRNA gene-based PCR primers, namely CAR-1 and CAR-2, were previously designed (Osorio et al. 1999) for the specific detection of any *Photobacterium damselae* isolate. However, a subspecies-specific gene was needed in order to design subspecies-specific primers that could discriminate between *P. damselae* subsp. *piscicida* and subsp. *damselae*. In this sense, another set of primers directed toward the urease gene were devised in this work, in order to develop a multiplex PCR assay which enables such discrimination.

Using this multiplex PCR assay, with 16S rRNA gene and *ureC* gene binding primers, all the *Photobacterium damselae* subsp. *damselae* strains used in the present study, regardless of their geographical origin and source of isolation, yielded 2 amplification products of 267 and 448 bp (corresponding to an internal fragment of the 16S rRNA gene and one of the *ureC* gene, respectively). In the same assay, all the *P. damselae* subsp. *piscicida* strains showed amplification of the 267 bp 16S rRNA gene fragment, but no amplification of partial urease gene occurred (Table 1). Fig. 2 shows these differences in the multiplex PCR patterns for the strains of the 2 subspecies of *P. damselae* included in this study.

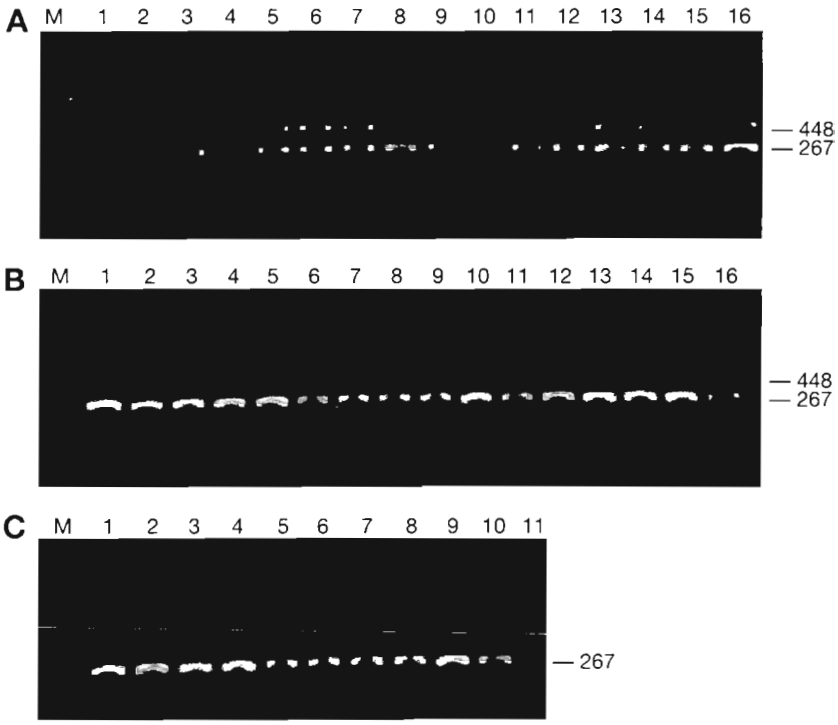

Fig. 2. Agarose electrophoresis of the multiplex-PCR products obtained for the different *Photobacterium* strains studied. Lanes: M, molecular weight marker 100 bp Molecular Ruler (Bio-Rad); (A) 1 to 16, *P. damselae* subsp. *damselae* strains; (B) 1, *P. histaminum* isolate; 2 to 16, *P. damselae* subsp. *piscicida* strains; (C) 1 to 10, *P. damselae* subsp. *piscida* strains; 11, negative control (no DNA). Numbers on the right indicate the size of the amplification products, corresponding to the 448 and 267 bp internal fragments of the *ureC* and 16S rRNA genes, respectively

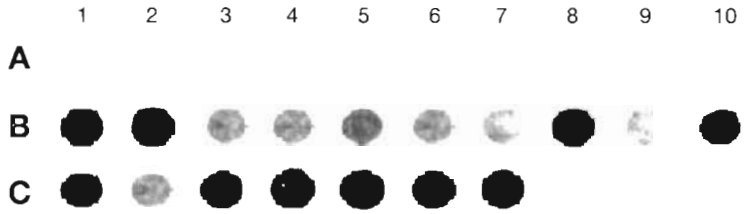

Fig. 3. Hybridization of the different *Photobacterium* strains with the *ureC*-targeted DNA probe. Genomic DNA (200 to 400 ng) was blotted onto nylon and membranes were exposed to the digoxigenin-labelled probe derived from a internal coding region of the *ureC* gene of *P. damselae* subsp. *damselae* (accession number U40071). Lanes: A1 to A10, *P. damselae* subsp. *piscicida* strains; B1 to B10 and C1 to C6, *P. damselae* subsp. *damselae* strains; C7, *P. histaminum*; C8 and C9, negative controls (PBS and 16S rRNA gene amplification product, respectively); C10, no sample

As PCR misamplification and/or mismatches in the priming sites could have prevented the *ureC* gene from being amplified in *Photobacterium damselae* subsp. *piscicida* strains, a hybridization was performed with an *ureC* gene digoxigenin-labelled DNA probe in order to corroborate the subspecies-specific occurrence of the urease gene within *P. damselae* strains. As illustrated in Fig. 3, positive hybridization was obtained for all the subsp. *damselae* strains tested, whereas no subsp. *piscicida* isolate reacted with the *ureC* gene probe. These results clearly demonstrate the absence of the ureC gene in *P. damselae* subsp. *piscicida*.

Its is interesting to note that the *Photobacterium histaminum* reference strain showed the same PCR amplification pattern as all the *P. damselae* subsp. *damselae* isolates (Table 1; Fig. 2B, Lane 1), and also showed positive reaction in the hybridization experiments with the *ureC* probe (Fig. 3).

DISCUSSION

The inclusion of the causative agent of fish pasteurellosis within the genus *Photobacterium*, and the sharing of species level with *P. damselae* subsp. *damselae* implies that any molecular tool intended to serve as a diagnosis procedure for fish pasteurellosis must be tested with an array of strains of these subspecies, in order to make sure that no cross-reaction occurs. Though 16S rRNA genes have been widely used as a target for PCR-based detection procedures of an important number of fish pathogens (Magnússon et al. 1994, Toyama et al. 1994, Hiney & Smith 1998, Gibello et al. 1999), in the case of fish pasteurellosis there is a limitation imposed by the total homology at the 16S rRNA gene sequence level found between *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* (Gauthier et al. 1995, Osorio et al. 1999). Promising approaches, based either on DNA fragments from a *P. damselae* subsp. *piscicida* DNA library (Zhao & Aoki 1989, Aoki et al. 1995) or on plasmid sequences (Aoki et al. 1997), have been reported for detection of this fish pathogen. However, we have recently noted the failure of these methods to detect all the possible isolates of the causative agent of fish pasteurellosis and/or to discriminate between *P. damselae* subsp. *piscicida* and subsp. *damselae* strains (Osorio 1998). This can be explained by the fact that when some of these methods were designed, the causative agent of pasteurellosis disease was considered within the family Pasteurellaceae and, hence, no strains of *P. damselae* subsp. *damselae* were included as controls in the specificity experiments.

One of the phenotypical traits shown to be characteristic of *Photobacterium damselae* subsp. *damselae* isolates as opposed to subsp. *piscicida* is the ability to hydrolize urea. Though rarely occurring within the Vibrionaceae, urease genes are present in several species of both *Vibrio* and *Photobacterium*. Ureases from Gram-negative bacteria are reported to contain 3 nonidentical subunits (average 70, 11 and 9 kDa) (Mobley et al. 1995). Using conserved regions of the *ureC* gene from *Proteus mirabilis* (GenBank database accession No. U40071), an internal fragment of this gene in *P. damselae* subsp. *damselae* was amplified and sequenced. Taking this information together with our previous exhaustive knowledge of the 16S rRNA gene sequence of a large number of *P. damselae* isolates (Osorio et al. 1999), we designed a multiplex PCR protocol which allowed us to discriminate, in a single step, *P. damselae* subsp. *piscicida* from any of the other bacterial species that may occur in fish or seawater.

As the 16S rRNA gene belongs to a multigenic family in Vibrionaceae, the amplification product of this gene is normally more intense than the one obtained for the *ureC* gene, which, to the best of our knowledge, is a single-copy gene. In previous work, it was demonstrated that 16S rRNA gene primers can be used to detect as little as 1 pg to 10 fg of *Photobacterium damselae* purified DNA (Osorio et al. 1999). Nevertheless, the sensitivity of the multiplex PCR should not be

affected by the difference in gene copy numbers, since very good results and low detection levels are usually obtained for other fish pathogens when a single-copy gene is used as a target for PCR (Brown et al. 1994).

In addition, the multiplex PCR and hybridization assays performed in this study firmly demonstrated that the inability of *Photobacterium damselae* subsp. *piscicida* to hydrolyze urea is due to the lack of the *ureC* gene in its genome, and not to any inactivating mechanism for this gene. The possibility of the *ureC* gene being plasmid-borne can be ruled out since no plasmid DNA is present in some of the *P. damselae* subsp. *damselae* strains used in this work (Cutter & Kreger 1990, Fouz et al. 1992). Although at the moment this multiplex protocol has been tested only with pure cultures of the 2 subspecies of *P. damselae*, the subspecies-specific occurrence of the urease gene opens a door to further studies focused on diagnosis procedures based on PCR and/or DNA probes that can be applied directly to fish tissues.

The astonishing degree of similarity between these 3 taxa (both subspecies of *Photobacterium damselae* and *P. histaminum*), as revealed by sequencing of 16S rRNA gene as well as other DNA regions, indicates the need to reevaluate the taxonomy of the genus *Photobacterium*. In the concrete case of *P. histaminum*, the near total identity with *P. damselae* subsp. *damselae* in 16S and 5S rRNA, *ureC*, *gyrB* and other genes (Osorio et al. 1998, and unpubl. data) raises the question of its possible reclassification as *P. damselae* subsp. *histaminum*.

The results also point out the idea that one of the steps in *Photobacterium damselae* speciation process may include gain/loss events associated with the *ure* operon. This raises new questions as to how 2 subspecies related so closely at the ribosomal operon level have diverged so dramatically in certain phenotypic characters.

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