Short communication

A Novel PCR-RFLP Genotyping of *Flavobacterium psychrophilum* Targeting the *gyrB* Region

Shotaro Izumi^{1*}, Hajime Arai², Kyuma Suzuki²
and Futoshi Aranishi³

¹School of Marine Science and Technology, Tokai University, Shimizu, Shizuoka 424-8610, Japan

²Gunma Prefectural Fisheries Experiment Station, Shikishima, Maebashi, Gunma 371-0036, Japan

³Institute of Agricultural and Life Sciences, Shimane University, Matsue, Shimane 690-8504, Japan

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ABSTRACT—A novel PCR-RFLP typing method was developed using PCR primers specific for the B subunit of DNA gyrase gene (*gyrB*) of *Flavobacterium psychrophilum* and restriction endonuclease *Bsp*119I. With this PCR-RFLP, 300 isolates of *F. psychrophilum* were classified into two genotypes (197 and 103 isolates), and the respective genotypes were correlated with the host fish species from which the isolates were derived. These isolates were further subdivided into 12 genotypes by combining with three previous PCR-RFLPs. It is suggested that this PCR-RFLP typing system can be a useful tool in epizootiological studies on bacterial cold-water disease.

Key words: *Flavobacterium psychrophilum*, PCR-RFLP, *gyrB*, bacterial cold-water disease

Flavobacterium psychrophilum is the etiologic agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome in freshwater fishes. Regarding the genotypes of *F. psychrophilum*, restriction fragment length polymorphism of PCR amplified fragments (PCR-RFLP) based on point mutations of *PPIC* gene (Izumi *et al.*, 2003; Yoshiura *et al.*, 2006), *parE* (Izumi *et al.*, 2003; Izumi and Suzuki, 2018) and *gyrA* (Izumi *et al.*, 2007) have been reported. By these 3 PCR-RFLP typings, *F. psychrophilum* isolates have been divided into genotypes A and B, genotypes R and S, and genotypes QR and QS, respectively. In this study, we developed a novel PCR-RFLP typing method of *F. psychrophilum* referred to the B subunit of DNA gyrase gene (*gyrB*) sequences, which were determined in our previous study (Izumi and Suzuki, 2018). Further, we aimed to

examine the genetic typing of *F. psychrophilum* with higher resolution by combining multiple PCR-RFLPs including the method of this study.

Of 300 *F. psychrophilum* isolates employed in this study, 199 isolates from ayu *Plecoglossus altivelis*, 69 isolates from salmonid fishes (coho salmon *Oncorhynchus kisutch*, rainbow trout *O. mykiss*, red-spotted masu salmon *O. masou ishikawae*, masu salmon *O. masou masou*, chum salmon *O. keta*, Japanese char *Salvelinus pluvius*, grayling *Thymallus thymallus*, and other unidentified salmonids), 28 isolates from cyprinid fishes (common carp *Cyprinus carpio*, gimbuna *Carassius auratus langsdorffii*, pale chub *Zacco platypus*, Japanese dace *Tribolodon hakonensis*, hon-moroko *Gnathopogon caeruleus*, and tench *Tinca tinca*), and 4 isolates from other fishes (numachichibu *Tridentiger brevispinis*, mosquitofish *Gambusia affinis*, Japanese eel *Anguilla japonica*, and largemouth bass *Micropterus salmoides*) were included (Table 1).

All isolates were cultured at 18°C on modified cytophaga agar medium (Wakabayashi and Egusa, 1974). From individual isolates, whole genomic DNA was prepared using chelating resin as described in our previous works (Izumi and Wakabayashi, 1997; Izumi *et al.*, 2003). For PCR amplification, a pair of PCR primers Fp-GB3F (5'-TAGGCGAAACGACTAAACGT-3') and Fp-GB3R (5'-GCAAAATTGCCTGAAAAGCC-3') were used for specific amplification of the partial region of *gyrB* of *F. psychrophilum* (Izumi and Suzuki, 2018). PCR amplification was carried out with a total reaction volume of 20 µL using a Techgene thermal cycler (Techne, UK). The reaction mixture contained 2 µL of template DNA, 1.6 µL of dNTP mixture (2.5 mM each), 2 µL of each primer (10 µM each), and 0.1 µL of Ex *Taq* DNA polymerase (5 units/µL, Takara, Japan). The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 20 s, and extension at 72°C for 60 s. Preheating at 94°C for 5 min and a final extension at 72°C for 5 min were included. After PCR amplification, an aliquot of PCR amplicons was electrophoresed using 1% Gene pure LE agarose gel (ISC Bio Express, USA) in TAE buffer at room temperature for 30 min. Following gel electrophoresis, the amplicons were stained with Midori Green solution (Nippon Genetics, Japan) and visualized with a blue LED transilluminator.

Prior to RFLP assay, restriction endonuclease cleavage patterns were predicted from the 27 DNA sequences determined in our previous study (accession numbers: AB326024 ~ AB326050, Izumi and Suzuki, 2018). As a result, we nominated an endonuclease of *Bsp*119I (Thermo Fisher Scientific, USA) to digest the PCR amplicons. The unpurified amplicons were digested with 0.3 µL of *Bsp*119I (10 units/µL) in a total volume of 20 µL for 2–3 h at 37°C. The digested amplicons were electrophoresed on 3% Gene pure LE agarose gel in TAE buffer at room temperature for 1 h and detected in the same manner as described above. As

* Corresponding author
E-mail: izumis@tokai-u.jp

Table 1. Relationship between PCR-RFLP genotypes of *Flavobacterium psychrophilum* and host fish species.

Host fish family	Host fish species	Number of isolates exhibiting PCR-RFLP genotypes of		Total
		C	D	
Plecoglossidae	Ayu <i>Plecoglossus altivelis</i>	183	16	199
	Coho salmon <i>Oncorhynchus kisutch</i>	0	10	10
Salmonidae	Rainbow trout <i>O. mykiss</i>	0	37	37
	Red-spotted masu salmon <i>O. masou ishikawae</i>	1	7	8
	Masu salmon <i>O. masou masou</i>	0	8	8
	Chum salmon <i>O. keta</i>	0	1	1
	Japanese char <i>Salvelinus pluvius</i>	0	1	1
	Grayling <i>Thymallus thymallus</i>	0	2	2
	unidentified salmonids	0	2	2
Cyprinidae	Common carp <i>Cyprinus carpio</i>	3	2	5
	Gin-buna <i>Carassius auratus langsdorfii</i>	2	0	2
	Pale chub <i>Zacco platypus</i>	3	11	14
	Japanese dace <i>Tribolodon hakonensis</i>	0	1	1
	Honmoroko <i>Gnathopogon caerulescens</i>	1	4	5
	Tench <i>Tinca tinca</i>	1	0	1
Gobiidae	Numachichibu <i>Tridentiger brevispinis</i>	1	0	1
Poeciliidae	Mosquitofish <i>Gambusia affinis</i>	1	0	1
Anguillidae	Japanese eel <i>Anguilla japonica</i>	0	1	1
Centrarchidae	Largemouth bass <i>Micropterus salmoides</i>	1	0	1
Total		197	103	300

Fig. 1. PCR-RFLP of *Flavobacterium psychrophilum*. Lane M, 100 bp DNA Ladder (AS ONE, Japan); Lane C, genotype C; Lane D, genotype D. Arrowheads in Lanes C and D indicate the digested fragments (473 and 409 bp) and undigested PCR product (882 bp), respectively.

predicted, two distinct cleavage patterns of genotypes C and D were obtained in RFLP analysis (Fig. 1). The cleavage pattern of genotype C was estimated to contain 473 bp and 409 bp DNA fragments. Genotype D had no cleavage site and was a single DNA fragment of 882 bp. The difference between genotypes C and D was due to a silent mutation of the codon encoding phenylalanine that was a 949th amino acid of GyrB (Fig. 2).

Using this PCR-RFLP genotyping, 300 isolates of *F. psychrophilum* could be divided into 197 isolates of genotype C and 103 isolates of genotype D (Table 1). The

Genotype C

gyrB sequence AAA **TTC** GAA
 GyrB sequence Lys⁹⁴⁸ Phe⁹⁴⁹ Glu⁹⁵⁰

Genotype D

gyrB sequence AAA **TTT** GAA
 GyrB sequence Lys⁹⁴⁸ Phe⁹⁴⁹ Glu⁹⁵⁰

Fig. 2. Relationship between nucleotide sequences of *gyrB*, amino acid sequences of GyrB, and RFLP genotypes in *Flavobacterium psychrophilum*. In genotype C, the recognition site and cutting site of *Bsp*119I are highlighted and indicated by an arrowhead, respectively.

Cramer's coefficient of association (Cramer's V) between host fish families such as Plecoglossidae, Salmonidae, Cyprinidae, Gobiidae, Poeciliidae, Anguillidae, and Centrarchidae and the PCR-RFLP genotypes of these 300 *F. psychrophilum* isolates was calculated to be 0.82. On the other hand, the localities of isolation were classified into seven regions consisting of six Japanese regions of Tohoku, Kanto, Chubu, Kansai, Chugoku, and Kyushu-Shikoku, and other than Japan*. The Cramer's V between localities of isolation and the

* The Tohoku region includes Aomori, Iwate, Miyagi, and Yamagata prefectures. The Kanto region includes Gunma, Tochigi, Tokyo, and Kanagawa prefectures. The Chubu region includes Fukui, Nagano, Gifu, Yamanashi, and Aichi prefectures. The Kinki region includes Shiga, Kyoto, Hyogo, Osaka, and Wakayama prefectures. The Chugoku region includes Tottori, Yamaguchi, Okayama, and Hiroshima prefectures. Kyushu-Shikoku region includes Tokushima and Kumamoto prefectures.

Table 2. Relationship between combined PCR-RFLP genotypes of *Flavobacterium psychrophilum* and host fish species.

Host fish family	Host fish species	Number of isolates exhibiting PCR-RFLPs genotypes of												Total
		A/R/Q/R/C ^{*2}	A/R/Q/R/D	A/R/Q/S/C	A/R/Q/S/D	A/S/Q/R/C	A/S/Q/R/D	A/S/Q/S/C	A/S/Q/S/D	B/R/Q/R/C	B/R/Q/R/D	B/R/Q/S/C	B/R/Q/S/D	
Plecoglossidae	Ayu <i>Plecoglossus altivelis</i>	5	17	132	12	3				2		16	1	199
Salmonidae	Coho salmon <i>Oncorhynchus kisutch</i>										5		5	10
	Rainbow trout <i>O. mykiss</i>								5		30		2	37
	Red-spotted masu salmon <i>O. masou ishikawae</i>										1		1	8
	Masu salmon <i>O. masou masou</i>										1		7	8
	Chum salmon <i>O. keta</i>												1	1
	Japanese char <i>Salvelinus pluvius</i>												1	1
	Grayling <i>Thymallus thymallus</i>										1		1	2
	unidentified salmonids										1		1	2
Cyprinidae	Common carp <i>Cyprinus carpio</i>												3	5
	Gin-buna <i>Carassius auratus langsdorffii</i>												2	2
	Pale chub <i>Zacco platypus</i>											1	3	14
	Japanese dace <i>Tribolodon hakonensis</i>												1	1
	Honmoroko <i>Gnathopogon caeruleus</i>												1	5
	Tench <i>Tinca tinca</i>												1	1
Gobiidae	Numachichibu <i>Tridentiger brevispinis</i>	1												1
Poeciliidae	Mosquitofish <i>Gambusia affinis</i>	1												1
Anguillidae	Japanese eel <i>Anguilla japonica</i>													1
Centrarchidae	Largemouth bass <i>Micropterus salmoides</i>				1									1
Total		7	ND ^{*3}	17	ND	133	12	3	ND	ND	5	2	39	300

*2: "A/R/Q/R/C" representing the genotypes A (PPIC gene), R (*parE*), QR (*gyrA*), and C (*gyrB*).

*3: not detected.

PCR-RFLP genotypes was calculated to be 0.57. The PCR-RFLP genotyping system developed in this study was thus suggested to have strong relationships with the host fish species. In addition, combined with the three previously reported PCR-RFLPs (Izumi *et al.*, 2003; Yoshiura *et al.*, 2006; Izumi *et al.*, 2007), these 300 isolates could be further subdivided into 12 genotypes. Of the 16 possible PCR-RFLP genotypes, four genotypes of A/R/Q/R/D, A/R/Q/S/D, A/S/Q/S/D, and B/R/Q/R/C were not observed among the isolates used in this study (Table 2).

Many typing methods such as multilocus sequence typing (Fujiwara-Nagata *et al.*, 2013; Nilsen *et al.*, 2014) and whole genome RFLP by pulsed-field gel electrophoresis (Arai *et al.*, 2007) were also developed and used for epizootiological studies and population structure analysis of *F. psychrophilum*. These methods allow detailed typing but require special equipment or incur a high cost when process a large number of samples. In contrast, PCR-RFLP typings need only the equipment and technology already used for the detection and identification of the causative agents of fish infectious diseases extensively at the prefectural fishery experimental stations in Japan. Further, since the combination of four PCR-RFLPs including this PCR-RFLP theoretically types *F. psychrophilum* isolates into 16 genotypes, the PCR-RFLP typing system provided in this study can be a useful tool in infectious epizootiological studies of BCWD.

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