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PROOF RESEARCH PAPER

Simultaneous Detection of *Flavobacterium psychrophilum*, *Pseudomonas plecoglossicida*, and *Vibrio anguillarum* by a Multiplex PCR Targeting the gyrB Region

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Abstract

We have developed a multiplex PCR to detect bacterial disease agents of Flavobacterium psychrophilum, Vibrio anguillarum, and Pseudomonas plecoglossicida. Three primer pairs designed based on determined nucleotide sequences of the gyrB regions of these three bacteria were used. The detection limits and stringency of this method were higher enough for specific detection of these three fish diseases. Using this multiplex PCR, the rapid and simultaneous diagnosis of three major bacterial fish diseases caused by these bacteria from the organs of diseased fishes was successful.

Keywords: *Flavobacterium psychrophilum*, *Pseudomonas plecoglossicida*, *Vibrio anguillarum*, Multiplex PCR, gyrB.

Introduction

Bacterial cold-water disease (BCWD), bacterial haemorrhagic ascites (BHA), and fish vibriosis are major bacterial diseases of ayu, *Plecoglossus altivelis* (Temminck & Schlegel), salmonids, or other fresh water fishes. The etiological agents of BCWD, BHA, and fish vibriosis are *Flavobacterium psychrophilum*, *Pseudomonas plecoglossicida*, and *Vibrio anguillarum*, respectively (Wakabayashi, Horinouchi, Bunya, & Hoshiai, 1991; Wakabayashi, Sawada, Ninomiya, & Nishimori, 1996; Nishimori, Kita-Tsukamoto, & Wakabayashi, 2000; Muroga & Egusa 1988). These pathogens have been common worldwide and given severe economic losses to the fish farming industry and natural water ecosystem (Sako & Kusuda 1978; Actis, Tolmasky, & Crosa, 1999; Ganzhorn 2005).

In recent years, mixed infections of two or three out of these three diseases are often observed in ayu culture. It is very important for fishery operators to know correctly which and how many kind of disease agents are affected in disordered fishes. Because a kind of treatment effective for a disease is sometimes not effective or associated with exacerbations of another disease. For instance, a salt-bathing treatment for BCWD could exacerbate fish vibriosis seriously, and oxolinic-acid (OXA) dosing effective for fish vibriosis is ineffective for BCWD since most *F. psychrophilum* isolates are resistant to OXA (Izumi & Aranishi 2004, Izumi, Ouchi, Kuge, Arai, Mito, Fujii, Aranishi, & Shimizu, 2007).

Therefore, in the present study, we have developed a multiplex PCR amplification technique to detect *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum* simultaneously. For the specific detection, the target of PCR primers was in the *gyrB* region of each pathogenic bacterium. The application of this multiplex PCR procedure for the direct detection of pathogenic bacteria from gill, kidney and ulcerous body surface lesion of ayu were also described.

Materials and Methods

Bacterial Isolates and DNA Extraction

Thirty-six isolates of *F. psychrophilum*, 18 isolates of *P. plecoglossicida*, 13 isolates of *V. anguillarum*, 2 isolates of other *Flavobacterium* species, 4 isolates of other *Pseudomonas* species, 24 isolates of other *Vibrio* species, 19 isolates of fish disease bacterial species, and 33 unidentified yellow pigmented isolates grown on the tryptone-yeast-extract (TYE) agar were used (Table 1). All the isolates were routinely cultured at appropriate temperatures on heart-infusion (HI) agar, Luria-Bertani (LB) ager, TYE agar, tryptic-soy (TS) agar, or TS agar supplemented with 1.5% NaCl (Table 1). Bacterial DNA was extracted according to the previously described method using a chelating resin, Chelex100 (Sigma, MO, USA) (Walsh, Metzger, &

Table 1. Bacterial isolates, growth condition, and *gyrB* nucleotide sequence accession number

Bacterial species (parentheses indicate the number of isolates)	Isolate no.	Isolated from	Isolation year	Isolation locality	Growth medium	incubation temperature	*gyrB* accession no.
Flavobacterium psychrophilum (36)	NCIMB1947*	Coho salmon	Unknown	United States	TYE	18 °C	AB326024
	FPC826	Coho salmon	1980	United States			AB326025
	FPC828	Coho salmon	1990	Miyagi, Japan			AB326026
	FPC840	Ayu	1987	Tokushima, Japan			AB326027
	FPC924	Ayu	1992	Wakayama, Japan			AB326028
	FPC931	Ayu	1993	Hiroshima, Japan			AB326029
	AA9401	Ayu	1994	Aichi, Japan			AB326030
	FPC956	Ayu	1994	Shiga, Japan			AB326031
	YMA9609	Ayu	1996	Yamanashi, Japan			AB326032
	GFA9604	Ayu	1996	Gifu, Japan			AB326033
	KNA9801	Ayu	1997	Kanagawa, Japan			AB326034
	OKA9806	Ayu	1998	Okayama, Japan			AB326035
	IP980601	Ayu	1998	Iwate, Japan			AB326036
	MG980922-1	Ayu	1998	Miyagi, Japan			AB326037
	YMA980608	Ayu	1998	Yamagata, Japan			AB326038
	FPC814	Rainbow trout	1991	Tokyo, Japan			AB326039
	FPC942	Rainbow trout	1994	Yamagata, Japan			AB326040
	YMR9615	Rainbow trout	1996	Yamanashi, Japan			AB326041
	OKR9801	Rainbow trout	1998	Okayama, Japan			AB326042
	OKR9802	Rainbow trout	1998	Okayama, Japan			AB326043
	FKR9801	Rainbow trout	1998	Fukui, Japan			AB326044
	FPC958	Amago	1994	Tottori, Japan			AB326045
	FKM9801	Amago	1998	Fukui, Japan			AB326046
	OKM9801	Amago	1998	Okayama, Japan			AB326047
	FPC945	Oikawa	1993	Hiroshima, Japan			AB326048
	OY99Oik-1	Oikawa	1999	Okayama, Japan			AB326049
	YMY9604	Yamame	1996	Yamanashi, Japan			AB326050
Pseudomonas plecoglossicida (18)	GMA4-65	Ayu	2004	Gunma, Japan	LB	25 °C	
	GMA3-30	Ayu	2003	Gunma, Japan			
	GMA3-32	Ayu	2003	Gunma, Japan			
	GMA3-35	Ayu	2003	Gunma, Japan			
	GMA3-37	Ayu	2003	Gunma, Japan			
	GMA3-38	Ayu	2003	Gunma, Japan			
	GMA3-41	Ayu	2003	Gunma, Japan			
	GMA3-45	Rainbow trout	2003	Gunma, Japan			
	GMA3-47	Rainbow trout	2003	Gunma, Japan			
	FPC941	Ayu	1994	Shiga, Japan			
	Shiga1	Ayu	1991	Shiga, Japan			
	FPC337	Ayu	1991	Tokushima, Japan			
	N95845	Ayu	1991	Nagano, Japan			
	FPC976	Ayu	1987	Wakayama, Japan			
	PE951219	Pejerrey	1991	Kanagawa, Japan			
	YS970506	Ayu	1993	Yamanashi, Japan			
	TB9602	Ayu	1992	Tochigi, Japan			
	Pj9536	Pejerrey	1991	Tochigi, Japan			
	SG941028	Ayu	1990	Shiga, Japan			
	SG950106	Ayu	1991	Shiga, Japan			
	SG950330	Ayu	1991	Shiga, Japan			
	SG960929A	Ayu	1992	Shiga, Japan			
	SG970718	Ayu	1993	Shiga, Japan			
	SG990810A	Ayu	1995	Shiga, Japan			
	SG000621	Ayu	1996	Shiga, Japan			
	SG010806	Ayu	1997	Shiga, Japan			
	SG020710	Ayu	1998	Shiga, Japan			
	ATCC 19264*	Cod	1956	Unknown			
Vibrio anguillarum (13)	GMA5-5	Ayu	2005	Gunma, Japan	HI	25 °C	
	GMA5-80	Ayu	2005	Gunma, Japan			
	GMA5-144	Rainbow trout	2005	Gunma, Japan			
	GMW-45	Rainbow trout	2006	Gunma, Japan			
	GMW-48	Rainbow trout	2006	Gunma, Japan			
	GMW-51	Whitespotted char	2006	Gunma, Japan			
	GMA5-4	Ayu	2005	Gunma, Japan			
	GMA5-81	Ayu	2005	Gunma, Japan			
	GMA5-143	Rainbow trout	2005	Gunma, Japan			
	GMW-46	Rainbow trout	2006	Gunma, Japan			
	GMW-47	Rainbow trout	2006	Gunma, Japan			
	GMW-50	Whitespotted char	2006	Gunma, Japan			
Flavobacterium columnare	IAM14301	Chinook salmon	1955	United States	TYE	18 °C	AB326053
Flavobacterium branchiophilum	ATCC35035	Yamame	1977	Gunma, Japan			AB326054
Pseudomonas aeruginosa	IAM1514	Unknown	Unknown	Unknown	LB	25 °C	

Pseudomonas anguilliseptica	FPC48	Eel	Unknown	Unknown			
Pseudomonas fluorescens	IAM12022	Pre-filter tanks	Unknown	UK			
Pseudomonas putida	FPC333	Unknown	Unknown	Unknown			
Listonella pelagia	IAM14408	Seawater	Unknown	United States			
Vibrio parahaemolyticus	NBRC12711	Boiled and dried juveniles of Japanese anchovy	Unknown	Japan			
Photobacterium damselae subsp. damselae	NBRC15633	Damselfish	Unknown	United States			
Vibrio aestuarianus	NBRC15629	Oyster	Unknown	United States			
Vibrio alginolyticus	NBRC15630	Horse mackerel	Unknown	Japan			
Vibrio campbellii	NBRC15631	Seawater	Unknown	Unknown			
Vibrio harveyi	NBRC15634	Luminescing amphipod	Unknown	United States			
Vibrio mediterranei	NBRC15635	Coastal sediment	Unknown	Spain			
Vibrio natriegens	NBRC15636	Salt marsh mud	Unknown	United States			
Vibrio orientalis	NBRC15638	Seawater	Unknown	China	TS with NaCl	25 °C	
Vibrio penaeicida	NBRC15640	Kuruma prawn	Unknown	Kagoshima, Japan			
Vibrio tubiashii	NBRC15644	Hard clams	Unknown	Unknown			
Vibrio vulnificus	NBRC15645	Human	Unknown	United States			
Vibrio ichthyoenteri	NBRC15847	Japanese flounder	Unknown	Japan			
Vibrio diazotrophicus	IAM14402	Sea urchin	Unknown	Canada			
Vibrio fluvialis	IAM14403	Human	Unknown	Bangladesh			
Vibrio gazogennes	IAM14404	Saltwater marsh	Unknown	United States			
Vibrio metschnikovii	IAM14406	Fowl	Unknown	Unknown			
Vibrio nereis	IAM14407	Seawater	Unknown	Unknown			
Vibrio proteolyticua	IAM14410	Dark beetle	Unknown	Unknown			
Vibrio haliotico	IAM14596	Abalone	Unknown	Hokkaido, Japan			
Vibrio equitatus	IAM14957	Unknown	Unknown	Unknown			
Vibrio superstes	IAM15009	Abalone	Unknown	Australia			
Vibrio ordalii	ATCC33509	Coho salmon	Unknown	United States			
Aeromonas bestiarum	GMW-35	Whitespotted char	2006	Gunma, Japan	HI		
Aeromonas hydrophila	GMW-4	Carp	2004	Gunma, Japan	HI		
Aeromonas hydrophila	GMW-12	Carp	2004	Gunma, Japan	HI		
Aeromonas hydrophila dhakensis	GMW-10	Japanese crucian carp	2004	Gunma, Japan	HI		
Aeromonas salmonicida masoucida	1-a-1	Masu salmon	Unknown	Unknown	TS		
Aeromonas salmonicida salmonicida	FPC367	Unknown	Unknown	Unknown	TS		
Aeromonas salmonicida salmonicida	GMW-31	Yamame	2006	Gunma, Japan	HI		
Aeromonas salmonicida salmonicida	GMW-33	Whitespotted char	2006	Gunma, Japan	HI	25 °C	
Aeromonas salmonicida salmonicida	GMW-38	Whitespotted char	2006	Gunma, Japan	HI		
Aeromonas sobria	GMW-20	Carp	2004	Gunma, Japan	HI		
Aeromonas sp	GMW-23	Carp	2004	Gunma, Japan	HI		
Aeromonas sp	GMW-40	Rainbow trout	2006	Gunma, Japan	HI		
Edwardsiella tarda	JCM1656	Human feces	Unknown	Unknown	LB		
Escherichia coli	IAM1239	Unknown	Unknown	Unknown	TS		
Klebsiella oxytoca	GMW-15	Carp	2004	Gunma, Japan	HI		
Pseudomonas fluorescens	GM2311	Ayu	1998	Gunma, Japan	HI		
Pseudomonas putida	GMW-37	Goldfish	2006	Gunma, Japan	HI		
Shewanella baltica	GMW-27	Topmouth gudgeon	2005	Gunma, Japan	HI		
Shewanella xiamenensis	GMW-5	Carp	2004	Gunma, Japan	HI		
unidentified yellow pigmented bacterium (33)	GMA3-49	Ayu	2003	Gunma, Japan	TYE	18 °C	AB326055
	GMA3-59	Ayu	2003	Gunma, Japan			AB326056
	GMA3-60	Ayu	2003	Gunma, Japan			AB326057
	GMA4-01	Ayu	2004	Gunma, Japan			AB326058
	GMA4-02	Chum salmon	2004	Gunma, Japan			AB326059
	GMA4-03	Chum salmon	2004	Gunma, Japan			AB326060
	GMA4-05	Japanese fluvial sculpin	2004	Gunma, Japan			AB326061
	GMA4-47	Ayu	2004	Gunma, Japan			AB326062
	GMA4-50	Ayu	2004	Gunma, Japan			AB326063
	GMA4-55	Ayu	2004	Gunma, Japan			AB326064
	GMA4-56	Ayu	2004	Gunma, Japan			AB326065

GMA4-66	Ayu	2004	Gunma, Japan	AB326066
GMA4-73	Ayu	2004	Gunma, Japan	AB326067
GMA4-76	Ayu	2004	Gunma, Japan	AB326068
GMA4-80	Rainbow trout	2004	Gunma, Japan	AB326069
GMA4-81	Rainbow trout	2004	Gunma, Japan	AB326070
GMA4-83	Ayu	2004	Gunma, Japan	AB326071
GMA4-85	Ayu	2004	Gunma, Japan	AB326072
GMA4-88	Ayu	2004	Gunma, Japan	AB326073
GMA4-89	Ayu	2004	Gunma, Japan	AB326074
GMA4-90	Ayu	2004	Gunma, Japan	AB326075
GMA4-92	Ayu	2004	Gunma, Japan	AB326076
GMA4-95	Ayu	2004	Gunma, Japan	AB326077
GMA4-118	Ayu	2004	Gunma, Japan	AB326078
GMA4-129	Ayu	2004	Gunma, Japan	AB326079
GMY-1	Ayu	2004	Gunma, Japan	AB326080
GMY-2	Ayu	2004	Gunma, Japan	AB326081
GMY-3	Ayu	2004	Gunma, Japan	AB326082
GMY-6	Carp	2004	Gunma, Japan	AB326083
GMY-13	Carp	2004	Gunma, Japan	AB326084
GMY-14	Carp	2004	Gunma, Japan	AB326085
GMY-15	Carp	2004	Gunma, Japan	AB326086
GMY-17	Japanese crucian carp	2005	Gunma, Japan	AB326087

*, type strain
TYE, tryptone-yeast-extract; HI, heart-infusion; LB, Luria-Bertani; TS, tryptic-soy; TS with NaCl, tryptic-soy supplemented with 1.5% NaCl
NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen (UK); ATCC, American Type Culture Collection, Manassas (USA); IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo (Japan); NBRC, NITE Biological Resource Center, Tokyo (Japan); JCM, Japan Collection of Microorganisms, Ibaraki (Japan); FPC, National Research Institute of Aquaculture, Mie (Japan); GM, Gunma Prefectural Fisheries Experimental Station, Gunma (Japan); SG, Shiga Prefectural Fisheries Experiment Station, Shiga (Japan).
All isolates without these abbreviations are from National Research Institute of Aquaculture. Mie (Japan).

Higuchi, 1991; Izumi & Wakabayashi 1997). Extracted DNA solutions were used as the template for PCR amplification without further purification.

Determination of *gyrB* Sequence of *Flavobacterium* and Yellow Pigmented Isolates

The *gyrB* sequences of *F. psychrophilum* isolates (n = 27), *F. columnare* isolate (n = 1), *F. branchiophilum* isolate (n = 1), and unidentified yellow bacterial isolates from diseased fishes (n = 33) were determined. A universal degenerated primer pair, FL-G1F and FL-G1R, was designed based on the deposited *gyrB* sequences of *F. aquatile* (GenBank accession number AB034225), *F. salegens* (GenBank accession number AB034227), *F. uglinosum* (GenBank accession number AB034224), *F. johnsoniae* (GenBank accession number AB034222), and *F. ferrugineum* (GenBank accession number AB048188) and used. The oligonucleotide sequences of FL-G1F and FL-G1R were GTYTCSGGNGGWCTKCACGG and CTSCCRTCNACRTCGGCATC, respectively. The PCR conditions with the primer pair were, a preheating 94 ^{o}C for 5 min, 35 cycles of amplification consisting of denaturation at 94 ^{o}C for 15 sec, annealing at 56 ^{o}C for 20 sec, and extension at 72 ^{o}C for 1 min, and a final extension at 72 ^{o}C for 5 min. The PCR amplification was performed in a total reaction volume of 10 µL with a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). The reaction mixture contained 2 µL of template DNA, 0.1 nmol of each dNTP, 10 pmol of each primer, and 0.25 unit of *Taq* DNA polymerase (Takara, Shiga, Japan). The direct sequencing of these PCR products obtained with the universal primer pair was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on a Model 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's directions.

Multiplex PCR Amplification

The specific primers for *F. psychrophilum*, Fp-GB3F and FpGB3R, were newly designed based on the determined *gyrB* nucleotide sequences of *F. psychrophilum* and unidentified yellowish bacterial isolates. The specific primers for *V. anguillarum* (Va-GBF1, Va-GBR1) and *P. plecoglossicida* (PL-G2F and PL-G2Rm) were from our previous reports (Izumi & Suzuki, 2016, Izumi *et al.*, 2007). The multiplex PCR amplification was performed using QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany). The reaction mixture contained 1 µL of template DNA, 5 µL of 2×QIAGEN Multiplex PCR Master Mix, 1 µL of Q-Solution, 2 µL of RNase-free water, and 1 µL primer mixture of 6 primers (Fp-GB3F, FpGB3R, Va-GBF1, Va-GBR1, PL-G2F, and PL-G2Rm). The primer mixture is containing each primer at 2 µM. The PCR amplification was performed in a total reaction volume of 10 µL with a GeneAmp PCR System 9700 (Applied Biosystems). The oligonucleotide sequences of the 6 primers and the multiplex PCR conditions were shown in Table 2.

Specificity of the Multiplex PCR

To evaluate the specificity of the multiplex PCR, total genomic DNA of *Flavobacterium psychrophilum* NCIMB1947, *Pseudomonas plecoglossicida* shiga1,

Vibrio anguillarum ATCC19264, and ayu kidney were prepared with PureLink DNA Extraction kit (Invitrogen, CA, USA). These DNA solutions were mixed and used as templates of the multiplex PCR. Further, 36 isolates of *F. psychrophilum*, 18 isolates of *P. plecoglossicida*, 13 isolates of *V. anguillarum* were used as positive controls. Two strains of other *Flavobacterium* species, 4 strains of other *Pseudomonas* species, 24 strains of other *Vibrio* species, 19 strains related to bacterial fish disease, and 33 strains of unidentified yellow bacteria listed in Table 1 were used as negative controls.

Sensitivity of the Multiplex PCR

F. psychrophilum NCIMB1947, *P. plecoglossicida* shiga1, and *V. anguillarum* ATCC19264, were used to estimate the sensitivity of the multiplex PCR. Total genomic DNA solutions of these strains were prepared with PureLink DNA Extraction Kit (Invitrogen) and of which the optical densities (wave length = 260 nm) were measured to calculate the weight of DNA included in the solutions. To determine the sensitivity of the multiplex PCR, a serial 10-fold dilution of extracted each bacterial DNA were used as template ranging from 10 ng to 1 fg per PCR tube.

Multiplex PCR Diagnosis of the Experimentally Infected Fish

Thirty cultured ayu (3.8 g ± 0.5) were experimentally infected by *F. psychrophilum* GMA0330, *P. plecoglossicida* shiga1, and *V. anguillarum* GMA0504 individually by immersion method. During 21 days breeding in 70 L aquariums with running water (water temperature = 16 ^{o}C), the gill, kidney and ulcerous body surface of infected ayu were aseptically removed from died ayu. The PCR templates from gill washings were prepared with Chelex100 (Sigma). Those from gill, kidney, and ulcerous body surface were prepared with PureLink DNA Extraction Kit (Invitrogen, CA, USA).

Multiplex PCR Diagnosis of the Naturally Diseased Fish

Two kinds of samples were used. The one is disordered ayu that had been caught in rivers of Gunma Prefecture, Japan. The other is dead ayu that had been cultured in a private fish farm in Gunma Prefecture. The gill washings (n = 137) and body surface lesions (n = 67) were collected from the former samples. The kidney homogenates were prepared from the latter (n = 25). The template DNAs of gill washing and body surface lesion were prepared according to our previous paper (Izumi & Wakabayashi 1997). The template DNAs of kidney were prepared with PureLink DNA Extraction Kit (Invitrogen).

Results and Discussion

The *gyrB* Sequence Determination and Primer Design

The *gyrB* nucleotide sequences of *Flavobacterium psychrophilum* isolates (n = 27), *F. columnaris* isolate (n = 1), *F. branchiophilum* isolate (n = 1), and unidentified yellow bacterial isolates (n = 33) were successfully determined. They were deposited in DDBJ/EMBL/GENBANK under the accession number listed in Table1. With degenerated primers targeting the *gyrB* of *Flavobacterium* species, FL-G1F and FL-G1R, we could amplify the *gyrB* region from the unidentified yellow isolates. This suggest that these unidentified yellow isolates were also belonging to the genus *Flavobacterium* or related bacterial genera. The molecular phylogenetical analysis of these determined sequences support this suggestion (data not shown). Based on the *gyrB* sequences determined in this study and deposited in the international DNA database, we designed novel specific primers for the *gyrB* of *F. psychrophilum*, Fp-GB3F and Fp-GB3R. The reason why we did not use the previously reported *gyrB* primers specific for *F. psychrophilum* is that the region amplified by these previous primers was actually not the *gyrB* but the *parE* region (Izumi & Wakabayashi 2000). For *Pseudomonas plecoglossicida*, previously reported primer specific for *gyrB* of this bacterium was used, but in the reverse primer, PL-G2Rm, 2 bp was added on the 5' end to adjust the annealing temperature of multiplex PCR (Izumi, Yamamoto, Suzuki, Shimizu,

Table 2. The multiplex PCR condition and oligonucleotide sequences of primers used in this study. A preheating 95 ^{o}C for 15 min, a final extension at 72 ^{o}C for 10 min and 35 cycles of denaturation, annealing and extension were included

F. psychrophilum	*P. plecoglossicida*	*V. anguillarum*	Detection from			
			Gill	Body Surface Lesion	Kidney	Total
+	+	+	0	0	0	0
+	+		0	1	0	1
+		+	0	0	1	1
	+	+	0	0	0	0
+			33	50	15	98
	+		0	0	0	0
		+	0	0	1	1
			104	16	8	128
	Total		137	67	25	229

& Aranishi, 2007).

Specificity and Sensitivity of the Multiplex PCR

Three primer pairs of Fp-GB3F/Fp-GB3R, PL-G2F/PL-G2Rm, and Va-GBF1/Va-GBR1, could amplify the expected sized PCR product (889 bp, 522 bp, and 346 bp, respectively) from 36 isolates of *F. psychrophilum*, 18 isolates of *P. plecoglossicida*, and 13 isolates of *V. anguillarum*, respectively. On the other hand, no PCR amplifications occurred from all the other strains than *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum* listed in Table 1. Using mixed DNA solutions of ayu kidney, *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum* as templates, we could confirm the amplified PCR products according to the combination of DNA mixture (Figure 1). The detection limits of the multiplex PCR were 10 fg for *F. psychrophilum* genomic DNA, 100 fg for *P. plecoglossicida* genomic DNA, and 10 fg for *V. anguillarum* genomic DNA per reaction (Figure 2). Comparing with the conventional singleplex PCR, these values of each detection limit were thought to be enough to detect these pathogenic bacteria with higher sensitivity (Suzuki, Arai, Kuge, Katagiri, & Izumi, 2008).

Diagnosis of the Experimentally Infected and Naturally Diseased Fishes

Ayu that had been challenged by *F. psychrophilum*, *P. plecoglossicida*, or *V. anguillarum* with immersion method were precisely diagnosed as BCWD, BHA, and fish vibriosis by the multiplex PCR, respectively (Figure 3). The naturally disordered ayu in the rivers and a private fish farm were also diagnosed by the multiplex PCR. Totally from 229 samples, more than half of the samples (n = 128) were negative for three infections, 98 samples were single infection of BCWD, 1 sample was infected with vibriosis, and mixed infections of BCWD/BHA (n = 1) and BCWD/vibriosis (n = 1) were also observed (Table 3). These results indicates that our multiplex PCR method is sufficiently practical and can provide more detailed epidemiological information with the same efforts as before in routine fish disease diagnosis.

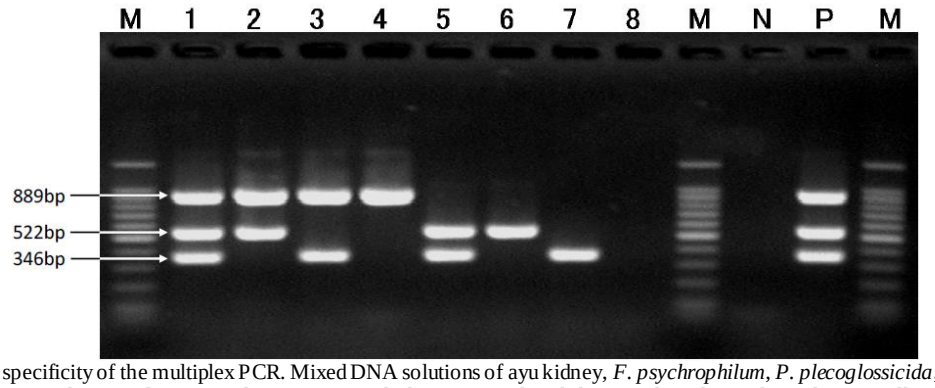

Figure 1. The specificity of the multiplex PCR. Mixed DNA solutions of ayu kidney, *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum* were used as templates. Sample no. 1 was ayu kidney, *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum*, sample no. 2 was ayu kidney, *F. psychrophilum*, and *P. plecoglossicida*, sample no. 3 was ayu kidney, *F. psychrophilum*, and *V. anguillarum*, sample no. 4was ayu kidney and *F. psychrophilum*, sample no. 5 was ayu kidney, *P. plecoglossicida*, and *V. anguillarum*, sample no. 6 was ayu kidney and *P. plecoglossicida*, sample no. 7 was ayu kidney, and *V. anguillarum*, and sample no. 8 was ayu kidney. Lane N was the negative control of no DNAs. Lane P was the positive control of mixed DNAs of *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum*. Lanes M were 100 bp DNA ladder.

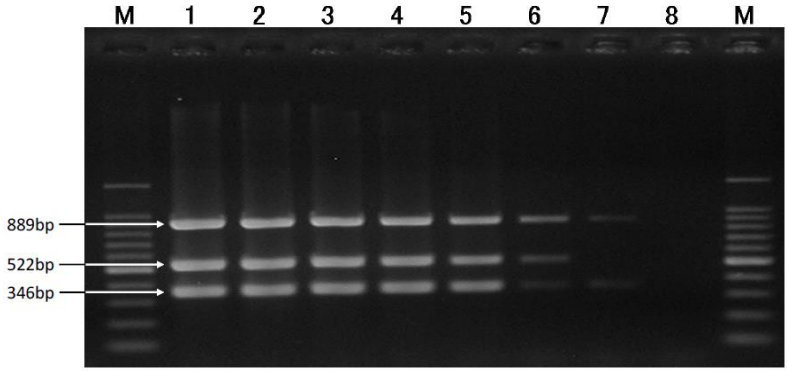

Figure 2. The sensitivity of the multiplex PCR. Various consentrations of mixed DNA solutions of *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum* were used as templates. The DNA consentration of Lanes 1, 2, 3, 4, 5, 6, 7, and 8 were 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg, respectively. Lanes M were 100 bp DNA ladder.

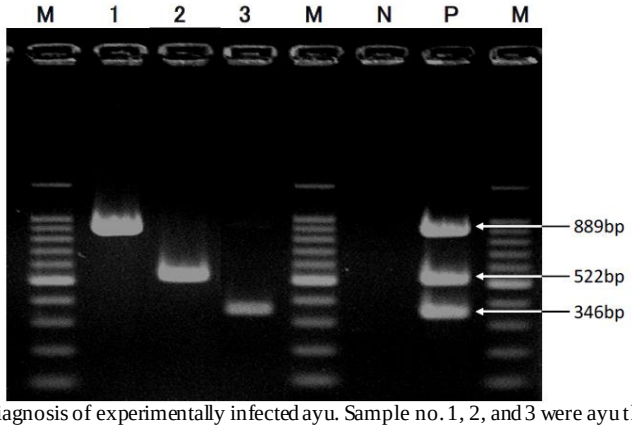

Figure 3. The multiplex PCR diagnosis of experimentally infected ayu. Sample no. 1, 2, and 3 were ayu that had been challenged by *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum*, and precisely diagnosed as BCWD, BHA, and fish vibriosis, respectively. Lane N was the negative control. Lane P was the positive control of mixed DNAs of *F. psychrophilum*, *P. plecoglossicida*, and *V. anguillarum*. Lanes M were 100 bp DNA ladder.

References

Actis, L. A., Tolmasky, M. E., & Crosa, J. H. (1999). Vibriosis. In P. T. K. Woo & D. W. Bruno (Eds.), *Fish Diseases and Disorders, Vol. 3, Viral, Bacterial and Fungal Infections*, (pp. 523–557). Wallingford, UK., CABI Publishing., 874 pp.

Ganzhorn, J. (1994). Vibriosis. In American Fisheries Society - Fish Health Section (Ed.) *Blue Book 2016 Edition, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*., Maryland, USA., American Fisheries Society Book Series. Retrieved from http://www.afs-fhs.org/perch/resources/14069220291.2.10vibriosis2014.pdf

Izumi, S., & Wakabayashi, H. (1997). Use of PCR to detect *Cytophaga psychrophila* from apparently healthy juvenile ayu and coho salmon eggs. *Fish Pathology*, 3: 169-173. http://dx.doi.org/10.3147/jsfp.32.169

Izumi, S., & Wakabayashi, H. (2000). Sequencing of *gyrB* and their application in the identification of *Flavobacterium psychrophilum* by PCR. *Fish Pathology*, 35: 93-94. http://dx.doi.org/10.3147/jsfp.35.93

Izumi, S., & Aranishi, F. (2004). Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. *Applied and Environmental Microbiology*, 70: 3968-3972. http://dx.doi.org/10.1128/AEM.70.7.3968-3972.2004

Izumi, S., Ouchi, S., Kuge, T., Arai, H., Mito, T., Fujii, H., Aranishi, F., & Shimizu, A. (2007). PCR-RFLP genotypings associated with quinolone resistance in *Flavobacterium psychrophilum*. *Journal of Fish Diseases*. 30: 141–147. http://dx.doi.org/10.1111/j.1365-2761.2007.00797.x

Izumi, S., Yamamoto, M., Suzuki, K., Shimizu, A., & Aranishi, F. (2007). Identification and detection of *Pseudomonas plecoglossicida* isolates with PCR primers targeting the *gyrB* region. *Journal of Fish Diseases*, 30: 391-397. http://dx.doi.org/10.1111/j.1365-2761.2007.00820.x

Izumi, S., & Suzuki, K. (2016). A Novel PCR detection method for major fish pathogenic bacteria of *Vibrio anguillarum*. *Turkish Journal of Fisheries and Aquatic Sciences*, 16: 571-578. http://dx.doi.org/10.4194/1303-2712-v16_3_09

Muroga, K., & Egusa, S. (1988). Vibriosis of Ayu: A Review. *Journal of the Faculty of Applied Biological Science, Hiroshima University*, 27: 1-17. http://dx.doi.org/0.15027/23482

Nishimori, E., Kita-Tsukamoto, K., & Wakabayashi, H. (2000). *Pseudomonas plecoglossicida* sp. Nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. *International Journal of Systematic and Evolutionary Microbiology*, 50: 83-89. http://dx.doi.org/10.1099/00207713-50-1-83

Sako, H., & Kusuda, R. (1978). Chemotherapeutic studies on trimethoprim against vibriosis of pond-cultured ayu. I. Microbiological evaluation of trimethoprim and sulfonamides on thecausative agnent *Vibrio anguillarum*. *Fish Pathology*, 13: 91-96. http://dx.doi.org/10.3147/jsfp.13.91

Suzuki, K., Arai, H., Kuge, T., Katagiri, T., & Izumi, S. (2008). Reliability or PCR methods for the detection of *Flavobacterium psychrophilum*. *Fish Pathology*, 43: 124-127. http://dx.doi.org/10.3147/jsfp.43.124

Wakabayashi, H., Horinouchi, M., Bunya, T., & Hoshiai, G. (1991). Outbreaks of cold-water disease in coho salmon in Japan. *Fish Pathology*, 26: 211-212. http://dx.doi.org/10.3147/jsfp.26.211

Wakabayashi, H., Sawada, K., Ninomiya, K., & Nishimori, E. (1996). Bacterial hemorrhagic ascites of ayu caused by *Pseudomonas* sp. *Fish Pathology*, 31: 239-240. http://dx.doi.org/10.3147/jsfp.31.239

Walsh, P. S., Metzger, D. A., & Higuchi, R. (1991). Chelex100 as a medium for simple extraction of DNA for PCR based typing from forensic material. *Biotechniques*, 10: 507-513.