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Prevalence of *Flavobacterium psychrophilum* Infection in Ayu (*Plecoglossus altivelis*) in Gunma Prefecture, Japan and Comparison of the *gyr B* Sequences of Isolates

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ABSTRACT

The prevalence of cold-water disease (CWD) in ayu (*Plecoglossus altivelis*) caused by *Flavobacterium psychrophilum* was investigated in 3 different sites along the Tone River in Gunma Prefecture, Japan. *F. psychrophilum* was isolated from 7 of 8 fishes from site A (88%), 15 of 101 from site B (15%), and 3 of 8 from site C (38%). The gene encoding DNA gyrase subunit B (*gyrB*) of *F. psychrophilum* in 16 isolates was partially sequenced. Among the 16 strains, 15 strains had gene sequences that completely matched those of a species type strain (NCIMB1947^T). Another strain confirmed one nucleotide substitution, however, the codon resulting from this substitution was synonymous with that in the type strain sequence. The results suggested that CWD was widespread in the Tone River of Gunma Prefecture, with little genetic divergence from the species type strain.

Key words: ayu, *Flavobacterium psychrophilum*, *gyr B*, sequence analysis

INTRODUCTION

Ayu (*Plecoglossus altivelis*) which belongs to family *Osmeridae* and genus *Plecoglossus*, is a highly popular food and game fish in Japan. *Flavobacterium psychrophilum* causes cold-water disease (CWD) in various fish including ayu, coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*Oncorhynchus mykiss*) (Wakabayashi *et al*., 1991; Holte *et al*., 1993; Lorenzen *et al*., 1997; Kondo *et al*., 2001; Mata *et al*., 2002; Madetoja and Wiklund, 2002). After *F. psychrophilum* was first isolated from ayu in Japan with CWD in 1987, the organism has rapidly spread (Wakabayashi *et al*., 1991). Outbreaks of CWD caused by *F. psychrophilum* killed not only a large number of fries but also adult fishes, making the disease a serious problem for the fishing industry in several countries (Lorenzen *et al*., 1997; Madetoja *et al*., 2002; Michel and Garcia, 2003), including Gunma Prefecture, Japan. However, much of the epidemiology of CWD and *F. psychrophilum* remains poorly understood.

F. psychrophilum can be detected and analyzed using culture methods, Polymerase Chain Reaction -Restriction Fragment Length Polymorphism (PCR-RFLP) analysis, and type-specific PCR methods (Toyama *et al*., 1994; Izumi and Wakabayashi, 1997; Nilsson and Strom, 2002;

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Izumi *et al*., 2003). Previous report showed DNA gyrase subunit B (*gyrB*) gene had relatively genetic diversity using PCR-RFLP, suggesting that this gene is a useful tool for molecular epidemiologic analysis of *F. psychrophilum* (Izumi *et al*., 2003). Thus, the sequence analysis of *gyrB* gene in isolates and the prevalence of CWD caused by *F. psychrophilum* in ayu in Gunma Prefecture, Japan were investigated.

MATERIALS AND METHODS

A total of 117 ayu samples were collected from 3 sites along the Tone River in Gunma Prefecture, Japan, between May and June 2002. The geographic locations of the 3 sites are shown in Figure1. Dead or dying ayu fishes having focal ulceration of muscle, bleeding over areas of the body surface, and gill pallor were collected. Ayu suspected to have CWD were stored at 4°C and analyzed within 8 h after obtaining from the river.

Isolation and identification of *F. psychrophilum* were carried out. Ulcerated areas were dissected from muscle, gill, and kidney, and inoculated in a modified cytophaga agar (MCA; liter, 13.0 g agar, 10.0 g tryptone, 2.5 g beef extract, 2.0 g yeast extract, 0.2 g CH_3COONa, 0.2 g $CaCl_2$ - $7H_2O$, and 0.2 g $MgSo_4$- $7H_2O$, pH 7.2) (Wakabayashi and Egusa, 1974). MCA inoculated with gill specimens was incubated for 7 days at 10° C. The MCA inoculated with other specimens was incubated for 5 days at 18°C. Isolates grown in MCA were identified using an antiserum slide agglutination method with a rabbit antiserum and 2 kinds of PCR methods targeting 16S ribosomal RNA gene and *gyrB* gene from *F. psychrophilum.* The antiserum was obtained from the Japan Fisheries Resource Conservation Association (Tokyo, Japan). The primers of PCR method targeting 16S ribosomal RNA gene were 5'-CGATCCTACTTGCGTAG-3' and 5'-GTTGGCATCAACACACT-3', producing a theoretical amplicon of 1,089 nucleotides (Toyama *et al*., 1994), and the primers targeting *gyrB* gene were 5'-TGCAGGAAATCTTACACTCG-3' and 5'-GTTGCAATTACAATGTTGT-3', producing

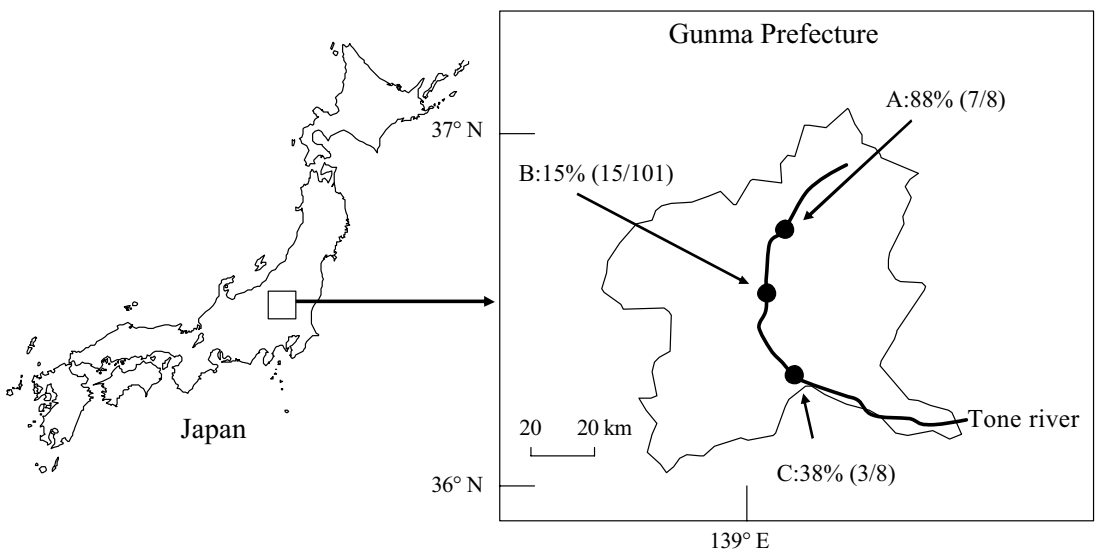

Figure 1 Locations of sampling site within Gunma Prefecture and prevalence of *Flavobacterium psychrophilum* among dead or dying ayu having typical signs of cold-water disease.

the amplicon of 1,017 nucleotides(Izumi and Wakabayashi, 1997). After identification of the isolates as *F. psychrophilum*, the *gyrB* in 16 *F. psychrophilum* isolates was partially sequenced (Table 1. As shown in Table 2, new primers were designed and used to amplify a portion of the *gyrB* gene from *F. psychrophilum* (theoretical size, 400nt). The PCR reaction was performed using PCR Master Mix (Promega, Madison, WI) following the condition: 94°C, 5min; 30 cycles of 94°C, 30 sec, 51°C, 90 sec, and 72°C, 2 min; an additional 5 min at 72°C in the last cycle. Amplicons were electrophoresed on a 1.5% agarose gel. After purification of DNA fragments with a QIAquick PCR purification kit (Qiagen, Germany), the nucleotide sequence was determined using an automated DNA sequencer (ABI 310 DNA sequencer, Applied Biosystems, USA) and a Dye

Table 1 *F. psychrophilum* isolates in Gunma Prefecture and compaired strains in this study.

Isolate[a]/Strain	Source		No.of *gyrB* sequence
	Host fish[b]	Locality	
G02-02,G02-03,G02-04, G2-05	Ayu	Gunma,Japan(site A)	AB111950[c]
G02-01	Ayu	Gunma,Japan(site B)	AB111949
G02-06, G02-07, G02-08, G02-09 G02-10, G02-11, G02-12, G02-13, G02-14, G02-15, G02-16	Ayu	Gunma,Japan(site B)	AB111950[c]
	Ayu	Gunma,Japan(site C)	AB111950[c]
NCIMB 1947[T]	Coho salmon	Washington,U.S.A	AB034732
FPC840	Ayu	Tokushima,Japan	AB012860
FPC817	Coho salmon	Miyagi, Japan	AB034733
FPC814	Rainbow trout	Tokyo,Japan	AB034740
FPC945	Oikawa	Hiroshima,Japan	AB034745
FPC956	Ayu	Shiga,Japan	AB034736
OKA9805	Ayu	Okayama,Japan	AB034737
OKR9802	Rainbow trout	Okayama,Japan	AB034741
TG-P01/88	Rainbow trout	Brittany,France	AB034739

[a] 16 strains(G02-01-G02-16) were sequenced.

[b] The scientific names of fishes are *Oncorhynchus kisutch* for coho salmon, *O. mykiss* for rainbow trout, *Peoglossus altivelis* for ayu and *Zacco platipus* for oikawa.

[c] The *gyrB* sequences of G02-02 and the other 15 isolates are sharing the accession number of AB111950

[T] The type strain of the species.

Table 2 Nucleotide sequences of primers used.

Primer	Position[1/]	Nucleotide sequences (5'-3')
Fpsy-F	611-630	GAACCCGTTTTCGAAAGTCA
Fpsy-R	1010-991	TACCACGCAAGCTAAACACG

[1/] Positions indicated represent the *gyrB* gene sequences of *Flavobacterium psychrophilum* NCIMB1947[T] (GenBank accession number AB034732).

Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) (Kojima *et al*., 2002). DNA sequences of *gyrB* were analyzed phylogenetically using CLUSTAL W program on the DNA databese of Japan(DDBJ) (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) and TreeView program. The *gyrB* sequences of *F. psychrophilum* species type strain NCIMB1947^{T} (GenBank accession no. AB034732) , FPC840 (AB012860), FPC817 (GenBank accession no. AB034733), FPC956 (AB034736), OKA9805 (AB034737), FPC945 (AB034745), FPC814 (AB034740), TG-P01/88 (AB034739), and OKR9802 (AB034741) and other species of *Flavobacterium* such as *F. aquatile* (AB034225), *F. ferrugineum*(AB073076), *F. johnsoniae*(AB034222), *F. salegens*(AB034227), and *F. uglinosum*(AB034224) previously accessed on GenBank were determined simultaneously. Evolutionary distances were estimated using Kimura's two-parameter method (Kimura, 1980) and phylogenetic trees were constructed using neighbor-joining(N-J) method selecting *F. aquatile* as the outgroup. Reliability of the tree was estimated using 1,000 botstrap replications.

RESULTS AND DISCUSSION

As shown in Figure 1, *F. psychrophilum* was isolated and identified from 7 of 8 fishes from site A (88%), 15 of 101 fishes from site B (15%), and 3 of 8 fishes from site C (38%). Thus, CWD caused by *F. psychrophilum* appeared to be widespread in the Tone River of Gunma Prefecture, Japan from May to June, 2002. All isolates were only obtained from kidney specimens.

A portion of *gyrB* gene was successfully sequenced in 16 *F. psychrophilum* isolates and *F. psychrophilum* species type strain NCIMB1947^{T} (GenBank accession no. AB034732) and strain FPC840 (accession no. AB012860). No insertions or deletions were observed; indeed, 15 strains had partial *gyrB* sequences (400 nt) completely matching with those of a species type strain (NCIMB1947 ^{T}) and strain FPC840. Only one nucleotide substitution (position 773; A to C) was found in strain G02-01. However, the codon resulting from this substitution was synonymous with that in the NCIMB1947 ^{T} and FPC840 strain sequences. In contrast, 9 nucleotide substitutions and 8 amino acids substitutions were detected among 8 *gyrB* sequence of *F. psychrophilum* strains accessed on GenBank (Table 3). All *F. psychrophilum* strains had 99.8 to 100% nucleotide sequence homology and 99.3 to 100% amino acid sequence homology (Table 3). Phylogenetic trees constructed by N-J method in the strains and *Flavobacterium* bacteria are shown in Figure 2. In the rooted tree, about 10% of genetic diversity could be seen in the *rpoB* gene among all strains, and these strains were divided into two clusters, I and II. *F. aquatile*, *F. johnsoniae*, *F. uglinosum*, *F.ferrugineum*, and *F. salegens* belong to cluster I, while strains of *F. psychrophilum* belong to cluster II. Only 1% genetic diversity was seen within cluster II. Thus, our strains were genetically closely related to NCIMB1947 ^{T}, although comparing only partially sequences of *gyrB* genes (Table 3, Figure 2).

At three sampling points, *F. psychrophilum* was detected in 15% to 88% of dead or dying ayu having typical signs of CWD. The results suggested that CWD caused by *F. psychrophilum* was widespread in the Tone River of Gunma Prefecture, Japan. CWD caused by *F. psychrophilum* in ayu was recognized in 1987, and the pathogen has rapidly spread in Japan (Wakabayashi *et al*., 1991). In 1994, CWD was found in small populations of ayu in Gunma prefecture, but *F. psychrophilum* was not isolated and identified at that time. Subsequently, a large number of ayu were killed by CWD and *F. psychrophilum* was detected in that outbreak (Nakano *et al*., 2001). The results are consistent with those described in an earlier report (Wakabayashi *et al*., 1991). However, the reason for the recent increasing of CWD remains unknown, therefore, additional studies are required.

F. psychrophilum was isolated from kidney tissue only. A recent study using *in situ* hybridization demonstrated that *F. psychrophilum* can infect and be detected in various tissues from ayu including gill, heart, and muscle as well as kidney (Liu *et al*., 2001). An agar plate cultivation method was used to isolate *F. psychrophilum*. Thus, one possible difference in this detection failure in exstrarenal organs could be a low sensitivity of agar cultivation comparing to *in situ* hybridization. Further studies should be performed to detect the *gyrB* gene of *F. psychrophilum* in various tissues from ayu with lesions of CWD.

The *gyrB* gene encodes the subunit B protein of DNA gyrase; variation in this subunit has been used for taxonomic classification of various bacteria (Fukushima *et al*., 2002). Sequences of the *gyr B* genes imply that the rate of molecular evolution is greater than that as determined by 16S rRNA sequences (Fukushima *et al*., 2002; Yamamoto and Harayama, 1998). Izumi *et al.* (2003) analyzed *gyrB* genes from various fishes including ayu, coho salmon, rainbow trout using PCR-RFLP method. They demonstrated that restricting fragments gave 4 electrophoresed patterns on agarose gel and the fragments patterns from ayu differed from the type strain (NCIMB1947^T) (Izumi *et al*., 2003). According to the report by Izumi *et al.*(2003) the type strain was type S and type R had one nucleotide mutation (accession no. AB034732, type strain position 163, C to T). Another sequence position (position 611-1010) was used. In this study, part of the gyrB was sequenced and compared to nucleotides and amino acids sequences of the gene. The gyrB and amino acids sequences of these isolates were genetically similar to the gene of *F. psychrophilus* NCIMB1947^T. The results suggested that in

Table 3 Nucleotide divergences and the supposed amino acid changes found in *gyrB* of *F. psychrophilum* isolates and strains.

Isolate/Strain	Nucleotide(amino acid) divergence				
	Position[a]			Divergence[b]	
G02-02(14)[c]			None[d]		
G02-01	C 773	(Arg)		A	(Arg)
FPC814	G 797	(Lys)		A	(Glu)
FPC840			None[d]		
FPC817	A 808	(Leu)		G	(Leu)
FPC945	T 840	(Ala)		C	(Val)
FPC956	A 933	(Ser)		C	(Tyr)
OKA9805	G 902	(Arg)		A	(Ala)
	C 1046	(Ser)		T	(Pro)
	C 1086	(Phe)		T	(Ser)
OKR9802	T 831	(Pro)		C	(Leu)
TG-P01/88	T 834	(Glu)		A	(Asp)

[a] Nucleotide and the position in *gyrB* were according to those of NCIMB 1947^T(AB034732). Supposed amino acid was enclosed in parenthesis.

[b] Nucleotide and supposed amino acid changes in position.

[c] The number in parenthesis is the number of *F. psychrophilum* isolates which have identical *gyrB* sequences with G02-02.

[d] No nucleotide change was detected.

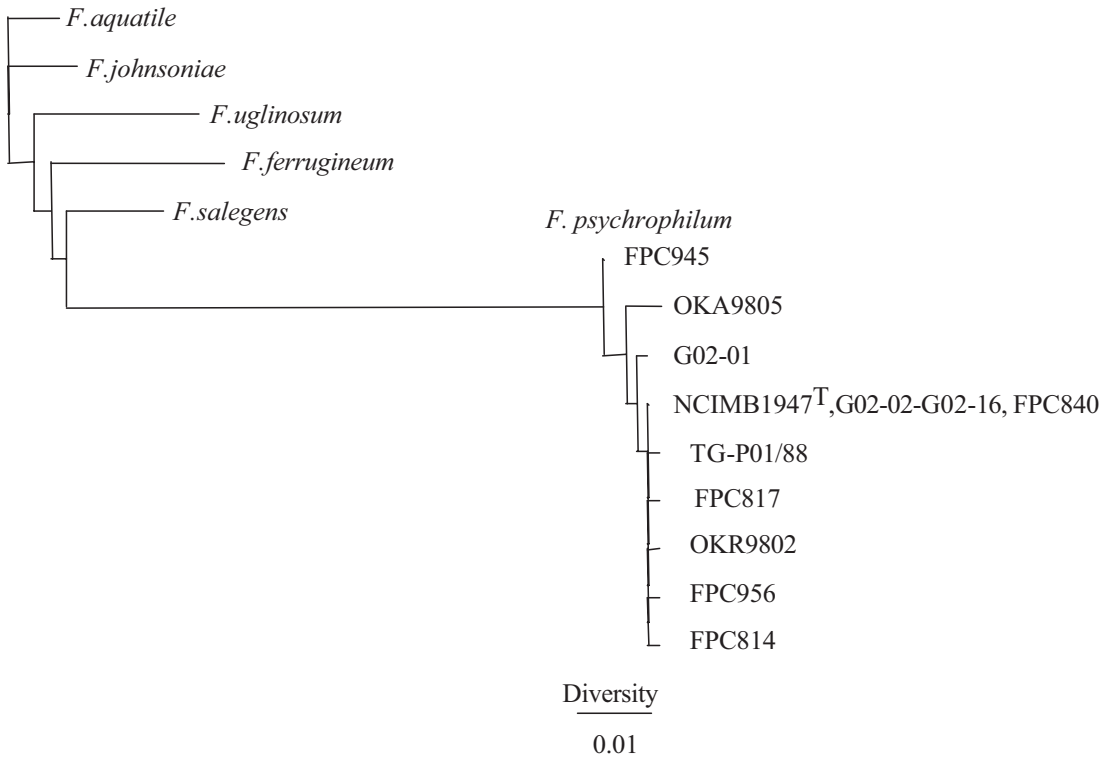

Figure 2 Phylogenetic rooted tree of the *gyrB* gene in *Flavobacterium* spp. constructed by the neighbor-joining method.

Gunma Prefecture, ayu with CWD might be infected with *F. psychrophilum* which was genetically related to NCIMB1947^T. The species type strain, NCIMB1947 ^{T}, was isolated from coho salmon in the state of Washington, USA (Borg, 1960). Together with the preliminary results, this close identity suggested common genetic characteristics between the strains acquired and this USA type strain, which may represent a major pathogen of CWD in ayu in Gunma Prefecture. Better understanding of the epidemiology of CWD and *F. psychrophilum* will require further molecular epidemiologic studies.

CONCLUSION

Flavobacterium psychrophilum causing cold-water disease (CWD) in various fishes including ayu (*Plecoglossus altivelis*) was isolated in 3 different sites (site A, B and C) along the Tone River in Gunma Prefecture, Japan. The prevalence of *F. psychrophilum* was 88%(7/8) in site A, 15%(15/101) in site B, and 38%(3/8) in site C. Sequence analysis of *gyr B* gene in 16 isolates of *F. psychrophilum* was investigated and compared to the species type strain (NCIMB1947^T). It was found that 15 strains had gene sequences completely matched the species type strain. The result suggested that CWD was widespread in the Tone River of Gunma Prefecture, with little genetic divergence from the species type strain.

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