Multilocus sequence analysis of the genus *Aliivibrio*: Identification and phylogeny of *Aliivibrio* species isolated from cultured walleye pollock (*Gadus chalcogrammus*) in Korea

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We performed MLSA (multilocus sequence analysis) and phenotypic characterization of *Aliivibrio* species isolated from walleye pollock (*Gadus chalcogrammus*) maintained in 3 different facilities of Gangwon Province, the east coast of Korea. Of 38 *Aliivibrio* species identified by 16S rDNA sequences, 12 strains were randomly selected and MLSA was conducted with 5 house-keeping genes (*gap*A, *gyr*B, *pyr*H, *rec*A and *rpo*A) and 16S rDNA gene. Phylogenetic analysis and homology of the concatenated sequences (4,580 bp) with other *Vibrionaceae* genera revealed that 4 strains (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1) were identified as *Aliivibrio logei* and one strain (GSGc16.1) as *A. wodanis*. One strain (GSGc17.1) was tentatively identified as *A. logei*, but needs further analysis because it did not belong to the same clade with *A. logei* type strain. 6 strains (GSGc17.2, GNGc16.2, GSGc16.2, GSGc17.3, GSGc18.2, GSGc17.4) need further investigation as potential novel species. Either phenotypic characterization or 16S rDNA sequence alone did not provide enough information for identification of *Aliivibrio* strains at the species level. *A. logei* and *A. wodanis* are generally known as non-pathogenic bacteria, but also known as opportunistic or secondary pathogens of cold water fishes. Cares should be taken to prevent potential outbreaks due to these bacteria, although there was no outbreaks during the sampling period.

Key words: *Aliivibrio*, *Vibrionaceae*, MLSA, Multilocus sequence analysis, Walleye pollock, *Gadus chalcogrammus*.

Introduction

The family *Vibrionaceae* consists of a large number of ecologically diverse species and is widely distributed in the aquatic environment. They are either ubiquitous inhabitants of aquatic environments or symbiotic with marine life, but some of them are pathogenic to other organisms (Thompson et al., 2005; Soto and Nishiguchi, 2014). As of 2017, the family *Vibrionaceae* is classified into 8 genera:

Vibrio, *Photobacterium*, *Catenococcus*, *Grimontia*, *Echinimonas*, *Salinivibrio*, *Enterovibrio* and *Aliivibrio* (Doi et al., 2017).

The genus *Aliivibrio* was established with the species previously belonged to the genera *Photobacterium* and *Vibrio*, based on phylogenetic and phenotypic differences from them (Urbanczyk et al. 2007), and currently comprises 6 species: *Aliivibrio fischeri* (Beijerinck, 1889), *Aliivibrio logei* (Bang et al., 1978), *Aliivibrio salmonicida* (Egidius et al., 1986), *Aliivibrio wodanis* (Lunder et al., 2000), *Aliivibrio finisterrensis* (Beaz-Hidalgo et al., 2010) and *Aliivibrio sifiae* (Yoshizawa et al., 2010). Within the genus

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Aliivibrio, 3 species (*A. fischeri, A. logei, A. sifiae*) are known for bioluminescence (Dunlap et al., 2007; Kaeding et al., 2007; Yoshizawa et al., 2010). On the other hand, *A. salmonicida* and *A. wodanis* are known to cause cold water vibriosis (CV), Hitra disease and winter ulcer in cold water fish species such as Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) (Egidius et al., 1986; Lunder et al., 2000; Khider et al., 2018). Moreover, the bioluminescetic *A. logei* is also known as a potential pathogen of cod larvae (Reid et al., 2009). Therefore, clear identification of the genus *Aliivibrio* to the species level is important for precise diagnosis and appropriate control strategies in aquaculture farms.

In general, identification of *Aliivibrio* species can be attempted by amplification of 16S DNA fragment. However, it is difficult to identify them with a single 16S DNA gene, because the homology of this gene among the species in the family *Vibrionaceae* is very high (Urbanczyk et al., 2007). Recent advances in genetic analysis technology have enabled genus *Alivibrio* to be distinguished from other *Vibrionaceae* genera at the genome-level, but due to the presence of various phenotypes in the genus *Aliivibrio*, the clear distinction among species is still difficult (Ast et al., 2009).

Various methods have been applied to clearly identify Vibrios to the species level, and in particular, identification using MLSA (Multilocus Sequence Analysis) has been actively used for Vibrios taxonomy (Sawabe et al., 2007; Thompson et al., 2009). MLSA is a method for delineating bacterial species within a genus and obtaining a higher resolution of the phylogenetic relationships among species or genera, by combining 16S rDNA sequences commonly used for bacterial identification with a number of housekeeping gene sequences (Glaeser and Kämpfer, 2015). Several MLSA schemes have been reported for identification and phylogenetic relationships of bacterial genera and species within genera (e.g., genus *Aeromonas*, genus *Edwardsiella*, genus *Photobacte-*

rium and genus *Vibrio)* (Cano-Gomez et al., 2011; Abayneh et al., 2012; Labella et al., 2018; Wamala et al., 2018).

Walleye pollock (*Gadus chalcogrammus*) is one of the most commercially valuable fish species in Korea. The annual catch of walleye pollock has been drastically decreased since 2000s, and not fully recovered yet (Kang and Kim, 2015). Recently artificial propagation of walleye pollock was succeeded (Seo and Kwon, 2017), but still there is insufficient information available for successful recovery for walleye pollock resources in Korea. In general, mortalities due to infection of pathogens are thought to be one of the main obstacles against successful mass production of fish fry. Thus, it is necessary to investigate potential pathogens in walleye pollock. Currently, several pathogens such as *Ichthyophonus*, viral hemorrhagic septicemia virus (VHSV), nervous necrosis virus (NNV) and *Vibrio anguillarum* have been reported in walleye pollock (Meyers et al., 1999; White et al., 2014; Jeon et al., 2016; Nam et al., 2017; Seo et al., 2018), but no mass mortality associated with these pathogens has been reported yet.

During the routine monitoring of bacterial diseases in artificially produced walleye pollock from aquaculture facilities in Gangwon Province, the east coast of Korea, bacterial strains suspicious of the genus *Aliivibrio* were constantly obtained. The presence of *Aliivibrio* species in seawater was reported in Korea (Suh et al., 2015), but not identified at the species level. In this study, we identified the genus *Aliivibrio* isolated from walleye pollock at the species level by MLSA, together with phenotypic characteristics.

Materials and methods

Sampling
A total of 191 fish (average total length 27.9 cm, average body weight 220.2 g) were randomly collected from 3 different locations (A, B, C) during February 2016 and November 2018 (Table 1). The

Table 1. Sampling information of walleye pollock (*Gadus chalcogrammus*)

Date (month)	facility A	facility B	facility C
2016.02.	18	-	-
03	14	4	7
04	16	4	6
05	15	3	6
07	12	5	7
08	6	-	-
09	6	-	-
10	6	-	-
11	6	-	-
12	6	-	-
2017.01	4	-	-
03	4	-	-
04	2	-	-
05	2	-	-
06	2	-	-
07	2	-	-
10	1	-	-
12	7	-	-
2018.02	3	-	-
04	4	-	-
06	6	-	-
11	7	-	-
Total number of fish	149	16	26
Average T/L[a](cm)	29.8	25.2	25.0
Average B/W[b](g)	262.6	158.5	137.0

a: total length, b: body weight

aquaculture facility A used FRP round tank filled with deep sea water and surface water to maintain the water temperature constant (approximately 10℃). The facility B used marine netpen cages and the water temperature was fluctuated seasonally (8-20℃). The facility C used FRP tank, but the water temperature was fluctuated during sampling period (10-20℃). The fish in the facility B and C were maintained until June and July, due to the high water temperature in summer. We also collected seawater flowing into the fish tank of the facility C.

Isolation of bacteria

The fish samples were taken to the laboratory im-mediately after sampling, and euthanized by an over-dose of anesthetics (2-phenoxyethanol, Sigma, USA). After measuring the body size of the samples, the presence of any external abnormal signs were checked with naked eyes. Then, the abdominal cavity of the fish was opened and the internal organs were examined. The sterilized loop were deeply stabbed into the spleen and kidneys to plate on TSA medium (Trypticase soybean agar, Difco, USA) supplemented with 1.5% NaCl and the plate cultured at 20℃ for 24 to 48 h. Approximately 50 mg of the upper part of the small intestine was also cut and homogenized with 450 μl of PBS. Then they were diluted to 10^2 folds and plated on TSA medium as above. The most dominant colonies were transferred to TCBS medium (Thiosulfate citrate bile salts sucrose, Difco, USA) and incubated at 20℃ for 24-48 h. 200 μl of filtered seawater with a membrane filter (pore size 5.0 μm, Advantec, Japan) was applied to the TSA medium, and then bacterial isolation was attempted as above.

Genomic DNA extraction

All of the 124 colonies appeared in TCBS medium were cultured in TSB medium (Trypticase soybean broth, Difco, USA) supplemented with 1.5% NaCl at 20℃ for 24 h. The cultured bacteria were pelleted by centrifugation at 1,764 ×*g* for 10 min, washed 2-3 times with PBS, and centrifuged again under the same conditions. After that, genomic DNA was extracted according to the QIAamp DNA mini kit (Qiagen, Germany). 180 μl of ATL buffer and 20 μl of Proteinase K (10 mg / ml) were added to the bacterial pellet and lysed at 60℃. And 200 μl of AL buffer was added and reacted at 70℃ for 10 min. The DNA was precipitated by adding 200 μl of ethanol, transferred to a column tube, washed at 5,018 ×*g*, and then 200 μl of AE buffer was added to extract the genomic DNA.

PCR amplification and phylogenetic analysis using MLSA

Conventional PCR was conducted with 124 bacterial strains, using 16S rDNA primer set (Table 2). The amplified PCR product was electrophoresed using 1.5% agarose gel and the target band was confirmed under a UV transilluminator. The target band was purified using gel purification kit (Bioneer, Korea), and sequenced with ABI Prism 3730 XL DNA analyzer (PE applied biosystems, USA). The obtained 16S rDNA sequences were compared with those of the family *Vibrionaceae* in the GenBank by Blast search (NCBI, USA). Of the *Aliivibrio* species identified by 16S rDNA sequences, 12 strains (8 in A, 2 in B, 2 in C) were randomly selected for further MLSA. Additional primer sets were selected for MLSA : DNA gyrase subunit B (*gyr*B), glyceraldehyde-3-phosphate dehydrogenase (*gap*A), uridylate kinase (*pyr*H), recombinase A (*rec*A) and RNA polymerase alpha subunit (*rpo*A) genes. Information on all the primer sets and PCR conditions are shown in Table 2. The PCR products were sequenced by the same method as above.

Multiple alignment was performed with the reference sequences of the family *Vibrionaceae* (Ast et al., 2009; Yoshizawa et al., 2010), to analyze systematic information of the genus *Aliivibrio* bacteria isolated from walleye pollock in this study. We removed all gaps and missing sequences among multiple sequences. Neighbor joining tree (Saitou and Nei, 1987) was constructed using the Kimura two-parameter model of the MEGA 7 program (Kimura, 1980; Kumar et al., 2016), and the bootstrap values were based on 1,000 times.

Phenotypic characterization

Biochemical and physiological characteristics of the 12 strains used for MLSA were also investigated. API 20E and API ZYM test (Biomerieux, France) were conducted for biochemical characterization. Conventional gram staining and catalase test were also conducted. In addition, these strains were cultured in TSB (Trypticase soybean broth, Difco, USA) medium to observe growth by different salinity condition (0.5, 5, 7% NaCl) and different temperature condition (4, 10, 22, 25, 30℃). The results was compared with those of other known genera in the family *Vibrionaceae*.

Results and Discussion

A total of 38 strains were identified as *Aliivibrio* species by 16S rDNA sequencing of 124 strains produced in TCBS medium. They showed 99.4-100.0%

Table 2. Oligonucleotide primers used for PCR identification of isolated bacteria

Target	Primers	Sequence(5′-3′)	Condition	References
16S rDNA	fD2 rP1	AGAGTTTGATCATGGCTCAG GGTTACCTTGTTACGACTT	95℃(30s)-51℃(1min)- 72℃(2min), 30cycle	Weisburg et al., 1991
DNA gyrase subunit B (*gyr*B)	22fVf 1240rVf	GAAGTTATCATGACGGTACTTC AGCGTACGAATGTGAGAACC	95℃(30s)-51℃(1min)- 72℃(1min), 30cycle	Ast et al., 2007
glyceraldehyde-3-phosphate dehydrogenase (*gap*A)	gapAfor1 gapArev1	AAGAGCGCAATGATATTGAAGTTG TAGCATCGAATACTGAAGTTTGAG	95℃(30s)-51℃(1min)- 72℃(1min), 30cycle	Ast et al., 2004
uridylate kinase (*pyr*H)	pyrH-04-F pyrH-02-R	ATGASNACBAAYCCWAAACC GTRAABGCNGMYARRTCCA	95℃(30s)-43℃(1min)- 72℃(1min), 30cycle	Thompson et al., 2005
recombinase A (*rec*A)	recA-01-F recA-02-R	TGARAARCARTTYGGTAAAGG TCRCCNTTRTAGCTRTACC	95℃(30s)-50℃(1min)- 72℃(1min), 30cycle	Thompson et al., 2005
RNA polymerase alpha subunit (*rpo*A)	rpoA-01-F rpoA-03-R	ATGCAGGGTTCTGTDACAG GHGGCCARTTTTCHARRCGC	95℃(30s)-50℃(1min)- 72℃(1min), 30cycle	Thompson et al., 2005

homology with all other *Aliivibrio* species registered in the GeneBank (data not shown), and it was difficult to clearly identify them at the species level with a single gene. Because each *Aliivibrio* species are known to have highly homologous genes among each other (Urbanczyk et al., 2007; Ast et al., 2009), MLSA is required for clear identification of *Allivibrio* strains at the species level. In this study, we randomly selected the 12 *Aliivibrio* species identified by 16S rDNA gene sequencing, and brought them to MLSA by combining the nucleotide sequences of 6 genes (*gyr*B, *gap*A, *pyr*H, *rec*A, *rpo*A and 16S rDNA). Then, these concatenated sequence data (4,580 bp) were compared with those of genus *Aliivibrio*, genus *Photobacterium* and genus *Vibrio* for the species identification and phylogenetic analysis.

The *Aliivibrio* concatenated alignment tree resolved *A. fischeri, A. salmonicida, A. logei, A. sifiae* and *A. wodanis* as distinct clades with strong bootstrap support. *A. thorii* and *A. figgae* also fell into 2 distinct clades, although their species name are not officially accepted yet (Fig. 1). 6 of 12 strains were closely clustered with *A. logei* ATCC 29985[T] (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1), *A. logei* ATCC 15382[T] (GSGc17.1) and *A. wodanis* ATCC BAA104[T] (GSGc16.1), while the other 6 strains (GSGc17.2, GNGc16.2, GSGc16.2, GSGc17.3, GSGc18.2, GSGc 17.4) were clustered distinctly from other known *Aliivibrio* species and divided into 2 clusters (Fig. 1). Interestingly, GSGc17.1 was clustered with *A. logei* ATCC 15382[T], but they were independently clustered with the type strain *A. logei* ATCC 29985[T] and other strains (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1) (Fig. 1).

When the concatenated sequences (4,580 bp) of the 12 *Allivibrio* strains from walleye pollock and other *Aliivibrio* species used in MLSA were compared to investigate their homology, 4 strains (GNGc16.1, YYGc 16.1, YYGc16.2, GSGc18.1) showed 99.8% homology with *A. logei* type strain ATCC 29985[T], and 99.4-99.5% homology with *A. logei* WHSW1 (Table 3A).

GSGc16.1 showed 99.1% homology with *A. wodanis* type strain ATCC BAA-104[T], 97.4 homology with *A. figgae* SA12 and 96.8% homology with *A. logei* ATCC 15382[T] (Table 3B). GSGc17.1 showed 99.6% homology with *A. logei* ATCC 15382[T], 98.6% homology with *A. figgae* SA12 and 96.8% homology with *A. wodanis* ATCC BAA-104[T] (Table 3B). However, *A. logei* ATCC 15382[T] and GSGc17.1 showed only 95.2% and 95.0% homology with the type strain *A. logei* ATCC 29985[T] (Table 3B), respectively. Ast et al., (2009) demonstrated that 3 strains (*A. logei* ATCC 15382[T], *A. logei* SR6, *A. logei* SA12) belong to different clusters from type strain *A. logei* ATCC 29985[T], and mentioned these 3 strains might be incorrectly identified. Recently, Klemetsen (2016) reidentified 2 strains (*A. logei* SR6, *A. logei* SR12) as *A. figgae* by MLSA.

For MLSA, we only selected *Allivibrio* strains having all 6 genes (*gyr*B, *gap*A, *pyr*H, *rec*A, *rpo*A and 16S rDNA) sequences available in NCBI. Consequently, 5 different *A. logei* strains were found but only 1 *A. wodanis* strain and no *A. finisterrensis* strain were found for MLSA in our study. Moreover, Klemetsen (2016) reported 5 new *Aliivibrio* species (e.g., *A. magni*, *A. thrudae*, *A. modi*, *A. friggae* and *A. raniae*) but did not register their genetic information in NCBI. More information on those house-keeping genes of the *Aliivibrio* species mentioned above, particularly for *A. wodanis*, would be necessary to resolve their phylogenetic position and relationships with other known *Allivibrio* species.

Biochemical and physiological characteristics of 12 strains used in MLSA were also investigated. All strains were identified Gram- negative. Biochemical and physiological characteristics of the 4 strains (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1) were identical to those of *A. logei* ATCC 29985[T], except for 25℃ growth. For GSGc16.1 and GSGc17.1 strains, biochemical and physiological characteristics were identical to those of *A. wodanis* ATCC BAA-104[T] and *A. logei* ATCC 15382[T], respectively, except for
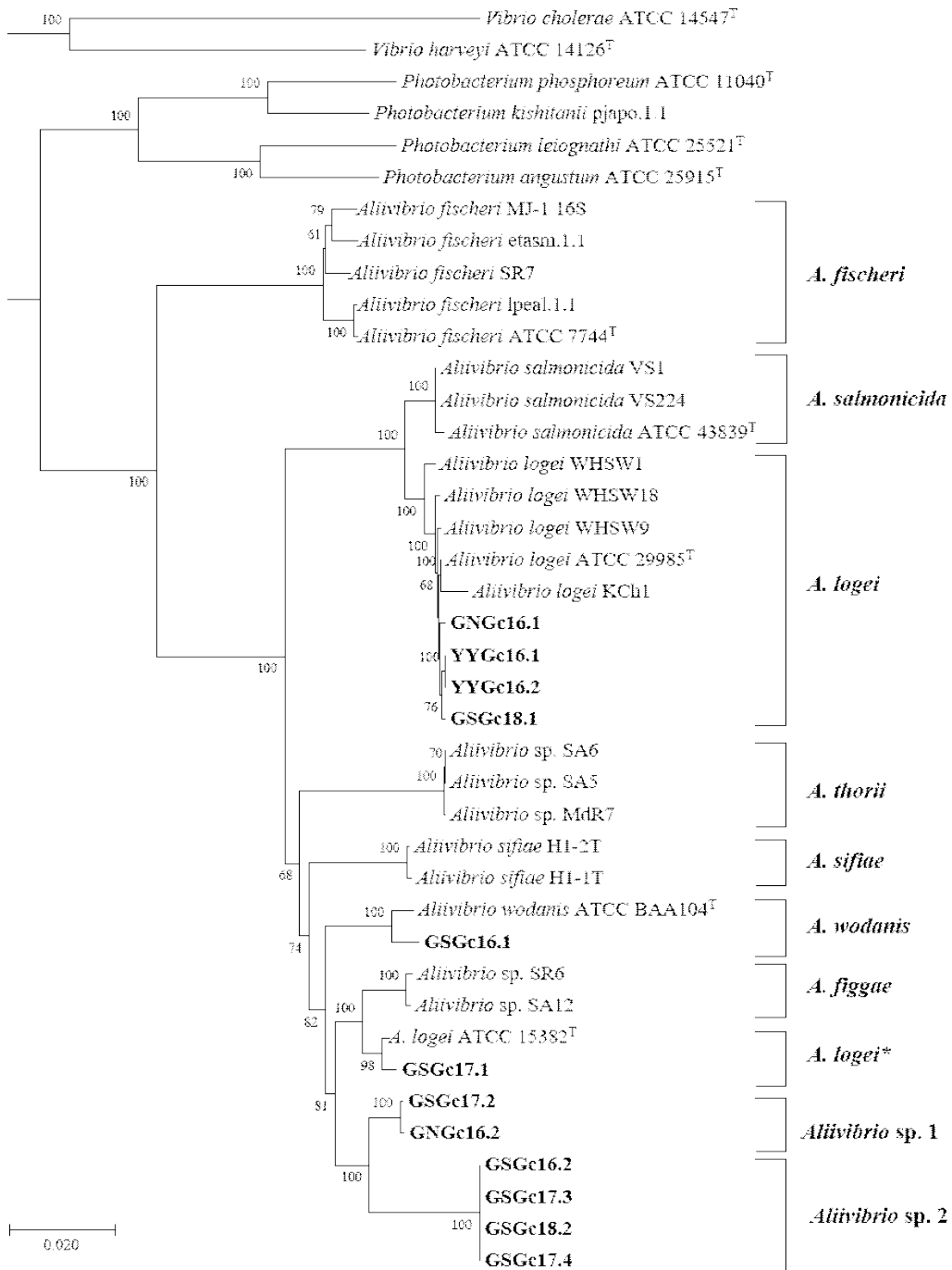
Fig. 1. Phylogenetic reconstructions based on six concatenated genes (*gap*A, *gyr*B, *pyr*H, *rec*A, *rpo*A, and 16S rDNA). Alignment was performed on all genes, and gaps and missing parts in the sequence were eliminated to make a total of 4,580 bp. The number after bacterial scientific name means strain number registered in the NCBI. This tree was constructed based on neighbor-joining method. Numbers at nodes denote the level of bootstrap based on 1,000 replicates. *A. thorii* and *A. figgae* have not been recognized as official bacterial species names yet.

Table 3. Estimation of evolutionary divergence among genus *Aliivibrio* species, based on 6 genetic loci sequences (*gapA*, *gyrB*, *pyrH*, *recA*, *rpoA* and 16S rDNA)

A

		1	2	3	4	5	6	7	8	9	10	11	12
1	GNGc16.1												
2	YYGc16.1	99.9%											
3	YYGc16.2	99.9%	100.0%										
4	GSGc18.1	99.8%	99.8%	99.8%									
5	A. logei ATCC 29985[T]	99.8%	99.8%	99.8%	99.8%								
6	A. logei WHSW1	99.4%	99.5%	99.5%	99.4%	99.5%							
7	A. logei KCh1	99.3%	99.3%	99.3%	99.3%	99.5%	99.0%						
8	A. salmonicida VS1	98.7%	98.6%	98.6%	98.6%	98.7%	98.9%	98.2%					
9	A. salmonicida ATCC 43839[T]	98.6%	98.5%	98.5%	98.5%	98.5%	98.7%	98.0%	99.8%				
10	A. logei ATCC 15382[T]	95.2%	95.1%	95.1%	95.1%	95.2%	95.3%	94.7%	95.1%	94.9%			
11	A. wodanis ATCC BAA104[T]	94.7%	94.6%	94.6%	94.7%	94.8%	94.7%	94.3%	94.6%	94.5%	97.0%		
12	A. fischeri ATCC 7744[T]	90.6%	90.5%	90.5%	90.6%	90.6%	90.8%	90.1%	90.8%	90.7%	91.2%	91.1%	

B

		1	2	3	4	5	6	7	8	9	10	11	12
1	GSGc16.1												
2	A. wodanis ATCC BAA104[T]	99.1%											
3	A. figgae SA12	97.4%	96.7%										
4	A. logei ATCC 15382[T]	96.8%	97.0%	98.4%									
5	GSGc17.2	96.7%	96.5%	97.4%	97.5%								
6	GSGc17.1	96.6%	96.8%	98.6%	99.6%	97.4%							
7	A. sifiae H1-1[T]	95.7%	95.7%	96.3%	96.4%	97.2%	96.3%						
8	GSGc16.2	95.4%	95.2%	96.1%	96.0%	97.3%	96.0%	95.0%					
9	A. thorii MdR7	94.6%	94.8%	95.3%	95.5%	95.0%	95.3%	95.3%	93.4%				
10	A. logei ATCC 29985[T]	94.5%	94.8%	94.6%	95.2%	94.9%	95.0%	94.7%	93.3%	93.9%			
11	A. salmonicida ATCC 43839[T]	94.1%	94.5%	94.5%	94.9%	94.5%	94.7%	94.4%	93.2%	93.9%	98.5%		
12	A. fischeri ATCC 7744[T]	91.0%	91.1%	91.1%	91.2%	91.4%	91.1%	90.9%	90.6%	91.1%	90.6%	90.7%	

A : Homology comparison based on other *Aliivibrio* species, 4 strains (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1) B : GSGc16.1, C : GSGc17.1, D : GSGc17.2 and GNGc16.2, E : 4 strains (GSGc17.2, GSGc16.2, GSGc17.3, GSGc18.2, GSGc17.4). And all positions containing gaps and missing data were eliminated. There were a total of 4,580 positions in the final dataset. Values means similarities of nucleotide sequences.

Table 4. Differential biochemical and physiological characteristics of *Aliivibrio* species isolated in this study and other *Aliivibrio* and *Vibrio* species

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth in NaCl:														
0.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	ND	+	+	-	+	+
5%	+	+	+	+	+	+	+	+	ND	+	+	ND	+	+
6%	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND
7%	-	-	-	-	-	-	-	v	ND	-	+	ND	-	+
8%	-	-	-	-	-	-	-	-	-	-	ND	-	-	ND
Growth at:														
4°C	+	+	+	+	+	+	+	+	+	+	-	+	+	-
15°C	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
22°C	+	+	+	+	+	+	+	+	ND	-	+	+	+	+
25°C	+	+	+	+	+	-	+	+	ND	-	+	+	+	+
30°C	-	+	+	-	-	-	-	-	+	-	+	+	+	+
35°C	-	-	-	-	-	ND	ND	-	ND	ND	+	-	ND	ND
37°C	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND
production of :														
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Lysine decarboxylase	+	-	+	v	+	+	-	-	+	-	+	+	-	-
Indole	-	-	-	-	-	-	-	+	+	-	-	-	+	-
Urease	-	-	-	-	-	-	+	-	-	-	+	+	-	+
Voges-Proskauer	-	-	-	-	-	-	-	-	ND	-	+	-	-	+
Gelatinase activity	-	-	-	-	-	-	-	+	ND	-	-	-	+	+

Table 4. Continue

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Production of acid from :														
D-Mannitol	V	-	V	-	-	+	-	-	+	+	+	-	+	+
D-Mannose	V	-	V	-	-	+	+	+	+	+	-	+	+	+
Sucrose	-	-	-	-	-	-	+	+	+	-	+	-	-	+
Fermentation of D-glucose	+	+	+	+	+	+	+	+	V	+	+	+	+	+
Oxidative production of acid from														
melibiose	-	-	-	-	-	-	-	-	ND	-	-	-	+	-
D-sorbitol	-	-	-	-	-	-	-	-	ND	-	-	-	-	+
Oxidase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
API ZYM test:														
Esterase (C4)	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Acid phosphatase	+	+	+	+	+	+	+	V	+	+	+	-	+	+
Naphthol phosphohydrolase	+	+	+	+	+	+	+	+	V	+	+	-	+	+
Alkaline phosphatase	+	+	+	+	+	+	+	+	ND	+	+	+	+	+

1, 4 stains (GNGc16.1, YYGc16.1, YYYGc16.2, GSGc18.1); 2, GSGc16.1: 3, GSGc17.1: 4, GSGc17.2 and GNGc16.2: 5, 4 isolates (GSGc16.2, GSGc17.3, GSGc18.2, GSGc17.4); 6, *A. logei* ATCC 29985[T]; 7, *A. logei* ATCC 15382[T]; 8, *A. wodanis* ATCC BAA-104[T]; 9, *A. sifiae* H1-1[T], 10, *A. salmonicida* ATCC 43839[T]; 11, *A. fischeri* ATCC 7744[T], 12, *A. finisterrensis* CMJ 11.1; 13, *V. splendidus* I ATCC 33125[T]; 14, *V.anguillarum* ATCC 14181[T] were adopted from Lunder et al.,(2000). +, ≥ 75% of strains Positive; −, < 25% of strains negative; V, Variable.

several parameters (e.g. 30℃ growth test, indole test, gelatinase activity test, D-mannose test, sucrose test for GSGc16.1, and 30℃ growth test, lysine-decarboxylase test, urease test for GSGc17.1). The 6 sreains (GSGc17.2, GNGc16.2, GSGc16.2, GSGc17.3, GSGc 18.2, GSGc17.4) shared several biochemical and physiological characteristics with other species of genus *Aliivibrio* (Table 4), but there was no parameter for clearly differentiating them at the species level. Urbanczyk et al. (2007) showed that 4 biochemical tests (Gelatinase activity, Voges-Proskauer test, Arginine dihydrolase, and Indole production) can distinguish genus *Aliivibrio* from genus *Vibrio*. However, phenotypic characterizations alone are thought to be insufficient to distinguish *Allivibrio* species; biochemical and physiological characteristics of 4 strains (GNGc16.1, YYGc16.1, YYGc16.2, GSGc18.1) and *A. logei* ATCC 29985[T] were almost identical, but GSGc16.1 and GSGc17.1 strains showed that at least 3 tests (Grow that 30°C, D-Mannitol, D-Mannose) did not coincide with other known *Allivibrio* species (Table 4).

From the results of MLSA, 5 of 12 strains from walleye pollock were clearly identified as *A. logei* or *A. wodanis*. Other 7 strains need re-identification (GSGc17.1) or further characterization as potential novel species (*Aliivibrio* sp. 1 for GSGc17.2, GNGc 16.2, and *Aliivibrio* sp. 2 for GSGc16.2, GSGc17.3, GSGc18.2, GSGc17.4). Our study suggested that MLSA provides an accurate and practical approach for routine identification of *Allivibrio* species.

Aliivibrio logei and *A. wodanis* are known as opportunistic or secondary pathogen of gadoid and salmonid fish (Brunvold et al., 2007; Reid et al., 2009; Karlsen et al., 2014; Hjerde et al., 2015), although *A. logei* has been extensively studied as bioluminescetic species (Konopleva et al., 2016). The 38 *Aliivibrio* strains in this study were obtained from the visceral organs (e.g., spleen, kidney, intestines) of fish maintained in 3 different facilities, but there was no considerable trends regarding the occurrence of

Table 5. Information on 12 *Aliivibrio* strains in this study

Strain	Date	location	Source
GSGc16.1	2016.02	facility A	spleen
GSGc16.2	2016.02	facility A	kidney
YYGc16.1	2016.03	facility B	spleen
YYGc16.2	2016.03	facility B	kidney
GNGc16.1	2016.03	facility C	spleen
GNGc16.2	2016.05	facility C	kidney
GSGc17.3	2017.01	facility A	spleen
GSGc17.4	2017.01	facility A	spleen
GSGc17.1	2017.04	facility A	intestine
GSGc17.2	2017.04	facility A	intestine
GSGc18.1	2018.02	facility A	water
GSGc18.2	2018.02	facility A	intestine

Aliivibrio species (Table 5). During the sampling period in our study, neither mass mortality nor abnormal clinical symptoms suspicious for bacterial infection were found. Therefore, currently the possibility of *Aliivibrio* species as a pathogen to walleye pollock is thought to be low. But continuous monitoring is necessary because they can cause outbreaks when the host fish is immunologically compromised or damaged by other pathogens (Karlsen et al., 2014).

Acknowledgments

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